Secondary metabolites in organic and conventional crops and diets, and their bioavailability in humans

Carotenoids, flavonoids, phenolic acids, and polyacetylenes

PhD thesis

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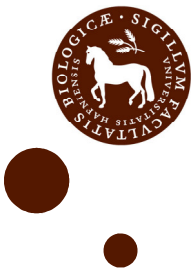

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Preface

This PhD study was conducted from 2007 to 2010 at the Department of Basic Sciences and Environment, Faculty of Life Sciences, University of Copenhagen, and the Division of Food Chemistry, National Food Institute, Technical University of Denmark.

This project was part of the research project OrgTrace, which was financially supported by the Ministry of Food, Agriculture and Fisheries, Denmark, and coordinated by the International Centre for Research in Organic Food Systems (ICROFS). The participants in the OrgTrace project were the National Food Institute, Technical University of Denmark; the Faculty of Life Sciences, University of Copenhagen; and the Faculty of Agricultural Sciences, Aarhus University, and I would like to thank all the participants for good collaboration and team-work.

I would like to thank my supervisors Professor John Nielsen and Senior Scientist Pia Knuthsen for giving me the opportunity to work with this project, and for encouragement and guidance during the PhD study. I am also grateful to Associate Professor Jan H. Christensen for his always open mind and support, when help was needed.

I also acknowledge Claudine Manach and Augustin Scalbert for giving me the opportunity to work three months at the National Institute for Agricultural Research (INRA, Clermont-Ferrand, France) in the summer 2008 and their inspiration during the stay. The results from the stay abroad are not included in this thesis because the work is still ongoing.

I would also like to thank my colleagues at the Division of Food Chemistry, National Food Institute, Technical University of Denmark, especially the group working with bioactive compounds, for assistance and help during the PhD study.

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Malene Søltoft

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Abstract

Organic agriculture promotes biodiversity and natural biological cycles, and minimizes the application of external inputs. Organic farming has increased progressively in the last two-three decades, especially in Scandinavia, and approximately 5 % of the food products were organic in Denmark in the end of 2007. The main reasons for buying organic food products are increased environmental protection, animal welfare, and human health as well as better taste and freshness. The health aspects could be related to, for instance, lower levels or lack of pesticide and medicine residues and a higher content of nutrients.

The fundamental differences between organic and conventional farming, especially in soil fertility management, have been proposed to induce differences in the content of nutrients and secondary metabolites of food products. However, it is still considered scientifically undocumented because a limited number of comparative studies have been carried out under comparable, valid, and well-controlled conditions. Secondary metabolites are non-essential, but play a role in the development, growth, and defence mechanisms in plants. They have been proposed to contribute in the prevention of diseases in humans, such as cancer and cardiovascular diseases, due to, for instance, their antioxidant properties and ability to bind proteins. Nevertheless, secondary metabolites have previously been given little attention and the number of valid and well-controlled studies of secondary metabolites in organic and conventional crops is limited, especially human intervention studies. The aim of this study was to investigate the impact of agricultural production system on the content of selected secondary metabolites (carotenoids, flavonoids, phenolic acids, and polyacetylenes) in vegetables and diets as well as on plasma status of carotenoids after a two-week intervention with organic and conventional food products.

A two-year field trial with cereals, beans, and vegetables was performed at various locations in Denmark. The study included three different growth systems: one conventional with application of pesticides and inorganic fertilizers, one organic with the use of animal manure, and another organic, where cover crops were used as nutrient supply. Furthermore, a two-year human intervention study was carried out based on diets produced from the cultivated crops from the three growth systems. The chemical analyses of carotenoids in crops, intervention diets, and plasma samples were based on routine procedures at the National Food Institute, Technical University of Denmark, while new rapid and effective chemical methods were developed and validated for analyses of flavonoids,

phenolic acids, and polyacetylenes in vegetables and intervention diets. The one-step pressurised liquid extraction method for analysis of flavonoids and phenolic acids showed considerable improvements and possibilities, especially the simultaneous extraction and clean-up by in-cell addition of C18-material. Furthermore, the extraction of polyacetylenes was based on a new rapid and effective methodology using an Ultrasonic Liquid Processor. The secondary metabolites were quantified using HPLC-UV and the identification was performed applying mass spectrometry.

Carotenoids (β-carotene, α-carotene, and lutein) were quantified in the carrots together with 5-*O*-caffeoylquinic acid (5-CQA) and polyacetylenes (falcarindiol, falcarindiol-3-acetate, and falcarinol). The most abundant phenolic acid in the potatoes was 5-CQA, while various different quercetin- and isorhamnetin-glycosides were quantified in the onions with the most abundant being quercetin-3,4'-diglucoside (Q-3,4'-diglu) and quercetin-4'-glucoside (Q-4'-glu). No systematic differences between the three growth systems across growth year were observed, except a higher content of 5-CQA in the organic growth system with cover crops, despite differences in fertilization strategy and levels. The compounds found in the intervention diets were similar to the vegetables and the major compounds were Q-3,4'-diglu, Q-4'-glu, and β-carotene. The growth system did not induce systematic differences between the intervention diets across growth year similarly to the vegetables, even though a few significant differences of specific compounds were observed. Furthermore, the storage and processing procedures used in the preparation of intervention diets affected the levels of secondary metabolites considerably and the amounts ingested were not necessarily comparable with the levels found in the food products. In conclusion, the agricultural production system did not have a general significant impact on the ability of vegetables to synthesise secondary metabolites or on the content in the diets from the human intervention study.

The health effects of secondary metabolites in organic and conventional food products depend on the amounts ingested and their bioavailability. The amounts ingested were generally insignificantly different and the plasma status of carotenoids increased after ingestion of the intervention diets from the three growth systems, but no systematic differences were observed between the diets from different growth systems, except for lutein. However, the small concentration differences of lutein are unlikely to be of significance for human health. Further research on the impact of growth system on the bioavailability of flavonoids, phenolic acids, and polyacetylenes is needed. Nevertheless, the expected health effects of organic compared with conventional food products do not seem to be related to the secondary metabolites investigated in this study and the perception about organic foods being healthier than conventional was not supported.

Abstract in Danish – Resumé

Økologisk landbrug fremmer biodiversiteten og de naturlige biologiske cykler samt minimerer eksterne tilførsler. Andelen af økologisk landbrug er steget progressivt gennem de sidste to-tre årtier, specielt i Skandinavien, og ca. 5 % af alle fødevarer var økologiske i Danmark i slutningen af 2007. Forbrugerne køber primært økologiske fødevarer pga. øget miljøbeskyttelse, dyrevelfærd og sundhed samt bedre smag og friskhed. Den øgede menneskelige sundhed kan for eksempel skyldes lavere niveauer eller ingen tilstedeværelse af pesticid- og medicinrester samt et øget indhold af næringsstoffer.

Det har været foreslået at de fundamentale forskelle mellem økologisk og konventionelt landbrug, specielt med hensyn til gødning af jorden, kan medføre forskelle i indholdet af næringstoffer og sekundære metabolitter i fødevarer. Det bliver dog stadigvæk ikke betragtet som videnskabeligt bevist, da et begrænset antal studier er udført under sammenlignelige og kontrollere forhold. Sekundære metabolitter er ikke essentielle for planter, men spiller en vigtig rolle i deres udvikling, vækst og forsvarsmekanismer. Det har været foreslået at de bidrager til forebyggelsen af sygdomme, som f.eks. kræft og hjertekarsygdomme, muligvis pga. deres antioxidative effekter og evne til at binde proteiner. Ikke desto mindre, har der tidligere ikke været særlig meget fokus på sekundære metabolitter og antallet af studier af sekundære metabolitter i økologiske og konventionelle afgrøder udført under kontrollerede forhold er begrænset, specielt antallet af humane interventionsstudier. Formålet med dette studie er at undersøge effekten af dyrkningssystem på indholdet af udvalgte sekundære metabolitter (karotenoider, flavonoider, fenoliske syrer og polyacetylener) i grøntsager og diæter samt effekten på plasma status af karotenoider efter to-ugers intervention med økologiske og konventionelle fødevarer.

Der blev udført et to-årigt markforsøg med korn, bønner og grøntsager på forskellige lokaliteter i Danmark. Studiet inkluderede tre forskellige dyrkningssystemer: et konventionelt med tilførsel af pesticider og uorganisk gødning, et økologisk med tilførsel af gylle samt et økologisk, hvor næringstilførslen kom fra efterafgrøder. Derudover blev der udført et to-årigt humant interventionsstudie, hvor diæterne var baseret på de dyrkede afgrøder fra markforsøget inklusiv de tre dyrkningssystemer. De kemiske analyser af karotenoider i afgrøder, interventionsdiæter og plasma prøver var baseret på standardprocedurer på Fødevareinstituttet på Danmarks Tekniske Universitet, mens der blev udviklet nye hurtige og effektive kemiske metoder til analyse af flavonoider, fenoliske syrer og polyacetylener i grøntsager og interventionsdiæter. Der var

betydelige forbedringer og muligheder med den nye højtryks væskeekstraktion udført i ét trin til analyse af flavonoider og fenoliske syrer, specielt kombinationen af ekstraktion og oprensning ved tilsætning af C18-materiale til ekstraktionscellerne. Derudover var ekstraktionen af polyacetylener baseret på en ny hurtig og effektive metode med anvendelse af en "Ultrasonic Liquid Processor". HPLC-UV blev anvendt til kvantificering af sekundære metabolitter, som blev identificeret ved hjælp af massespektrometri.

Karotenoider (β-karoten, α-karoten og lutein) blev kvantificeret i gulerødderne sammen med 5-*O*-kaffeoylquininsyre (5-CQA) og polyacetylener (falkarindiol, falkarindiol-3-acetat og falkarinol). 5-CQA var den primære fenoliske syre i kartofler, mens flere forskellige glykosider af quercetin og isorhamnetin blev kvantificeret i løg, primært quercetin-3,4'-diglukosid (Q-3,4'-diglu) og quercetin-4'-glukosid (Q-4'-glu). Der blev ikke fundet nogen systematiske forskelle mellem dyrkningssystemer på tværs af dyrkningsårene på trods af forskelle gødningsmetoder og niveauer, bortset fra et højere indhold af 5-CQA i det økologiske dyrkningssystem med efterafgrøder. De sekundære metabolitter i interventionsdiæterne lignede dem, der blev fundet i grøntsagerne, og de primære stoffer var Q-3,4'-diglu, Q-4'-glu og β-karoten. Dyrkningsystemerne medførte ingen systematiske forskelle mellem interventionsdiæterne på tværs af dyrkningsårene i lighed med grøntsagerne, selvom enkelte signifikante forskelle blev observeret for nogle af stofferne. Derudover blev niveauet af sekundære metabolitter påvirket betydeligt af opbevaring og behandling af grøntsager i forbindelse med forberedelse af interventionsdiæterne og indtagsmængderne var ikke nødvendigvis sammenlignelige med de fundne niveauer i fødevarerne. Det kan konkluderes, at dyrkningssystemet generelt ikke havde en signifikant effekt på grøntsagernes evne til at syntetisere sekundære metabolitter eller på indholdet i diæterne fra det humane interventionsstudie.

Sundhedseffekterne af sekundære metabolitter i økologiske og konventionelle fødevarer afhænger af indtaget og deres biotilgængelighed. Indtaget var ikke signifikant forskelligt og plasma status af karotenoider blev øget efter indtag af interventionsdiæterne. Der blev dog ikke fundet nogle systematiske forskelle mellem de forskellige diæter, bortset fra lutein, men det er ikke sandsynligt at de små koncentrationsforskelle har nogen sundhedsmæssig betydning. Der kræves yderligere undersøgelser for at bestemme effekten af dyrkningssystem på biotilgængeligheden af flavonoider, fenoliske syrer og polyacetylener. Ikke desto mindre ser de formodede sundhedseffekter af økologiske i forhold til konventionelle fødevarer ikke ud til at være relateret til de sekundære metabolitter undersøgt i dette studie, som heller ikke kan bekræfte opfattelsen af at økologiske fødevarer er sundere end konventionelle.

Publications

This PhD project is based on the publications listed below, which can be seen in full text at the end of the thesis. They are referred to by their Roman numerals in the text. Reprint of Paper I was made with permission from the publisher.

Paper I

Søltoft, M.; Christensen, J. H.; Nielsen, J.; Knuthsen, P. Pressurised liquid extraction of flavonoids in onions. Method development and validation. *Talanta* **2009**, 80, 269-278.

Manuscript I

Søltoft, M.; Eriksen, M. E.; Träger, A. W. B.; Nielsen, J.; Laursen, K. H.; Husted, S.; Halekoh, U.; Knuthsen, P. Comparing the content of polyacetylenes in organically and conventionally grown carrot roots using a new and fast ultrasonic liquid extraction method. *Journal of Agricultural and Food Chemistry* **2010** (Submitted).

Manuscript II

Søltoft, M.; Nielsen, J.; Laursen, K. H.; Husted, S.; Halekoh, U.; Knuthsen, P. Effects of organic and conventional growth systems on the content of flavonoids and phenolic acids in vegetables. *Journal of Agricultural and Food Chemistry* **2010** (Submitted).

Manuscript III

Søltoft, M.; Bysted, A.; Madsen, K. H.: Budek, A. Z.; Bügel, S. G.; Nielsen, J.; Knuthsen, P. Effects of organic and conventional growth systems on the content of carotenoids in carrot roots and on intake and plasma status of carotenoids in humans. *Journal of the Science of Food and Agriculture* **2010** (Submitted).

Abbreviations

ACN: acetonitrile

ANOVA: analysis of variance

BHT: butylated hydroxytoluene (2,6-bis(1,1-dimethylethyl)-4-methylphenol)

C: conventional growth system using inorganic fertilezers and pesticides

CA: caffeic acid

4-CQA: 4-*O*-caffeoylquinic acid

5-CQA: 5-*O*-caffeoylquinic acid

DMSO: dimethyl sulfoxide

dw: dry weight

EtOAC: ethyl acetate

EtOH: ethanol

FaDOH: falcarindiol

FaDOAc: falcarindiol-3-acetate

FaOH: falcarinol

fw: fresh weight

GC: gas chromatography

HPLC: high pressure liquid chromatography

I: isorhamnetin

I-4'-gly: isorhamnetin-4'-glycoside

I-3,4'-digly: isorhamnetin-3,4'-diglycoside

P: octanol-water partitioning coefficient

LOD: limit of detection

LOQ: limit of quantification

MAE: microwave-assisted extraction

MeOH: methanol

MS: mass spectrometry

MS/MS: tandem mass spectrometry

MTBE: methyl *tertiary*-butyl ether

n.a.: no information available

n.d.: not detected

NMR: nuclear magnetic resonance

OA: organic growth system using animal manure

OB: organic growth system using cover crops

PCA: principal component analysis

PDA: photodiode array detector

PLE: pressurised liquid extraction

PLS-DA: partial least squares discriminant analysis

Q: quercetin

Q-3-glu: quercetin-3-glucoside

Q-4'-glu: quercetin-4'-glucoside

Q-3,4'-diglu: quercetin-3,4'-diglucoside

Q-7,4'-diglu: quercetin-7,4'-diglucoside

Q-3,7,4'-trigly: quercetin-3,7,4'-triglycoside

RSD: relative standard deviation

sd: standard deviation

SPE: solid-phase extraction

THF: tetrahydrofuran

tr: traces found

ULP: ultrasonic liquid processor

UPLC: ultra performance liquid chromatography

UV: ultraviolet radiation

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1 Introduction and objectives

1.1 Introduction

Organic agriculture started as an alternative to the increasing intensification of agriculture, particularly the use of inorganic nitrogen fertilizers *(1)*. Organic farming has increased steadily over the last two-three decades and is now practiced more or less all around the whole world *(2, 3)*. Organic agriculture has also increased very rapidly in most parts of Europe, especially in the Scandinavian and Mediterranean countries *(3)*. In the end of 2007, Denmark had one of the highest spending on organic products per capita as well as one of the highest portions of total market share since approximately 5 % of the food products were organically produced *(4)*.

The increasing demand for organic products can be ascribed to the improved environmental protection and animal welfare, but the personal profits such as enhanced freshness, taste, and health benefits appear to have a more significant effect on the organic budget share *(5, 6)*. The health aspects are related to *e.g.* the products being free from chemical substances such as pesticide and medicine residues, and also a presumed higher content of nutrients and health-promoting secondary metabolites *(5, 7)*. Several consumers perceive that organic food products are more nutritious, healthier, and safer than conventional *(7-9)*, but it is still considered scientifically undocumented and more studies are needed to support or refute this perception *(7, 8)*.

The possible health effects of organic products can be related to the content of nutrients and secondary metabolites. However, previous comparative studies of organic and conventional food products have generated contradictory results *(9)* probably due to few well-controlled and valid comparisons performed under ideal conditions *(2, 7, 8, 10)*. Nevertheless, the content of nitrate generally appear lower in organically grown crops than in conventionally ones *(7, 8, 10)* and a higher content of vitamin C is often seen organic than conventional food products, especially in leafy vegetables *(7)*. Otherwise, the scientific evidence for significant differences in the nutrient content of organically and conventionally grown crops does not exist *(8-11)* and the data on secondary metabolites is generally scarce *(12, 13)*.

Secondary metabolites (also known as bioactive secondary metabolites) are natural non-nutrient constituents in plants, which are believed to have health-promoting and/or toxic effects *(14)*. They play an important role in the growth, development, and defence system of plants *(15)*, but they are non-essential to both plants *(16)* and humans *(12)* in contrast to primary metabolites such as amino

acids, carbohydrates, and lipids. Nevertheless, secondary metabolites have been proposed to play a role in the prevention of numerous human diseases *(12)* and they might contribute to the protective effects of fruits and vegetables *(17-20)* against *e.g.* cancer *(17, 21-23)* and cardiovascular diseases *(24)*.

Secondary metabolites are found widely in the plant kingdom. In particular, fruits and vegetables are good sources of secondary metabolites such as carotenoids, polyacetylenes, and polyphenols (*e.g.* flavonoids and phenolic acids) *(25-28)*. The concentration levels of secondary metabolites in plants can be affected by pathogen infection and pest attack because one of their functions in plants is as defence compounds *(29, 30)*, but also factors such as cultivar, season, climate, and geographical location are important *(10)*. Furthermore, the fundamental differences between organic and conventional agricultural production systems, particularly in soil fertility management, might affect the content of secondary metabolites *(29)*.

The health effects of secondary metabolites are dependent on the amounts ingested and their bioavailability *(26)*. The quantities of secondary metabolites reaching our plate might differ from the quantities found in the unprepared food products because storage and processing procedures can affect both the compound composition and content *(10, 31)*. The bioavailability also differs between secondary metabolites *i.e.* the fraction of the compounds reaching the systemic circulation varies from one compound to another. Thus, the most abundant in our diet might not have the best bioavailability and be the most active in our body because they are poorly absorbed, highly metabolised, or rapidly eliminated *(26)*. However, the impact of the agricultural production system on bioavailability of nutrients and secondary metabolites has so far received little attention in scientific studies *(8)* and especially human intervention studies are especially lacking. Data from these studies are limited and needed in order to evaluate the possible human health impacts of organically and conventionally grown food products *(7)*.

1.2 Objectives

The PhD project is a part of a larger research project called OrgTrace (http://www.orgtrace.elr.dk/uk). The OrgTrace project started in 2007 and is still on-going, but will finish in the end of 2010. The main objective of OrgTrace is to study the impact of different agricultural management practices relevant for organic farming on the ability of cereal and vegetable crops to absorb trace elements from the soil and to synthesise bioactive compounds

(secondary metabolites, vitamins, and phytates) with health-promoting effects. The project also includes a human intervention study employing diets based on the cultivated crops from the different agricultural growth systems and the bioavailability of trace elements and bioactive compounds is evaluated. Additionally, rats are used as models to study various health effects, *e.g.* immune system responses, of organically and conventionally produced crops. It is the first study, which follows selected bioactive compounds the whole way from the plant and soil system to the plate, and into the human body, where absorption occurs.

This PhD project focuses on secondary metabolites in organic and conventional crops and diets, and the content in human samples after consumption of organic and conventional based intervention diets. The overall objective is to study the impact of agricultural production system on the content of selected and presumably health-promoting secondary metabolites in vegetables with high abundance in the human diet. Another aim of the project is to study the content of selected secondary metabolites in the diets from the human intervention study and also discuss their bioavailability and possible health effects based on literature search and selected analyses of human samples from the human intervention trial.

The project can be divided into the following four specific objectives:

- To identify the most abundant carotenoids, flavonoids, phenolic acids, and polyacetylenes in carrots, onions, and potatoes, and to develop validated quantitative analytical methods for analysis of flavonoids, phenolic acids, and polyacetylenes in crops and diets *(Paper I, Manuscript I, II, and III)*.
- To evaluate the impact of growth systems (organic versus conventional) on the ability of carrots, onions and potatoes to synthesise selected secondary metabolites (carotenoids, flavonoids, phenolic acids, and polyacetylenes) *(Manuscript I, II, and III)*.
- To determine the content of selected secondary metabolites (carotenoids *(Manuscript III)*, flavonoids, phenolic acids, and polyacetylenes) in the human intervention diets produced of the cultivated crops from objective 2 and evaluate the impact of growth system, storage, and processing on the content of these secondary metabolites in the diets.

- To evaluate the impact of growth systems on the plasma status of carotenoids in a human intervention study employing the diets from objective 3 *(Manuscript III)*, and evaluate the impact of growth system on the bioavailability of flavonoids, phenolic acids, and polyacetylenes and the possible health effects of organic and conventional food products.

The hypothesis is that the agricultural production system affects the biosynthesis of secondary metabolites due to, for instance, differences in fertilization strategies and levels, nutrient availability as well as pathogen infection and pest attack. Thus, differences in the content of secondary metabolites in organic and conventional food products are expected and the health effects of the food products will probably differ. The PhD project focuses on development of quantitative and validated methods for analysis of selected secondary metabolites with the intention to apply them in the growing field of organic agriculture in order to gain further knowledge about possible health effects of organically compared to conventionally produced food products.

2 Background - Organic agriculture

2.1 Definition and prevalence

Organic agriculture is a production system, which promotes and enhances biodiversity, natural biological cycles, and soil biological activity. It is based on management practices, which maintain the ecological harmony and minimizes the application of external inputs *(32)*. Organic agriculture is based on the principle of health, ecology, fairness, and care *(33)* and has been defined by the International Federation of Organic Agriculture Movements (IFOAM) as the following:

'Organic agriculture is a production system that sustains the health of soils, ecosystems and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects. Organic agriculture combines tradition, innovation and science to benefit the shared environment and promote fair relationships and good quality of life for all involved.' (33)

The first movements within organic agriculture appeared in the 1930s and 1940s *(1, 3)*, but organic farming has especially increased rapidly since the 1980s and 1990s *(2, 3)*. The strongest growth in Europe has been seen in Scandinavia and the Mediterranean countries *(3) e.g.* a significant increase in the total organically managed agricultural land has been observed in Denmark from 1995 to 2002 (Figure 1) *(34)*.

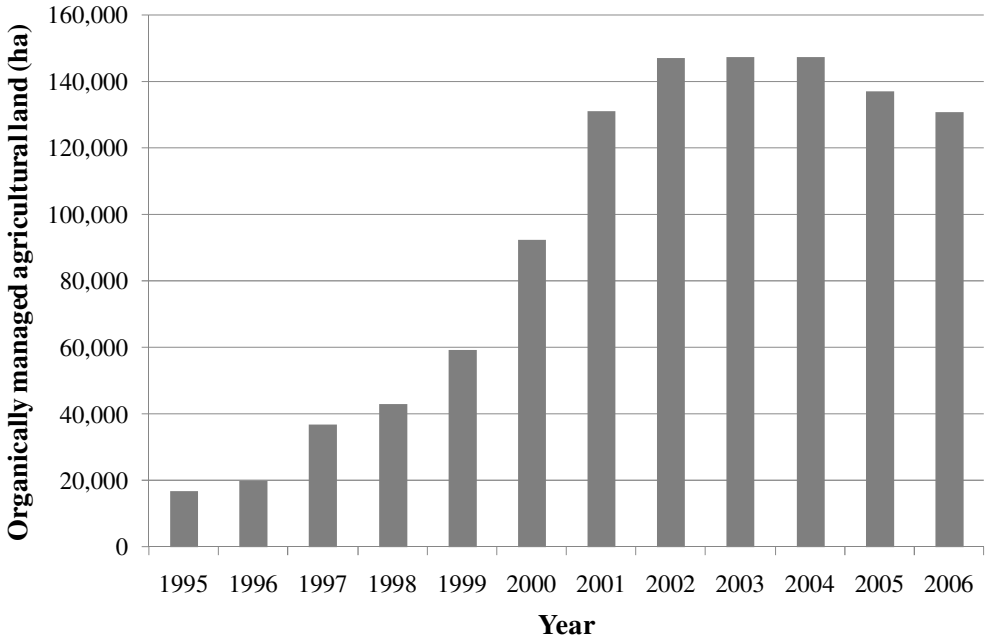


Figure 1. The area of organically managed agricultural land (hectare) in Denmark in 1995─2006 *(34)*.

Nowadays, organic agriculture is practiced almost all around the world *(3)* and the largest organically managed agricultural land is found in Oceania, Latin America, and Europe. In Europe, 1.9 % of the agricultural land is organic and regulated by the revised Regulation on Organic Production (EU Regulation (EC) 834/2007) from January 2009. The highest spending on organic products per capita and the highest portion of organic products of the total market share are seen in Austria, Denmark, and Switzerland. In these countries, around 5 % of the food products are organic *(4)*, but an increase to 9–10 % by 2015 is expected in Denmark *(35)*. Fruits and vegetables constitute respectively 5 % and 11 % of the total organic consumption in Denmark, where the main contributors are milk and cereals with about 50 % *(5)*.

The Danish organic consumer is characterised by having a high household income and education level. The organic budget share increases with age and is generally higher in families with children younger than 15 years, who live in urban areas *(5)*. A Norwegian study also showed that more women than men consume organic food products *(36)*.

The increased demand for organic products is mainly driven by the following preferences *(5)*:

- Animal welfare
- Environmental protection
- Health attributes
- Taste and freshness

The improved animal welfare is associated with good living conditions of farm animals *(37)*. The most clearly documented environmental benefits are improved biodiversity as well as water and soil conservation, while there is no clear evidence of reduced greenhouse gas emissions *(1, 32)*. The health attributes of organic products are associated with, for instance, no pesticide and medicine residues, the possibility of fewer bacteria, and a higher nutrient content *(5)*. Furthermore, some organic consumers state that the organic products taste better *(1)* and are fresher. The primary reasons for buying organic products varies between countries *(8)*, but the patterns are similar in the northern parts of Europe *(5, 6)*. The public good attributes (animal welfare and environmental protection) contribute to the organic budget share in Denmark, but the private good attributes (health, taste, and freshness) seem to be of greater signifance *(5)*.

2.2 Comparative studies of organic and conventional food products

2.2.1 Study designs

Comparative studies can be divided into three types: market-orientated supply, farm, and cultivation studies, which all have weaknesses and strengths, but they also complement each other. In the market-orientated supply studies, food products commercially available in shops are used. The studies are relatively easy to perform and a large number of samples can be included. However, only differences between products from various sources can be identified, but often the factors of significance cannot be determined because limited information about the cultivation is known and the cultivars might also be different. In comparison, farm studies are based on products from selected farms (often neighbouring) with suitable environmental factors, but the information regarding cultivation system comes from the farmers and the selection of farms can be difficult. Cultivation studies are viewed as the most accurate and valid form of comparative studies because the significance of the cultivation system can be determined. However, usually only a small number of samples can be examined and the results mainly apply to the specific location or farm. In general, many factors can affect the results from comparative studies and have to be taken into consideration *e.g.* the sampling, soil and climate conditions, pre-treatment, and chemical analyses *(8, 11, 38)*.

2.2.2 Crop studies

Fundamental differences in organic and conventional cultivation systems, especially in soil fertility management, may affect the content of nutrients as well as secondary metabolites in plants *(29)*, but few major differences between organically and conventionally produced crops have been found *(10, 11, 38)*. Furthermore, it is unlikely that the small differences observed in nutrient content will be beneficial to the consumer with regard to human health *(7, 9)*.

Nitrate appear to be one of the only nutrients, where consistent differences are observed as the content seems to be lower in organic crops than in conventional *(7, 8, 10, 11, 39)*. Organic crops also tend to have a higher content of phosphorous *(10)*, and the content of vitamin C appears higher in organic vegetables compared with conventional, especially in leafy vegetables *(7, 38)*, although Dangour *et al. (10)* did not discover significant differences in vitamin C in a review of numerous crops. Organic vegetables grown above ground might also have a higher dry matter content than

conventional *(38)* and the yield in organic agriculture is often about 10−15 % lower than in conventional agricultural systems *(1)*.

Little consideration has previously been given to secondary metabolites in organic and conventional crops *(13)*. However, higher contents of secondary metabolites in organically grown compared to conventionally grown food products have been indicated, but it is difficult to draw general conclusions due to few systematic studies *(13)*. Nevertheless, it has been stated that no consistent differences are evident in the content of polyphenols *(10)* and β-carotene *(39)* between organically and conventionally grown crops in reviews of comparative studies.

The inconsistency in results from comparative studies is also visible in Table 1, which shows the content of selected secondary metabolites of interest to this study in vegetables and cereals, but to my knowledge, no comparative crop studies have been made on polyacetylenes. Bourn and Prescott stated that fewer significant differences have been seen in farm studies than cultivation studies because numerous variables can affect the nutrient content in comparison with fertilizer treatments *(8)*. However, the opposite tendencies are seen in Table 1, where differences between organically and conventionally grown vegetables and cereals have been found in the farm studies, while very few differences have previously been seen in the cultivation studies. In farm studies, the content of carotenoids in carrots *(40, 41)* and of flavonoids in onions *(42)* grown organically was higher than in conventionally grown crops, while no differences were observed in comparable cultivation studies *(43, 44)*. However, the content of phenolic acids in wheat *(45, 46)* and oat *(47)* grown conventionally and organically was not different in neither farm or cultivation studies.

In general, it is difficult to draw general conclusions because few valid and high-quality comparative studies of crops have been carried out *(7, 8, 10, 12)*. The studies often lack background information, which could have explained the variability between studies. It is important that crops are grown, harvested, and stored under comparable and well-defined conditions (*e.g.* soil conditions, climate, cultivar and stage of ripeness) *(7, 8, 48, 49)* because otherwise numerous other factors than the agricultural production system might contribute to the differences observed.

Table 1. Carotenoids, flavonoids, and phenolic acids in organically and conventionally grown vegetables and cereals.

Group	Compound	Crop	Study design	Result	Reference
Carotenoids	β-carotene	Carrot	Farm study	Org ↑	*(40)*
	β-carotene	Carrot	Farm study	Org ↑	*(41)*
	β-carotene, lutein	Kale	Farm study	Org ↑	*(50)*
	β-carotene	Spinach	Farm study	Org ↓	*(51)*
	Lutein, zeaxanthin, total carotenoids	Wheat	Farm study	No difference	*(45)*
	α-carotene, β-carotene	Carrot	Cultivation study	No difference	*(43)*
	β-carotene, total carotenoids	Sweet peppers	Cultivation study	Org ↑	*(45)*
	Lutein, zeaxanthin	Wheat	Farm and cultivation study	No difference	*(52)*
Flavonoids	Quercetin-3-rhamnoside	Chinese cabbage, green pepper, onion, spinach	Farm study	Org ↑	*(42)*
	Myricetin	Onion, spinach	Farm study	Org ↑	*(42)*
	Quercetin, kaempferol, luteolin	Bell peppers	Cultivation study	No difference	*(53)*
	Apigenin,kaempferol, kaempferol-3-glucoside, luteolin, luteolin-3-glucoside, quercetin, quercetin-3-rutinoside	Collard, lettuce	Cultivation study	No difference (except for kaempferol-3-glucoside: Org ↑)	*(54)*
	Quercetin-3,4'-diglu, quercetin-4'-glu, quercetin	Onion	Cultivation study	No difference	*(44)*
	Total flavonoids	Chicory	Cultivation study	No difference	*(55)*
Phenolic acids	5-*O*-caffeoylquinic acid	Egg plant	Farm study	Org ↑ and ↓ [a]	*(56)*
	Caffeic acid	Onion	Farm study	Org ↑	*(42)*
	5-*O*-caffeoylquinic acid	Potato	Farm study	Org ↑	*(48)*
	Total phenolic acids	Wheat	Farm study	No difference	*(45)*
	Caffeic acid, gallic acid	Collard, lettuce	Cultivation study	No difference	*(54)*
	Total hydroxycinnamic acids	Oat	Cultivation study	No difference	*(47)*
	p-coumaric acid, ferulic acid, *p*-hydroxybenzoic, acid, sinapic acid, vanillic acid	Wheat	Cultivation study	No difference	*(46)*

[a] : cultivar difference

2.2.3 Animal studies

The majority of feeding trials have shown positive effects of organic feed on health parameters such as reproduction, immune responses, growth, and recovery from illness *(38, 57, 58)*. However, the differences in animal behaviour could also be due to other factors than cultivation system, for instance, the genetic background of the animal or the bioavailability of the nutrient *(38)*. The positive impact of organic feed on certain health-related biomarkers (*e.g.* day time activity and liver metabolic function) has been demonstrated in a previous rat study *(59)*. On the contrary, the bioavailability of minerals and metals was not affected by the growth system in another rat study investigating the effect of organic and conventional feed from a cultivation trial *(60)*. Preference tests with rats have also shown that organic feed is preferred in comparison with conventional *(57)*, which could be related to, for instance, a better nutritional content or taste *(38)*.

In general, the data from controlled animal studies investigating the impacts of feed based on organically and conventionally grown food products is extremely sparse *(7)*. Thus, further studies are needed to determined the effects of organic food products on health parameters in animal models *(57)*. However, it is important to be aware that the results from animal studies cannot be directly extrapolated to humans *(38)*.

2.2.4 Human intervention studies

It was stated recently in a review that the observational and clinical evidence supports the hypothesis that consumption of organically produced food is beneficial to human health *(58)*. An association between consumption of organic dairy products and lower eczema risk has been observed in infants, but no relation between the intake of organic meat, fruit, vegetables, eggs, or the proportion of organic food products within the total diet and the development of *e.g.* eczema or wheeze was observed *(61)*.

No significant differences were observed in the plasma status of carotenoids after 14 or 21 days ingestion of blanched carrots *(62)* or tomato puree *(63)*, respectively, based on organic and conventional products. However, the differences in the content of carotenoids between organically and conventionally produced carrots and tomato puree were also small or insignificant *(62, 63)*. In contrast, the urinary excretion of flavonoids (quercetin and kaempferol) was higher after intake (22 days) of an organic compared with a conventional diet produced from foods bought in supermarkets

or at farms. Furthermore, a difference in human markers of oxidation between the two cultivation system was observed, but the setup of the market-orientated supply study made it difficult to determine the significance of the cultivation system because the variation could, for instance, also be due to differences in cultivar *(64)*. Another intervention study with organically and conventionally grown apples did not show any effects of cultivation system on the antioxidant capacity and the study indicated similar antigenotoxic potential of apples from both cultivation systems *(65)*.

Generally, the data from human intervention studies on organic and conventional food products is sparse *(7)* and very few studies have been performed investigating the impact of growth system on the content of secondary metabolites. So, more human studies are needed to evaluate the potential health effects of organically in comparison with conventionally grown food products.

3 Background - Secondary metabolites

Plants produce a vast range of organic compounds, which can be divided into primary and secondary metabolites. The primary metabolites, *e.g.* phytosterols, amino acids, and organic acids, play an essential role in the photosynthesis, respiration, growth, and development of plants, while the secondary metabolites are other organic compounds also often accumulated in high concentrations *(16)*. Numerous secondary metabolites exist, but this PhD project focuses on the following four groups of secondary metabolites: carotenoids, flavonoids, phenolic acids, and polyacetylenes. These groups have been selected due to their potential health-effects and an expected high abundance in the cultivated crop included in the OrgTrace study. The following sections will give an overview of the four groups of secondary metabolites including their chemical properties, biosynthesis and functions in plant, the main dietary sources and intake as well as their bioavailability and biological functions in humans.

3.1 Carotenoids

3.1.1 Chemical properties

Carotenoids are a group of naturally occurring fat-soluble pigments consisting of more than 700 compounds *(25)*, which are mainly found in plants, algae, and several lower organisms *(66)*. Carotenoids are tetraterpenes formed by eight isoprenoid units and can be divided into two groups: carotenes and xanthophylls (Figure 2). Carotenes (*e.g.* α- and β-carotene) contain only carbon and hydrogen, while xanthophylls (*e.g.* lutein and zeaxanthin) are oxygenated carotenoids containing an alcohol, carbonyl or other functional groups *(67)*.

Carotenoids are very hydrophobic and usually found in the lipid parts of plant and animal tissues *(68)*. They have a large number of conjugated double bonds and can undergo *cis-trans* isomerism, but most of them occur naturally in the *trans*-form *(67)*. The majority of carotenoids have chiral centers (*e.g.* lutein), but they normally only occur in one of the possible enantiomeric forms because the biosynthesis is enantiomer-selective *(66)*. The carotenoids absorb visible light and have absorption maximum at 400-500 nm and a very characteristic colour (mainly yellow, orange and red) *(66)*. However, carotenoids are very susceptible to isomerisation and oxidation due to the

polyene chain *(67)*, which is responsible for their instability towards *e.g.* air oxidation, oxidising reagents as well as heat and light *(69)*.

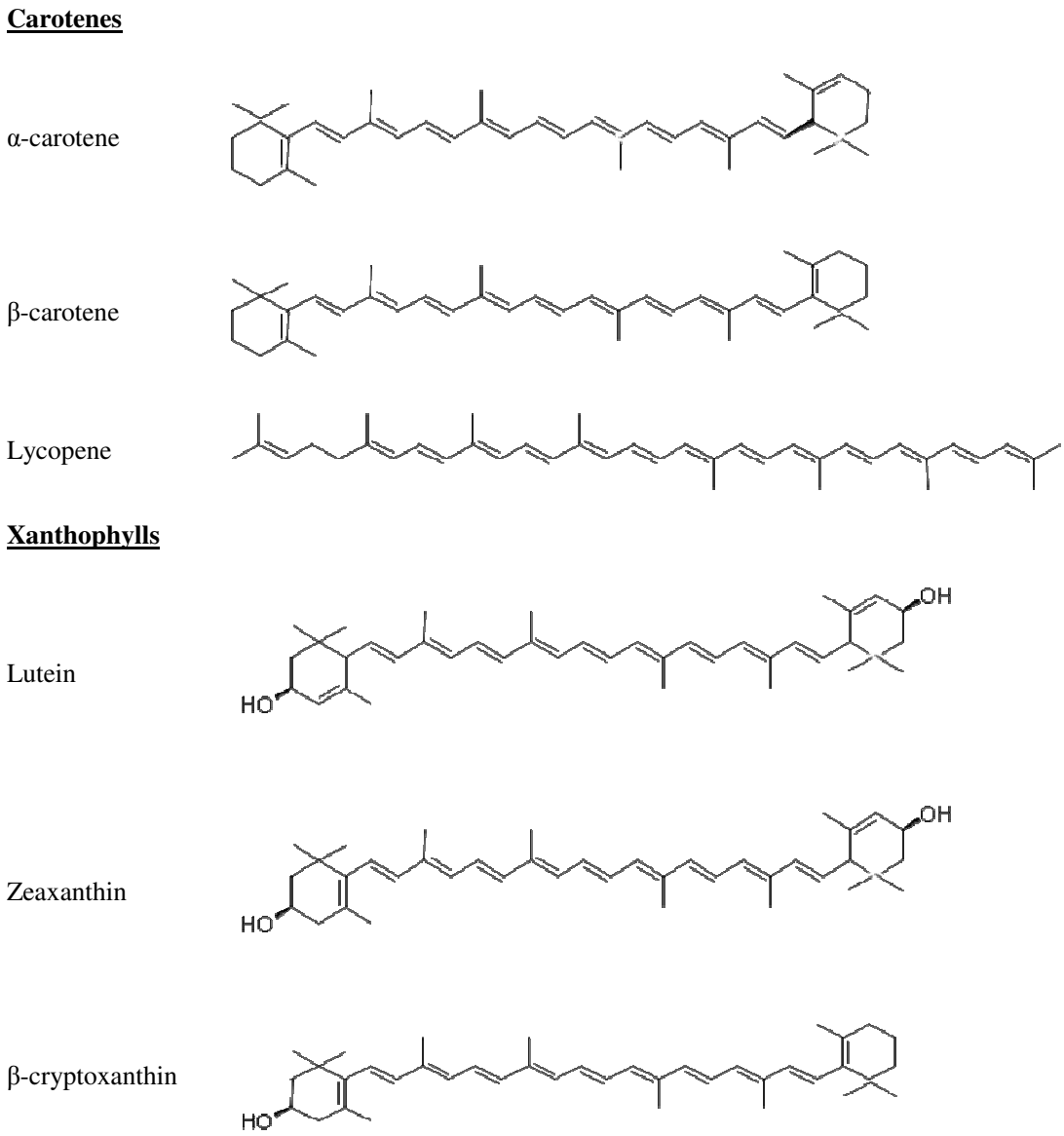


Figure 2. Chemical structures of major carotenoids.

3.1.2 Biosynthesis and functions in plants

Photosynthetic organisms are able to synthesise carotenoids *(69)*, which are products of the isoprenoid biosynthetic pathway. It takes place in the plastids via the non-mevalonate pathway leading to prenyl pyrophosphates. The specific biosynthetic pathway of carotenoids starts with the synthesis of geranylgeranyl diphosphate, which leads to the formation of lycopene through several

reaction steps *(66)*. Afterwards, other carotenoids are derived from the acyclic lycopene by, for instance, hydrogenation, cyclisation, oxygen addition, and chain elongation *(67)*. The xanthophylls are oxidation products of α- and β-carotene *(66)* (Figure 3).

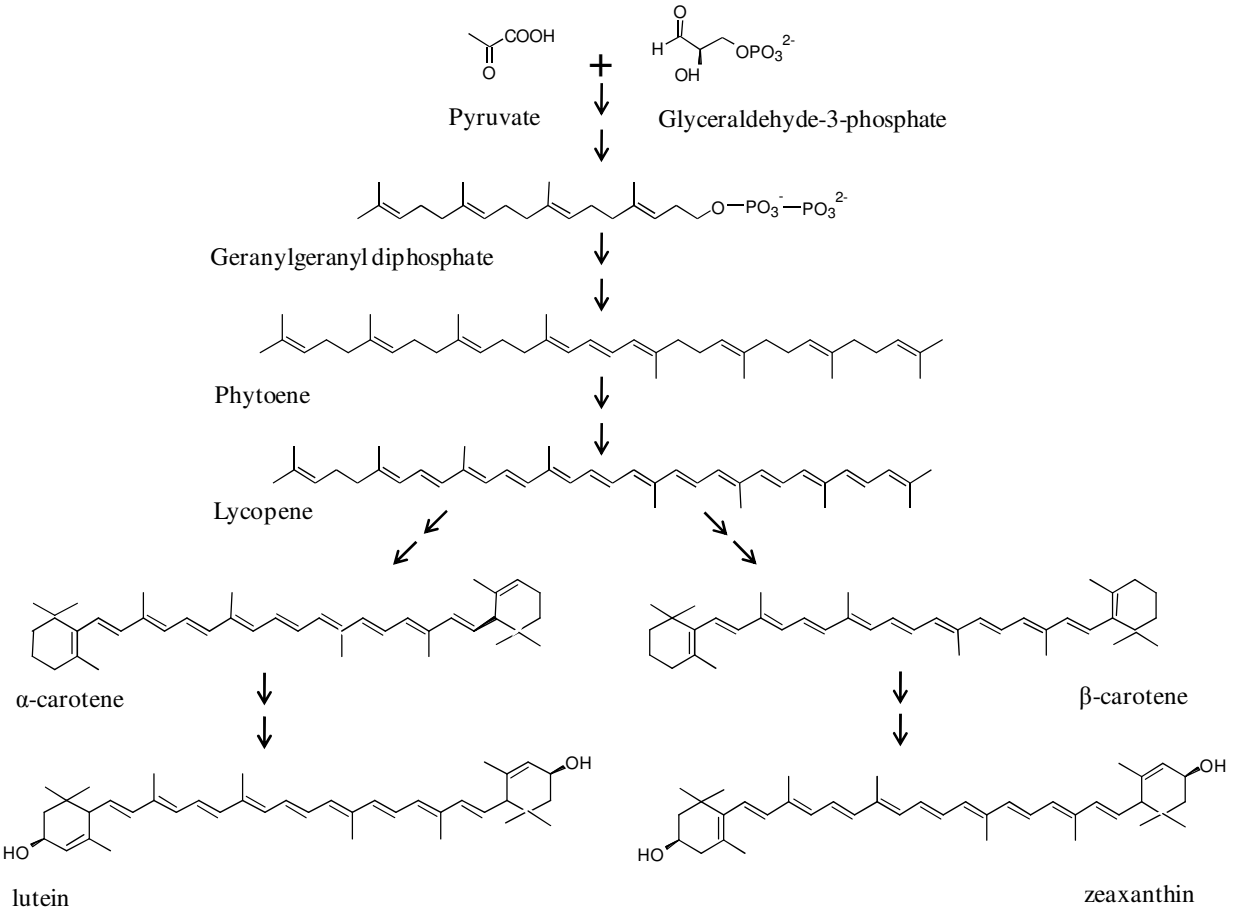


Figure 3. Main pathways in the biosynthesis of carotenoids in plants (modified from van den Berg *et al. (66)*).

The two major functions of carotenoids in photosynthetic organisms are to act as accessory pigments for light harvesting and participate in the prevention of photo-oxidative damage. Carotenoids are important in many plants due to their ability to protect cells from oxidative damage by quenching oxygen radical species and their function as antioxidants in plants shows interesting parallels with their potential role as antioxidants in foods and humans. Carotenoids also provide distinct pigmentation to flowers and fruits in order to attract animals for pollination and dispersal of the seeds *(66)*.

3.1.3 Dietary sources and intake

In developed countries, 80–90 % of the intake of carotenoids comes from fruits and vegetables *(25)*. Carotenoids are especially abundant in yellow-orange fruits and vegetables as well as dark green leafy vegetables *(25)*. The main dietary sources of carotenoids are green vegetables, carrots, tomatoes, and tomato products *(66, 70)*. Carrots and tomatoes were responsible for 47 % and 32 %, respectively, of the daily intake of carotenoids in Denmark, while other vegetables, fruit, dairy products, cereals, and fat and eggs contributed with 9 %, 1 %, 2 %, 5 %, and 4 % *(71)*.

Yellow and yellow-red vegetables and fruits, *e.g.* orange and corn, are some of dietary sources of β-cryptoxanthin *(72)* and zeaxanthin has also been found in corn *(73)*. However, the carotenoids of significance for the intake in Denmark are β-carotene (38 %), lycopene (33 %), α-carotene (17 %), and lutein (13 %) *(71)*. Carrots are the main dietary source of α-carotene and β-carotene together with spinach, green cabbage, and parsley, which also contain high amounts of β-carotene. In contrast, tomato and tomato products are the major sources of lycopene *(71, 74)*. The main carotenoids found in carrots are α-carotene (5.3–50 μg/g fresh weight (fw)), β-carotene (44–108 μg/g fw), and lutein (0–5.1 μg/g fw), while lycopene has not been detected in carrots (Table 2) *(25, 71, 72, 75-79)*. The other crops of relevance for this study only contribute with a few percentages to the total intake of carotenoids in the human intervention diets (Table 2).

The content and distribution of carotenoids in crops is affected by factors such as cultivar *(80, 81)*, climate *(41, 82)*, physiological age *(83)*, location *(81, 84, 84)*, and growth year *(81, 85)*. The growing conditions can also be an important factor *(25, 66)*, for instance, the fertilization level of nitrogen, which is one of the main nutrients required for plant growth *(86)*.

In diets, the content and distribution of carotenoids can be affected by processing of the food products due to changes in the structural integrity of the matrix *(66)*. The storage and thermal food processing (*e.g.* cooking, frying, and blanching) can both have a positive and negative impact on the content of carotenoids in food products *(25, 87)*. Oxidation processes and isomerisation can occur depending on the temperature, light, and amount of available oxygen *(88)*.

The average intake of carotenoids (sum of α-carotene, β-carotene, β-cryptoxanthin, lutein, and lycopene) in five European countries (France, Ireland, the Netherlands, Spain, and the UK) has been estimated to 14 mg/day *(74)*, while the average intake in Denmark has been estimated to 4.8 mg/day (sum of α-carotene, β-carotene, lutein, and lycopene) *(71)*. The intakes vary depending on *e.g.* the individual, region, country *(74)*, and season *(25)*. No recommendations on the daily intake of

carotenoids exist, except a recommended daily intake of provitamin A of 700-900 retinol equivalents per day in adults corresponding to 8.4–11 mg dietary β-carotene *(89)*.

Table 2. Content of the main dietary carotenoids (µg/g fresh weight) in the eatable parts of the crops used in the human intervention study.

	Carotenoids (µg/g fw)				
Crop	**α-carotene**	**β-carotene**	**Lutein**	**Lycopene**	**References**
Barley (flour)	< 0.01	< 0.01	n.d.	n.a.	*(75)*
Carrot	5.3─50	44─108	0─5.1	n.d.	*(25, 71, 72, 75-79)*
Faba bean	< 0.01	2.0	n.d.	n.d.	*(75)*
Oat	n.a.	< 0.01	1.8 [a]	n.a.	*(90)*
Onion	n.d.	0.01─0.069	0.02─0.16	n.d.	*(72, 76, 78)*
Potato	n.d.	0.01─0.05	0.12─1.0 [a]	n.d.	*(72, 76, 78)*
Rapeseed (oil)	n.a.	n.d.	n.a.	n.a.	*(91)*
Wheat (flour)	n.d.	0─0.043	0.76─2.2	n.d.	*(25, 71, 90)*
White cabbage	tr	0.21─0.66	0.80─1.5 [a]	n.d.	*(72, 76)*

n.d.: not detected, n.a.: no information available, tr: traces found.

[a] : including zeaxanthin

3.1.4 Bioavailability and biological functions in humans

Carotenoids can be absorbed by the small intestinal epithelium (enterocytes), when they have been incorporated into mixed micelles in the intestinal lumen. The uptake of carotenoids by the enterocytes occur by simple diffusion in similarity to many other dietary lipids *(92)*, but recently protein-mediated transport of carotenoids in enterocytes has also been suggested *(25)*. The critical steps in the absorption and transport of carotenoids are the release from the food matrix and the dissolution in the lipid phase *(93)*, and low amounts of dietary fat are required to ensure absorption of carotenoids (approximately 3-5 g/meal *(94)*). The absorption of β-carotene from cooked and raw carrots was 65 % and 41 %, respectively, in eight human ileostomy volunteers *(95)*

The bioavailabilty of carotenoids is the fraction of the compounds, which can be absorbed and is available for use or storage *(25)*. It is affected by factors such as the type of carotenoid, the food

matrix and processing, interactions with other carotenoids, fat and fibers, and the nutritional and physiological status of the consumer *(93, 94)*. The bioavailability of β-carotene is lower from vegetables than purified β-carotene (19−34 % from carrots), but processing of the food such as homogenisation or heat treatment can increase the bioavailability of carotenoids from vegetables. The relative bioavailability is normally measured as the increase in the content of carotenoids in plasma *(96)*. The percentage of carotenoids found in the plasma is 10 %, while the rest is found in the tissues *(67)*. Numerous carotenoids are present in human plasma, but the majority of carotenoids found are α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene, and zeaxanthin *(25, 75)*.

The most widely studied and understood biological functions of carotenoids in humans are their provitamin A activity *(25)*. Provitamin A carotenoids have an unsubsitituted β-ionene ring (*e.g.* α-carotene, β-carotene and β-cryptoxanthin) and can be cleaved into two molecules of retinol (vitamin A) *(67)*. Thus, they function as an important source of vitamin A *(97)*, which is an essential nutrient normally provided as preformed retinol (mainly as retinol esters) and provitamin A carotenoids *(98)*. Furthermore, their expected role as antioxidants is important in the protection of cells and tissues from damaging effects of free radicals and singlet oxygen *(25)*, which may otherwise lead to increased risk of several chronic diseases such as cancer, cardiovascular disease, and antherosclerosis *(85)*. Carotenoids are believed to contribute to the beneficial effects of increased vegetable consumption *(94)*. Epidemiological studies strongly suggest that consumption of carotenoid-rich foods reduce the incidence of several diseases such as cancer, cardiovascular disease, cataracts, and diseases related to low immune function *(67)*. An inverse association between consumption of carotenoids and breast cancer *(99)* as well as type 2 diabetes *(100)* has been indicated. However, no potential benefits from supplementation of β-carotene has been shown *(66)* and the protective effect on certain types of cancer can even be significantly attenuated or reversed in smokers *(101-103)*.

3.2 Flavonoids

3.2.1 Chemical properties

Polyphenols (also called polyphenolic compounds) are characterised by having at least one aromatic ring with one or more hydroxyl groups *(104, 105)*. Flavonoids are a group of naturally occurring polyphenols, which consists of more than 4000 different compounds *(106, 107)* with a C_6-C_3-C_6

skeleton *i.e.* two aromatic rings (A and B) connected by a three carbon bridge (Figure 4) *(104)*. The hydroxyl groups are normally present at the 4', 5, and 7-positions *(104)* and the majority of flavonoids exist naturally as glycosides *(104, 105)*. The major groups of flavonoids are flavonols, flavones, flavanones, flavanols, anthocyanidins, and proanthocyanidins *(106)*. The thesis will focus on flavonols, which have a wide distribution in the plant kingdom, except in algae and fungi *(104)*.

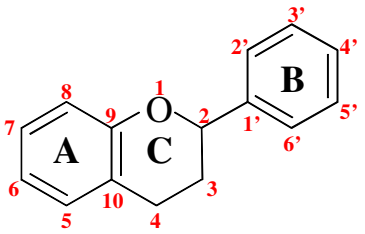


Figure 4. The flavonoid skeleton.

The solubility of flavonoids depends on the individual chemical structures, and their log P values range from 0.5 to 1.5 *(108)*. The water solubility is increased by the presence of sugar or hydroxyl substituents, while other substituents *e.g.* methyl groups can decrease the water solubility *(104)*. Many flavonoids are able to chelate metal ions, act as weak acids and are good donors in the formation of hydrogen bonds *(108)*. Generally, polyphenols have a white to yellow color *(109)* and at least one or more characteristic maxima in the UV-region 230-290 nm *(105)*. Flavonoids are susceptible to degradation, which is facilitated by the presence of oxygen, light as well as high temperatures *(110, 111)*.

3.2.2 Biosynthesis and functions in plants

The biosynthesis of flavonoids is a product of two separate biosynthetic pathways. The three carbon bridge and B-ring are synthesised from *p*-coumaroyl-CoA, which is produced from phenylalanine via the shikimic acid pathway as part of the biosynthesis of phenolic acids (Figure 8). The A-ring is a product of the condensation of three acetate units via the malonic acid pathway. The two pathways are combined yielding naringenin-chalcone, which is converted to naringenin. The different groups of flavonoids are products of naringenin, which is a central intermediate in the biosynthetic pathway of flavonoids *(16)*. The flavonols are synthesised from dihydroflavonols by introduction of a double bond between C2 and C3 (Figure 5) *(112)*. The biosynthesis of flavonoids in plants is highly sensitive and the production of flavonoids can be increased if the plant is exposed to stressed conditions *(108)*.

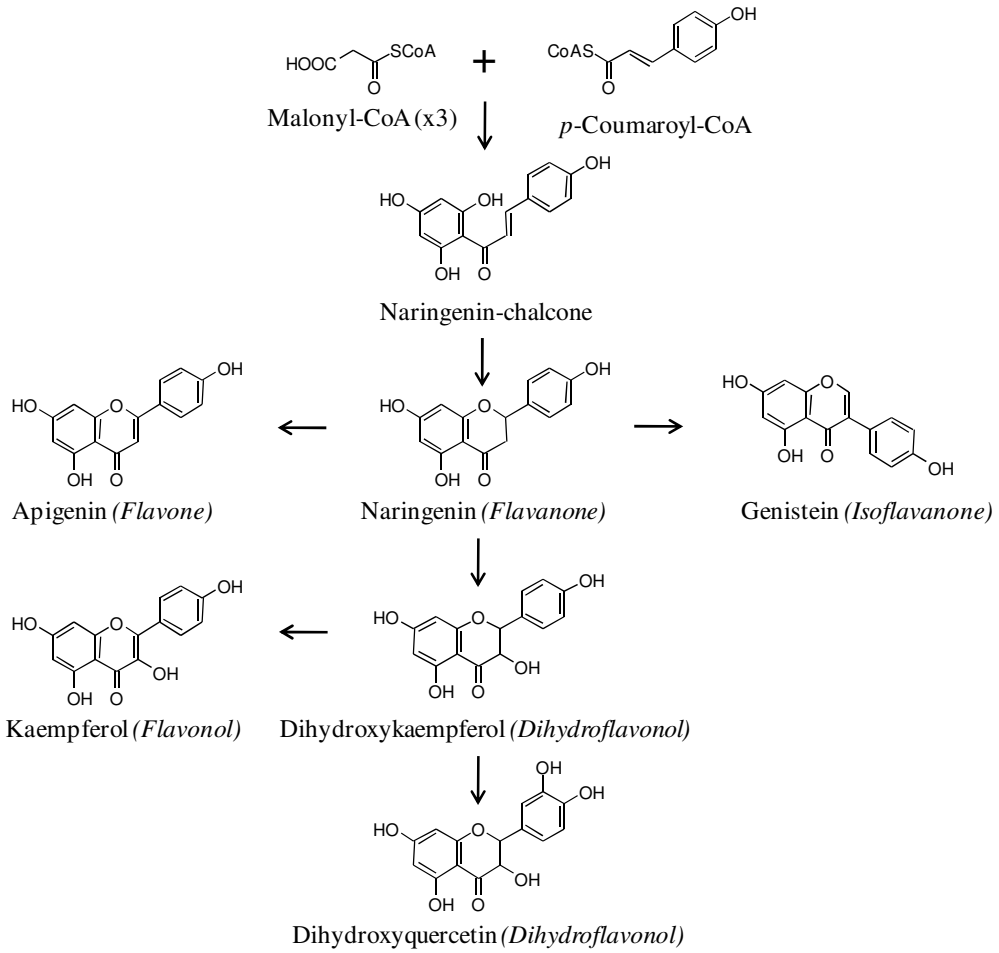


Figure 5. Main pathways in the biosynthesis of flavonoids in plants (modified from Crozier *et al. (16)*).

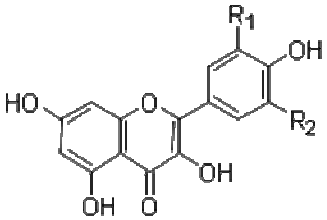
Flavonoids play an important role in the growth, development, and defence of plants *(15)*. One of their most important roles is as defence compounds against pathogen infection, attack from pests and herbivores. Flavonoids absorb UV light before photochemical damage in the plants occurs, especially flavones and flavonols are expected to be good UV protectants *(108, 113)*. They also act as attractants and signalling compounds in order to make flowers attractive to pollinating animals and to transmit signals to other parts of the plants. Flavonoids are good antioxidants and hydroxylation increase their antioxidant capacity *(108)*. Furthermore, they can act as plant growth hormones and regulators, and also enzyme inhibitors *(15)*.

3.2.3 Dietary sources and intake

Flavonoids are important constituents in the human diet *(114)* and the main dietary sources are beverages, fruits and vegetables *(26, 104)*. The most abundant group of flavonoids in food products is flavonols, which are found in, for instance, onion, curly kale, leeks, broccoli, and blueberries

(26). Onions are consumed in large amounts world-wide *(15)* and they are a major dietary source of flavonoids in *e.g.* Japan, Mexico, the Netherlands, and the US, where they constitute 17–46 % *(115-118)* of the total intake of flavonoids. Onions are also considered one of the main dietary sources of flavonols in the northern parts of Europe *(104)*, while tea and apples are some of the other dietary sources of flavonoids *(116, 117, 119)*.

The most abundant dietary flavonols are quercetin, kaempferol, isorhamnetin, and myricetin (Figure 6) *(104, 120)*. Quercetin is the dominating aglycone *(15, 120-122)* and constitute 56–76 % *(116-118)* in Japan, the Netherlands and US. Flavonoids are normally found as *O*-glycosides *(26, 104)* and the sugar moiety is often glucose, but also other sugars such as rhamnose and galactose can be the glycosidic constituent *(26, 120, 122)*. However, several papers analyse the content of aglycones after acid hydrolysis and the levels of the main dietary flavonols in the crops of interest for the human intervention study are shown in Table 3. The most abundant flavonols in onions are quercetin and kaempferol. Onions have the highest content of flavonols, while the contribution to the total intake of flavonols in our human intervention study is considerably lower from the other crops (Table 3). In general, the literature on flavonoids in cereals and rape seed is sparse. Few studies have previously investigated the content of flavonoids in oat *(123)* and barley mainly contain large amounts of flavanols and proanthocyanidins *(124, 125)*. Rape seed and wheat mainly contain phenolic acids *(126, 127)*, but flavones have also been detected in wheat *(126)*.



Compound	R_1	R_2
Quercetin	OH	H
Kaempferol	H	H
Isorhamnetin	OCH_3	H
Myricetin	OH	OH

Figure 6. Chemical structures of the main dietary flavonols.

Table 3. Content of main dietary flavonols (µg/g fresh weight) in the eatable parts of crops used in the human intervention study.

	Flavonols (µg/g fw)				
Crop	**Quercetin**	**Kaempferol**	**Isorhamnetin**	**Myricetin**	**References**
Barley	n.a.	n.a.	n.a.	n.a.	
Carrot	10–15	6.0	n.a.	4.0–10	*(121, 128, 129)*
Faba bean	200	<20	n.a.	260	*(129)*
Oat	n.a.	n.a.	n.a.	n.a.	
Onion	7.0–590	0.60–560	2.7–6.5	0.2–41	*(15, 44, 120, 121, 129-136)*
Potato	0.5	0.50	n.a.	0.01	*(135, 137)*
Rapeseed	n.a.	n.a.	n.a.	n.a.	
Wheat	n.a.	n.a.	n.a.	n.a.	
White cabbage	0.04–51	< 20	n.a.	n.d.	*(120, 121, 129, 135)*

n.d.: not detected, n.a.: no information available.

The content and distribution of flavonoids in crops is affected by, for instance, the cultivar *(15, 131, 133, 134, 138)*, physiological age *(108, 130)*, season *(129)*, growth year *(44, 131, 132)*, and geographical location *(130)*. Furthermore, the growing conditions (*e.g.* water *(129)* and nutrient availability *(139)*) can affect the content of flavonoids in plants. The distribution of flavonoids can also vary within the crops since a higher content has been found in the outer than the inner layers of onions *(135, 140)*.

In diets, the content and distribution of flavonoids is affected by storage and processing procedures, which can have a negative impact the content of flavonoids *(135, 141)*. The content of flavonoids can decrease during thermal cooking processes such as boiling, roasting, and blanching *(135, 140, 142, 143)* and pre-processing procedures (peeling, chopping etc.) *(144)*.

The dietary intake of flavonoids varies greatly between countries *(120)*. The average intake of flavonoids has been estimated to 17 and 23 mg/day in Japan *(118)* and Finland *(145)*, respectively, while the average intake of flavonols and flavones was 20–37 mg/day in Denmark, Mexico, the Netherlands, and the US *(115-117, 146)*. No recommendations on the daily intake of flavonoids have been found.

3.2.4 Bioavailability and biological functions in humans

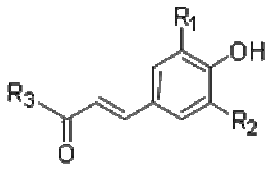
The intestine and liver are the most important organs involved in the metabolism of flavonoids, but also other organs contribute *e.g.* the kidney *(147)*. Glycosylated flavonoids must be hydrolysed by intestinal enzymes or the colonic microflora before absorption can occur. Flavonoids are extensively metabolised and conjugated (mainly methylation, sulfation, glucoronidation) in the small intestine and later in the liver *(26, 147)*, while breakdown to phenolic acid and non-phenolic metabolites occur in the large intestine *(26, 104)*. In general, the absorption of flavonoids is low with concentration levels below 10 µmol/L in the blood *(104)*. The sugar moiety is the most important factor determining the site and extent of absorption, while the position of the sugar affects the mechanisms involved in the intestinal uptake *(147)*. Flavonoids are mainly excreted in the bile or urine *(26)*, but only a small fraction is excreted in the urine with an intact flavonoid backbone due to the extensive metabolisation *(120, 147)*. The bioavailability of polyphenols varies greatly between the individual compounds. The most abundant dietary compounds might not necessarily have the best bioavailability due to poor absorption in the intestine, high metabolisation, or rapid elimination etc. *(26)*.

An inverse relationship between the intake of flavonoids and certain types of cancer (*e.g.* lung *(145, 148)* and prostate cancer *(145)*), dementia *(149)*, cardiovascular diseases *(118)*, type 2 diabetes, and asthma *(145)* has been shown in humans. Antioxidant activity, inhibition of enzymes by interactions with proteins, and interactions with specific receptor molecules in human cells are some of the suggested functions of flavonoids in humans *(108, 113)*. Flavonoids might contribute to the protective effects of a high fruit and vegetable consumption against cardiovascular diseases and cancer *(104, 150)* due to their antioxidant properties causing a lower concentration of free radicals, which decreases the probability of an initiation event *(108)*. The beneficial antioxidative effects of flavonoids have been shown in epidemiological and *in vitro* studies *(106)*, but similar effects are still undocumented *in vivo (113, 151)*. Polyphenols appear to have multiple functions and the antioxidant properties are probably only part of the human health effects, which could also be related to their ability to bind proteins, *e.g.* binding to cellular receptors and transporters, cell signalling and cell adhesion *(152)*. It seems reasonable considering the low plasma levels of polyphenols and the fact that lower plasma concentrations are required for polyphenols to affect the cell signalling pathways in comparison with antioxidative effects *(104)*.

3.3 Phenolic acids

3.3.1 Chemical properties

The most abundant phenolic acids in food products are hydroxybenzoic acids (*e.g.* gallic acids and tannins) and hydroxycinnamic acids, which belong to the group of polyphenols *(153)*. This PhD project will focus on hydroxycinnamic acids (also known as hydroxycinnamates). They are one of the most widely distributed group of plant products synthesised from phenylalanine *(154)* and contribute considerably to the daily intake of phenolic acids *(155)*. Hydroxycinnamic acids are *trans*-phenyl-2-propenoic acids with different ring substitutions *(156)* and caffeic (CA), *p*-coumaric, ferulic, and sinapic acids are the most abundant in the diet *(104, 156)* (Figure 7). Hydroxycinnamic acids are rarely found in the free form in food products, but usually ester-linked to sugars, organic acids, or lipids. Dimerisation also occurs, particularly in cereals *(157)*. Quinic acid conjugates of caffeic acid are the most common conjugates in fruits and vegetables *(16)*, and the most widespread is 5-*O*-caffeoylquinic acid (5-CQA), which is often referred to as chlorogenic acid (Figure 7) *(158)*.



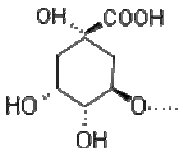
Compound	R_1	R_2	R_3
caffeic acid	OH	H	OH
p-coumaric acid	H	H	OH
ferulic acid	OCH_3	H	OH
sinapic acid	OCH_3	OCH_3	OH
5-*O*-caffeoylquinic acid	OH	H	

Figure 7. Chemical structures of the main dietary hydroxycinnamic acids.

Hydroxycinnamic acids can undergo *cis-trans* isomerism, but the natural forms are mainly *trans* isomers *(159)*. The solubility of phenolic acids is similar to flavonoids (log P: 0.5−1.5) *(108)*. As other polyphenols, they are able to chelate metal ions, act as weak acids, are good donors in the formation of hydrogen bonds *(108)*, and have at least one or more characteristic maxima in the UV-

region 230-290 nm *(105)*. Phenolic acids are susceptible to oxidation *(160)*, heat *(161)*, and light exposure *(162)*.

3.3.2 Biosynthesis and functions in plants

Phenolic acids are formed in the chloroplasts via the shikimic acid and phenylpropanoid pathway *(104, 108)*. The biosynthesis starts with the formation of 3-dehydroshikimic acid from carbohydrates and further conversion to L-phenylalanine, whereby the biosynthesis enters the phenylpropanoid pathway. L-phenylalanine is converted to cinnamic acid and further to *p*-coumaric acid, which can be metabolised to CA and ferulic acid via hydroxylation and methylation. 5-CQA and other caffeoylquinic acids are synthesised from *p*-coumaroyl-CoA (Figure 8), which is also an intermediate in the flavonoid biosynthesis (Figure 6) *(16)*.

Numerous compounds from the phenylpropanoid pathway are phytoalexins, *i.e.* compounds synthesised in response to, for instance, pathogen infection and pest attack of the plants *(30)*, but their protection can be selective and only inhibitory against certain pathogens and pests *(163)*. Hydroxycinnamic acids are also synthesised by plants as defence compounds against other stresses such as UV light and tissue injury *(157, 163)* and wounding can induce higher levels of phenolic acids in potatoes *(164)*. Phenolic acids also have antioxidant properties *(165)*, and inhibitory effects on fungi *(166)* and bacteria *(167)*. Synergistic effects between structurally related phenolic compounds in resistance against bacteria is possible *(163)*.

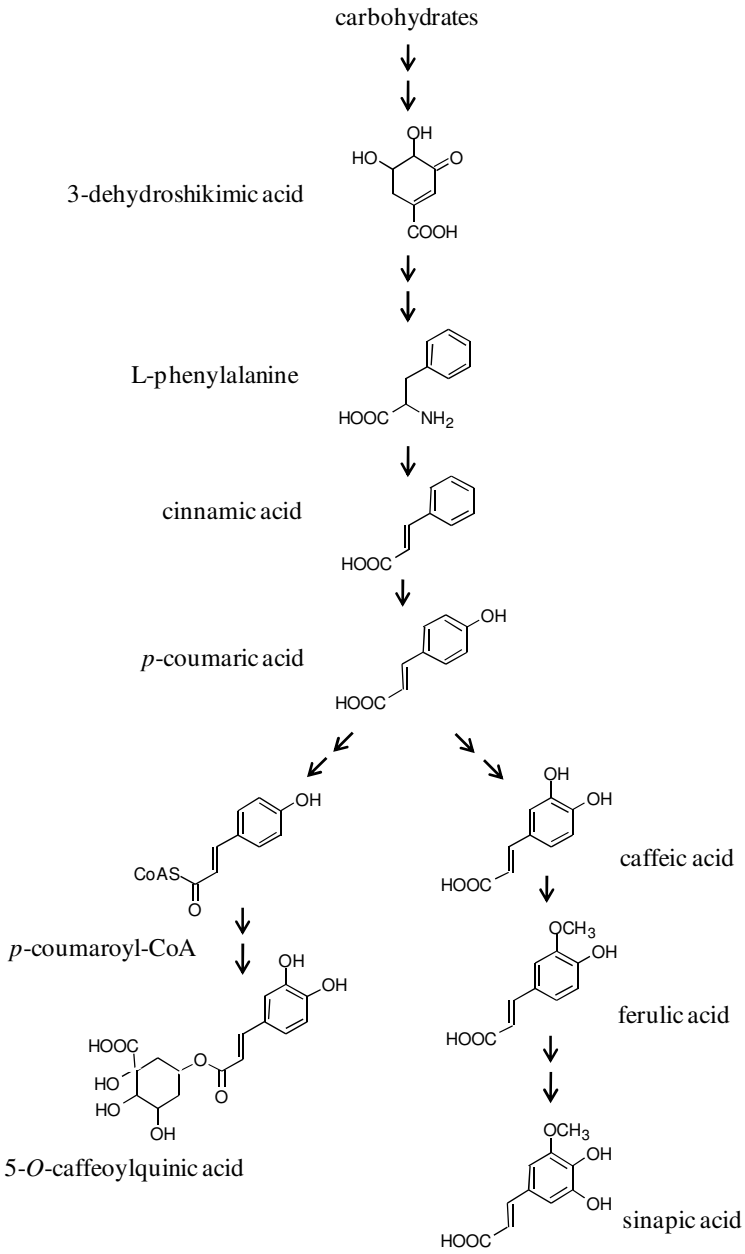


Figure 8. Main pathways in the biosynthesis of phenolic acids in plants (modified from Crozier *et al. (16)*).

3.3.3 Dietary sources and intake

Hydroxycinnamates are widely distributed in the plant kingdom and the major dietary sources are fruits, vegetables, beverages, and cereals *(27, 168)*. Coffee is one of the major dietary sources due to the high content of chlorogenic acids *(158)*. It accounted for 92 % of the total intake of caffeic acids in a German study *(155)* and 63 % of the total intake of polyphenols in a Finnish study, where also cereals contributed considerably (12 %) *(169)*. Potatoes and carrots are considered some of the

main vegetable sources of phenolic acids due a high content of, especially chlorogenic acids *(170, 171)*.

CA is the most abundant hydroxycinnamic acid in the diet *(104, 156)* and accounted for 65 % and 93 % of the total dietary intake of phenolic acids in Finland *(169)* and Germany *(155)*, respectively. The contributions from ferulic, *p*-coumaric, and sinapic acids were 19 %, 2.5 %, and 1.7 %, respectively, of the total intake of hydroxycinnamic acids in Finland *(169)*. The content of the main dietary hydroxycinnamic acids (aglycones, only free and soluble forms) in crops of interest for our study are shown in Table 4. Potatoes and carrots are the main dietary sources of 5-CQA in the human intervention study, while rape seeds contain a large amount of sinapic acid. In general, cereals are considered rich sources of ferulic acid *(165, 172)*, but the majority of polyphenols in grains are in the bound form *(172, 173)*.

Table 4. Content of the main dietary hydroxycinnamates (µg/g fresh weight, only soluble forms) in the eatable parts of crops used in the human intervention study.

	Hydroxycinnamates (µg/g fw)					
Crop	**caffeic acid**	***p*-coumaric acid**	**ferulic acid**	**sinapic acid**	**5-CQA**	**References**
Barley	n.a.	n.a.	1.3─6.9	n.a.	0.99─13	*(174)*
Carrot	19	6.8	n.d.	n.d.	9.6─100	*(80, 87, 170, 171, 175, 176)*
Faba bean	n.a.	n.a.	n.a.	n.a.	n.a.	
Oat	17	45	150	n.a.	n.d.	*(177)*
Onion	0.01	0.04	0.02	0.3	n.d.	*(143, 170)*
Potato	1.7─19	0─30	0─94	0─7.2	8.0─260	*(48, 137, 161, 164, 170, 175, 178-181)*
Rapeseed	n.a.	5.0─30	12─47	34─470	n.a.	*(127)*
Wheat	n.d.	16	52	n.a.	n.a.	*(173)*
White cabbage	2.9─8.0	2.1	4.0	n.d.	n.d.	*(170, 173)*

5-CQA: 5-*O*-caffeoylquinic acid, n.d.: not detected, n.a.: no information available, tr: traces found.

The content of phenolic acids is different in various parts of the plants and the outer layers contain the highest concentrations *(161, 180, 182)*. In the same way as other secondary metabolites, cultivar

(80, 161, 176), climate, growing year *(48, 54)*, and geographical location *(48)* has been shown to affect the content and composition of phenolic acids in crops.

Storage, preparation and processing of food products can affect the content and composition of phenolic acids *e.g.* the content of phenolic acids in potatoes and carrots was negatively affected by cooking processes such as baking, boiling, and frying *(161, 175, 183)* because food processing can lead to partial conversion of *trans* to *cis* isomers *(158)*, hydrolysis of chlorogenic acids into caffeic and quinic acids *(87)*, and loss of phenolic acids into the water during boiling *(175, 183)*. Hajslova *et al*. *(48)* also showed that the content of 5-CQA in carrots was decreased by 30 % after five month storage (darkness, constant humidity, 6–8^{o}C).

The dietary intake of hydroxycinnamic acids can vary from 0 to more than 1 g per day *(104, 156)*, especially depending on the consumption of coffee. A British study showed that a person drinking several cups of coffee a day and consuming cereals and citrus fruits could have an intake of 500–800 mg/day, while other persons might only ingest 25 mg/day if they do not drink coffee and only eat small amounts cereals, fruits, and vegetables *(158)*. No recommendations on the daily intake of phenolic acids have been found.

3.3.4 Bioavailability and biological functions in humans

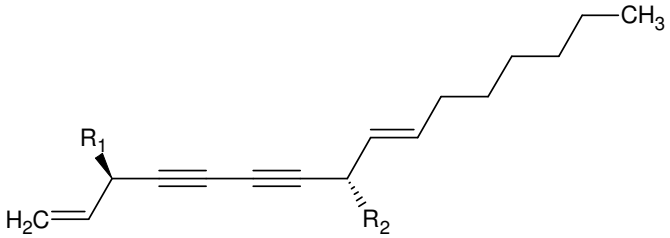
Hydroxycinnamic acids are rapidly absorbed, when they are ingested in the free forms *(16)*, and the absorption can occur along the entire gastrointestinal tract *(165)*. Phenolic acids are extensively metabolised in the body *(184)*, mainly in the liver *(165, 185)* and/or gut *(185)*, and the majority are conjugated with glucuronic acid or sulfates *(184)* in the same way as flavonoids. Most phenolic acids are excreted in the urine in the conjugated form and the excretion of unchanged phenolic acids in the urine is low *(168, 186)*, for instance, 11 % and 1.7 % of CA *(187)* and 5-CQA *(184)*, respectively, in humans, and 0.4─5.4 % and 1.2─2.3 % of ferulic acid *(185, 188)* and *p*-coumaric acid *(188)*, respectively, in rats. The distribution and range of metabolites varies between individuals and will depend on, for instance, the dose and route of administration *(189)*, but the degree of absorption varies between the specific compounds *(187, 188)*. The bioavailability of ferulic and *p*-coumaric acids was relatively low in a previous rat study *(188)* and the bioavailability of phenolic acids generally depends on *e.g.* the food matrix *(188)* and the metabolism by the gut microflora as shown for 5-CQA in a previous rat study *(190)*.

Inhibitory effects of phenolic acids on breast *(191)*, stomach, and colon cancer *(192)* have been demonstrated *in vitro* and a preventive effect of phenolic acids on the development of alzheimers disease *(193)* has also been observed *in vivo*. Phenolic acids have been shown to prevent oxidation of low-density lipoproteins *(194)*, which is thought to be the initial step in developing heart disease and stroke. Hydroxycinnamic acids are effective antioxidants, but the antioxidant activity is decreased with esterification and dimerisation. The nature of the linkage between hydroxycinnamates can also affect the antioxidant activity *(157)*. In general, the antioxidant capacity of phenolic acids is lower than flavonoids, but it is compensated by the relatively higher dietary intake *(157)*. Furthermore, their health effects might also be related to their ability to bind proteins as expected proposed for polyphenols in general *(152)*.

3.4 Polyacetylenes

3.4.1 Chemical properties

More than 2000 polyacetylenes are known *(195)* and the most common group of polyacetylenes isolated from food plants is aliphatic polyacetylenes *(196)*. Aliphatic C_{17}-polyacetylenes of the falcarinol-type are widely distributed in *Apiaceae* and *Araliaceae* species such as carrot, celery, parsnip, and parsley *(28, 195)*. Polyacetylenes have a carbon-carbon triple bond or alkynyl functional group *(195)*. The C_{17}-polyacetylenes contain a terminal 3-hydroxy(or 3-oxo)-hept-1-ene-4,6'-diyne moiety and the other terminal consist of a saturated aliphatic C_7H_{15}-moiety *(197)* (Figure 9). Aliphatic C_{17}-polyacetylenes will just be referred to as polyacetylenes in the following parts of the dissertation unless otherwise stated.



Compound	R_1	R_2
Falcarinol	OH	H
Falcarindiol	OH	OH
Falcarindiol-3-acetate	$OCOCH_3$	OH

Figure 9. Chemical structures of the main polyacetylenes in carrots.

Polyacetylenes have characteristic UV spectra with maxima in the region from 230 to 260 *(198, 199)*. They are unstable compounds and sensitive towards oxidative and/or enzymatic degradation, especially falcarindiol (FaDOH) *(200)*. The compounds are also thermally unstable and may undergo photodecomposition if exposed to daylight *(28, 201)*.

3.4.2 Biosynthesis and functions in plants

The polyacetylenes are synthesised from unsaturated fatty acids and built from acetate and malonate units in plants *(196)*. The departure from the primary metabolism are the three fatty acids crepenynic acid, stearolic acid, and tariric acid, which comprise the origin of most acetylenic compounds *(195)*. The polyacetylenes of the falcarinol-type follows the biosynthesis of other aliphatic C_{17}-polyacetylenes starting with dehydrogenation of oleic acid to the C_{18}-acetylenes crepenynic acid and dehydrocrepenynic acid. Afterwards, they are transformed to C_{17}-acetylenes by β-oxidation followed by oxidation and dehydrogenation, whereby aliphatic C_{17}-polyacetylenes of the falcarinol-type are produced. FaDOH is formed by oxidation of falcarinol (FaOH) and falcarindiol-3-acetate (FaDOAc) is synthesised from FaDOH (Figure 10). The biosynthesis of polyacetylenes occur in the endoplasmic reticulum of plants *(195)*.

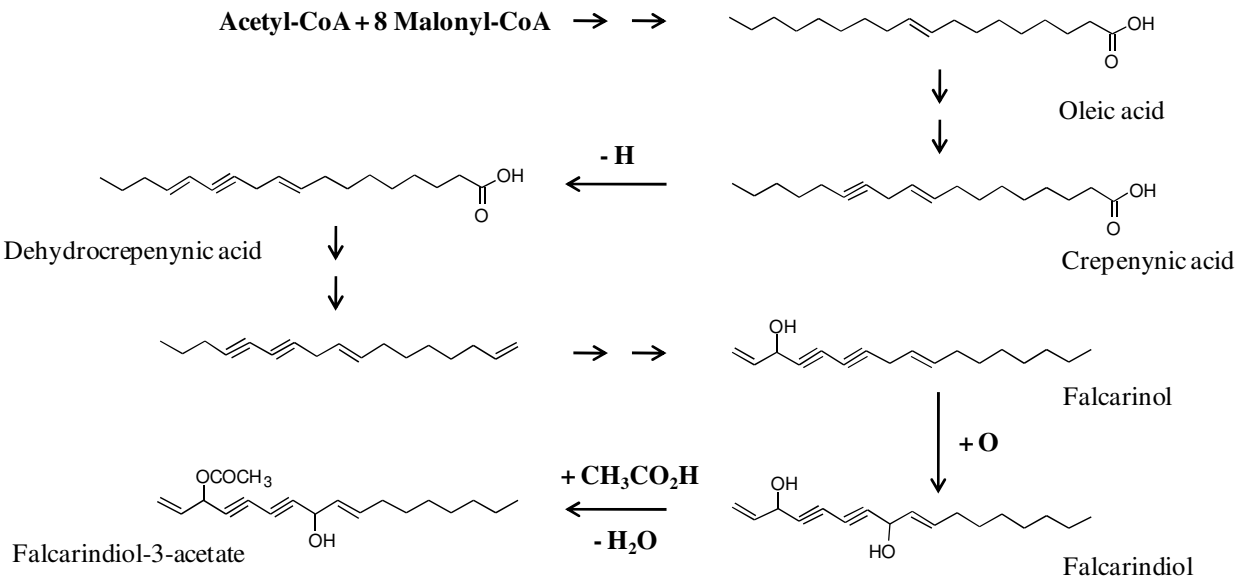


Figure 10. Main pathways in the biosynthesis of the main polyacetylenes in carrots (modified from Minto and Blacklock *(195)*, and Christensen and Brandt *(196)*).

Polyacetylenes play an important role as plant defence compounds *(202)* because they are highly toxic towards *e.g.* fungi and bacteria *(28, 203)*. Polyacetylenes also act as phytoalexins *(28)*, and FaOH and FaDOH seem to supplement each other in the defence against pathogens and pests *(197)*. FaDOH also contributes to the bitter taste of carrots *(171, 204)*, which is especially strong, when carrots have been exposed to abiotic stress during harvesting, transportation, storage, and processing *(204)*.

3.4.3 Dietary sources and intake

Carrots are the major dietary source of polyacetylenes, but they may also be supplied by other food sources *(28, 205)*. High concentrations of polyacetylenes have also been found in, for instance, ginseng *(206)*, parsnips and celery *(207)*, but carrots are expected to be the major dietary source due to the relatively large consumption *(207)*.

The concentration of polyacetylenes in plants differs considerably between various species *(208)* and the concentrations of the main polyacetylenes in peeled carrots are: FaDOH: 6.4─40 µg/g fresh weight *(171, 198, 203)*, FaDOAc: 5.1─26 µg/g fresh weight *(171, 198)* and FaOH: 2.8─67 µg/g fresh weight *(171, 198, 203)* (Table 5). The concentration of polyacetylenes in carrots depends on, for instance, the cultivar *(20, 171, 203, 209)*, root size, geographical location *(20)*, growth conditions (*e.g.* plant available water *(199)*), and storage conditions *(201)*. FaOH is uniformly distributed in the carrot root, whereas FaDOH and FaDOAc are primarily found in the peel *(171, 198, 203)*.

Table 5. Content of the main dietary polyacetylenes (µg/fresh weight) in carrots.

	Polyacetylenes (µg/g fw)			
Part of the carrot	**FaDOH**	**FaDOAc**	**FaOH**	**References**
Peel	32─360	66─145	1.3─100	*(198, 203, 209)*
Peeled	6.4─40	5.1─26	2.8─67	*(171, 198, 203)*
Unpeeled	16─84	7.0─110	4.4─60	*(20, 199, 204, 209-212)*

FaDOH: falcarindiol, FaDOAc: falcarindiol-3-acetate, FaOH: falcarinol.

The content and composition of polyacetylenes in the diets can be affected by the storage and processing conditions *(201)*, for instance, concentration of polyacetylenes in raw carrots decreased during long-term storage (1°C or frozen) *(20, 200, 201)* and peeling of carrot roots removed a major amount of FaDOH *(212)*. Boiling in water can also reduce the concentration of FaOH *(201)*, while blanching has caused both an increase *(20)* and decrease *(201)* in the content of FaOH as well as a decrease of FaDOH and FaDOAc *(201)*. The dietary intake of polyacetylenes is unknown and no recommendations on the intake have been found.

3.4.4 Bioavailability and biological functions in humans

The literature on absorption and bioavailability of polyacetylenes is scarce and to my knowledge, just one study on bioavailability has been made *(213)*. The study included 14 men, who were given 300, 600, or 900 mL of carrot juice containing 13.3 mg FaOH/L. FaOH was rapidly absorbed with a maximum concentration of 1–2.5 µg/L in plasma after 2–5 hours, which is in good agreement with the positive effects of polyacetylenes on cell cultures at 0.5–50 µg/L *in vitro (213)*.

High concentrations of polyacetylenes have previously been considered undesirable in food products due to their toxic effects such as allergic and irritant skin reactions *(214)*, and neurotoxicity *(215)*. However, they may be beneficial in low concentrations *(196)* and have been proposed to play an important role in the health-promoting properties of carrots *(20)*. Polyacetylenes also have anti-inflammatory effects *(216)* and cytoxocity against cancer cells *(211, 217, 218)*. The consumption of carrots reduced the risk of colorectal cancer in rats *(205)*, but the effects on cancer cells were concentration-dependent *in vitro (19, 201, 219)*.

4 Study design

The following paragraphs describe the study design, including the crop and human intervention study carried out as part of the OrgTrace project, where numerous chemical compounds were studied, but the description of the study design will focus on analysing the content of secondary metabolites in vegetables, intervention diets, and human samples as well as statistical analyses. The intention with these paragraphs is mainly to give an overview of the study design. Further details can be seen in the papers, manuscripts, and appendices mentioned within in each paragraph.

4.1 Crop study

4.1.1 Study design

The crops included in this study were: barley, carrot, faba bean, oat, onion, potato, rape seed, wheat, and white cabbage (Figure 11). They were grown in a field trial study undertaken in year one (2007) and year two (2008) at the locations shown in Figure 11. The carrots, onions, oat, and white cabbages were grown at one location in Denmark (Aarslev; 10°27'E, 55°18'N; sandy loam) in the VegQure rotation experiment (http://www.vegqure.elr.dk/uk/). The barley, faba beans, potatoes, rape seeds, and wheat were grown in the CropSys rotation experiment (http://www.cropsys.elr.dk/uk/) at the following three locations in Denmark: Flakkebjerg (11°23'E, 55°19'N; sandy loam), Foulum (09°34'E, 56°30'N; loamy sand) and Jyndevad (09°08'E, 54°54'N; sand). Further details on locations and soil profiles can be seen in Manuscript I and II.

The crops were grown in three different agricultural production systems at all locations: one conventional system (C) and two organic systems (OA and OB). The sequence of main crops was identical in the three growth systems with 8-year rotation in Aarslev and 4-year rotation at the other locations. In the conventional system, pesticides and inorganic fertilizer were used. The OA system relied on import of animal manure. In the OB system, nutrient supply was based on the use of cover crops (mainly legumes), but animal manure was also applied to onions and white cabbage as well as vinasse to barley, faba beans, potatoes, and wheat in order to facilitate a high nitrogen demand. In general, cover crops were grown in the autumn after the main crops and incorporated into the soil in the spring before the main crops were grown. The organic systems were managed in compliance with the Danish guidelines for organic farming administered by the Danish Plant Directorate

(http://pdir.fvm.dk). The levels of fertilization at the Aarslev and Foulum location are shown in Table 6, while the levels of fertilization of the potatoes from the Jyndevad and Flakkebjerg location can be seen in Manuscript II.

The carrots, onions, oat, and white cabbages from the Aarslev location were grown with three replicates of each growth system resulting in 9 plots per year (18 plots in total), while the other crops were grown with two replicates of each growth system resulting in 6 plots per year per location (36 plots in total). Each individual crop was harvested the same day for all growth systems *(Manuscript I, II and III)*.

Crop	Cultivar	Location
Barley	A mix of *Hordeum vulgare* cv. *simba*, *smilla* and *power*	Fl, Fo and Jy
Carrot	*Daucus carota* cv. *bolero*	Aar
Faba bean	*Vicia faba* cv. *columbo*	Fl, Fo and Jy
Oat	*Avena sativa* cv. *freddy*	Aar
Onion	*Allium cepa* cv. *hytech*	Aar
Potato	*Solanum tuberosum* cv. *sava*	Fl, Fo and Jy
Rape seed	*Brassica napus* cv. *elan*	Fl, Fo and Jy
Wheat	*Triticum* cv. *tommi*	Fl, Fo and Jy
White cabbage	*Brassica oleracea* cv. *impala*	Aar

Figure 11. Cultivated crops and locations in Denmark included in the crop study (map downloaded from http://maps.google.dk).

Table 6. Fertilization levels (N, P and K; kg/hectare) applied to the individual crops in the three growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) at the Aarslev (carrot, oat, onion, and white cabbage) and Foulum (barley, faba bean, potato, rape seed, and wheat) location. The amounts are an average of the growth years 1 and 2 *(Manuscript III)*.

		Growth system		
	Crop	**C** [a]	**OA** [b]	**OB** [c]
N	Barley	130	55	1
	Carrot	120	55	0
	Faba bean	0	1	1
	Oat	90	45	0
	Onion	170	110	70
	Potato	140	115	1
	Rape seed	185	95	0
	Wheat	165	105	1
	White cabbage	310	225	135
	Total	**1300**	**806**	**209**
P	Barley	20	10	0
	Carrot	20	5	0
	Faba bean	25	0	0
	Oat	15	5	0
	Onion	80	10	5
	Potato	30	25	0
	Rape seed	25	25	0
	Wheat	20	20	0
	White cabbage	45	15	10
	Total	**280**	**115**	**15**
K	Barley	95	65	45
	Carrot	60	20	0
	Faba bean	125	70	70
	Oat	45	15	0
	Onion	65	40	25
	Potato	175	145	90
	Rape seed	85	115	90
	Wheat	105	120	70
	White cabbage	145	85	50
	Total	**900**	**675**	**440**

[a] : applied as inorganic fertilizer.

[b] : applied as animal manure. K was supplemented as vinasse.

[c] : applied as vinasse, which also contained trace amounts of N. Animal manure was used for onions and cabbage.

4.1.2 Sample preparation

A representative sample was collected from each plot (5 kg of cereals and beans and 15 kg of the vegetables), but prior to that the potatoes, carrots, and onions were sorted with the following inclusion criteria both years (marketable quality): potatoes: 35-60 mm diameter, carrots: 50-250 g fresh weight and onions: 40-80 mm diameter. Only the eatable parts of the crops were analysed. They were washed in Milli-Q water, peeled, cut into 0.5 cm thick slices, and freeze-dried at a commercial freeze drying company (Danish Freeze Dry A/S, Kirke Hyllinge, Denmark). Afterwards, the samples were crushed, homogenised, and stored at −20 ^{o}C in an inert nitrogen atmosphere until chemical analyses *(Manuscript I, II and III).*

4.1.3 Chemical analyses

The chemical analysis of carotenoids was performed by extraction with tetrahydrofuran (THF) and ethanol (EtOH) (50:50, v/v) on an ultrasonication bath. The quantification was performed by HPLC-UV on a RP-C30 carotenoid column (4.6×250 mm, 5 µm; YMC, Wilmington, NC, US). The eluents were 96 % aqueous methanol (MeOH) and methyl *tertiary*-butyl ether (MTBE) mixed in the ratios 80:20 (v/v, A-eluent) and 30:70 (v/v, B-eluent). The concentrations of the carotenoids were calculated using an external standard curve of α-carotene and the specific molar extinction coefficients *(220)*. Further details on the chemical analysis of carotenoids can be seen in Manuscript III.

A new method for chemical analysis of flavonoids and phenolic acids was developed and validated as described in Paper I. The method involved pressurised liquid extraction with 65 % aqueous MeOH and addition of C18-material (Sepra E-C18, 50 µm; Phenomenex, Allerød, Denmark) to the extraction cells. The chemical compounds were quantified by HPLC-UV on a Prodigy RP-C18 column (4.6×250 mm, 5 µm, 30^{o}C; Phenomenex, Allerød, Denmark) with 0.1 % formic acid in Milli-Q water (v/v) and MeOH (90:10, v/v, A-eluent) and 100 % MeOH (B-eluent). The flavonoids and phenolic acids were quantified relative to quercetin, which is common practice in analysis of bioactive compounds *(221)*. The flavonoids and phenolic acids were identified by mass spectrometry (MS and MS/MS) and a comparison of retention times, UV and MS data with commercially available standards *(Paper I)*.

The chemical analysis of polyacetylenes involved extraction with ethyl acetate (EtOAC) using an Ultrasonic Liquid Processor based methodology. The polyacetylenes were quantified on HPLC-UV with a Prodigy RP-C18 column (4.6×250 mm, 5 µm; Phenomenex, Allerød, Denmark), and Milli-Q water and acetonitrile (ACN) as A- and B-eluent, respectively. The polyacetylenes were identified by UV and mass spectrometry (MS and MS/MS) and quantified relative to FaDOH, which was the only commercially available standard. The newly developed method was validated method and further details can be seen in Manuscript I.

4.2 Human intervention study

4.2.1 Study design

A double-blinded, cross-over, human intervention study was performed at The Department of Human Nutrition, University of Copenhagen (Denmark) for two consecutive years (January-April 2008 and 2009) with diets prepared from the crop study described above (year 1 and 2). The interventions were performed as 3×12 days dietary periods with wash-out periods of minimum 2 weeks (Figure 12) *(Manuscript III)*.

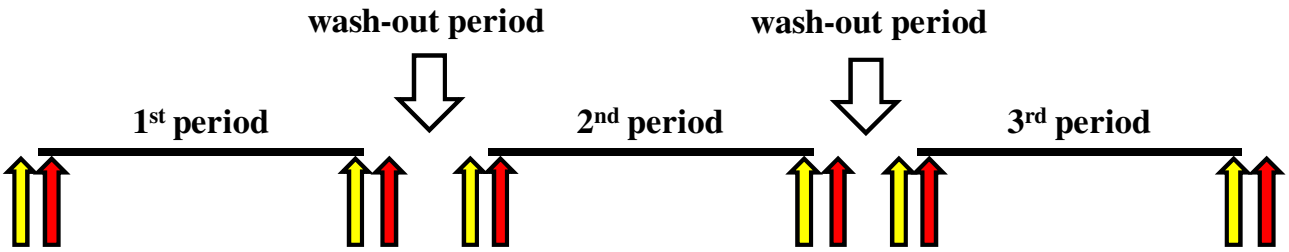


Figure 12. The design of the human intervention study with three dietary periods (12 days) and wash-out periods in between (minimum 2 weeks). Urine samples (⇑) were collected on day −1 and 12 and blood samples (⇑) on day 1 and 13.

The subjects (n = 18) consumed fully controlled diets during each intervention period and no other foods or drinks were allowed. The subjects consumed lunch at the department under supervision, while the other meals were distributed daily for consumption at home, except weekend meals, which were given out Friday afternoons. The subjects were instructed to consume all foods given out as well as report and also bring back any left overs to the department. Energy requirement for each individual subject was calculated from the reported physical activity, weight and age prior to

the beginning of the study *(222)*. During the wash out periods, the subjects were allowed to consume their regular diets without any restrictions *(Manuscript III)*.

The study protocol was approved by the Danish National Committee on Biomedical Research Ethics of the Capital Region of Denmark (J. no. H-C-2007-0078). The subjects, intervention diets and sampling of blood and urine will be described below. Further details on the human intervention study, except urine sampling, can be seen in Manuscript III.

4.2.2 Subjects

Adult men were recruited from the area of Copenhagen (Denmark) in the autumn before the start of the study each year. All subjects were healthy, omnivorous, non-smokers, did not exercise more than 10 hours per week or consume more than 21 units of alcohol per week. Neither of the subjects took any medication, vitamins, minerals or other supplements, and did not donate blood for at least 2 months prior to the study or during the study. The height of the subjects was measured at the beginning of the study. The weight was measured on day −1, 1, 4, and 12 during the first dietary period and on day 1 and 12 during the last two periods. The characteristics of the subjects at baseline can be seen in Table 7 *(Manuscript III)*.

Table 7. Characteristics of subjects at baseline (mean ± standard deviation) with the ranges in brackets *(Manuscript III)*.

	Year 1	**Year 2**
Number of subjects	18	18
Age (years)	25.1 ± 6.7 (18─40)	26.0 ± 7.1 (19─37)
Energy level (MJ/day)	13.5 ± 1.0 (12─15)	13.8 ± 1.1 (12─15)
Weight (kg)	73.7 ± 8.9 (57.0─92.3)	80.8 ± 8.6 (67.6─103)
Height (meter)	1.84 ± 0.07 (1.70─1.94)	1.83 ± 0.07 (1.76─1.93)
Body Mass Index (kg/meter ^{2})	21.8 ± 1.6 (19.8 ─25.0)	24.0 ± 2.3 (19.9─30.1)

4.2.3 Intervention diets

One conventional (C) and two organic (OA and OB) intervention diets were tested in the human study with two replicates of each diet resulting in six diets prepared from the cultivated food products at the Aarslev and Foulum location from the crop study described earlier. However, only

two of the three replicates from Aarslev were used in order to match the two replicates from Foulum. The six diets included two different menus consumed during each intervention period on day 1, 3, 5, 8, 10, and 12 (menu 1) and on day 2, 4, 6, 7, 9, and 11 (menu 2) (Table 8). The menus and the food quantities were identical in all diets. The content of carbohydrates, protein, and fat in the diets was 52 %, 15 %, and 33 % of total energy intake, respectively. The subjects were assigned by drawing lots to a pre-defined order in which the diets and replicates were distributed in a balanced way *(Manuscript III)*.

Table 8. Composition of the intervention diets (g/day) for 10MJ energy intake *(Manuscript III)*.

Meal	Component	Menu 1 (g d^{-1})	Menu 2 (g d^{-1})
Breakfast	Skimmed milk	385	385
	Carrot roll	160	160
	Carrot jam	52	52
	Butter	8	8
Lunch	Coleslaw salad	- [a]	145
	Barley and bean salad	100	- [a]
	Full grain bread	120	120
	Raw carrot	34	- [a]
	Meat balls	70	- [a]
	Hummus	- [a]	118
Snack	Carrot cake	93	- [a]
	Potato cake	- [a]	77
	Oat cookies	60	60
Dinner	Baked potatoes with carrot	286	- [a]
	Minced meat and vegetables	194	- [a]
	Fricassee with faba beans	- [a]	451
	Mashed potatoes	- [a]	275

[a] : not included in the menu

The average daily content of the cultivated crops in the diets is shown in Table 9. All other ingredients of the diets were kept at minimum, but the following food products were purchased from local distributors: low fat organic milk for drinking and cooking, minced pork meat, meat

balls, organic eggs, organic butter, sugar, lemon juice, baking yeast, salt, baking powder, gelatine powder, and pepper. The amounts and suppliers of the supplemented foods can be seen in Manuscript III. Subjects had free access to mineral water, table salt, and instant coffee, which was made by addition of mineral water. All meals were prepared shortly after harvest in the metabolic kitchen and stored at −20 °C until the day of consumption except coleslaw, barley and bean salads as well as raw carrots, which were stored at 2 °C until serving *(Manuscript III)*.

A 10 MJ portion of the six diets was collected, homogenised, freeze-dried, and stored in a nitrogen atmosphere at −80 ˚C until chemical analyses *(Manuscript III)*.

Table 9. Content of crops in the intervention diets (g/day) for 10MJ energy intake *(Manuscript III)*.

Crop	Menu 1 (g/d)	Menu 2 (g/d)	Average (g/d)
Barley	71	26	49
Carrot	162	184	173
Faba bean	30	102	66
Oat	45	45	45
Onion	80	42	61
Potato	195	264	230
Rapeseed oil	44	65	54
Wheat	92	83	88
White cabbage	28	100	64

4.2.4 Urine and blood sampling

Baseline urine samples (24-hour) were collected in plastic bottles on day −1 prior to each dietary period and end-time samples (24-hour) were collected on day 12 (Figure 12). The pH of the urine samples was adjusted to 3.7 and ascorbic acid added (0.4 %, v/v) after collection based on the methodology suggested by Nielsen *et al*. *(223)*. The samples were stored at −80°C.

Fasting baseline blood samples were collected in the morning on day 1 and end-time blood samples were taken in the morning after the last meal (day 13) in each dietary period (Figure 12). Fasting blood samples were drawn into EDTA tubes, centrifuged, and stored at −80 ˚C until chemical analyses *(Manuscript III)*.

4.2.5 Chemical analyses/methods

4.2.5.1 Intervention diets

The chemical analysis of carotenoids in the intervention diets followed the same principle as the analysis of crops *i.e.* liquid extraction with THF and EtOH (50:50, v/v) on ultrasonication and HPLC-UV quantification as described in further detail in Manuscript III.

A new analytical method for chemical analysis of flavonoids and phenolic acids was developed and validated as described in Appendix I. In general, the method followed the same principle as the analysis of crops including pressurised liquid extraction with 65 % aqueous MeOH and addition of C18-material to the extraction cells followed by HPLC-UV quantification.

The polyacetylenes were also analysed using a newly developed and validated method based on the same principle as the analysis of crops. A three times extraction of the intervention diets with EtOAc was performed using the ULP based methodology with HPLC-UV quantification (see further details in Appendix II).

4.2.5.2 Urine and blood samples

The plasma samples were extracted with petroleum ether three times under stirring for analysis of carotenoids. After an evaporation step, they were quantified on HPLC-UV with the chromatographic method also applied for analysis of carotenoids in crops and intervention diets (see details in Manuscript III).

Initially, it was intended to analyse the content of flavonoids in urine samples at baseline and end-time by applying HPLC-MS, but it was not within the time frame of the project. The analyses were initiated during a stay abroad at the National Institute for Agricultural Research (Clermont-Ferrand, France), but the analyses and data treatment is still ongoing and for that reason not included in the PhD thesis. Chemical analysis of phenolic acids and polyacetylenes in human samples was not intended to be included in this project.

4.3 Statistical analyses

One-way analysis of variance (ANOVA) was used to test for significant differences (5 % significance level) between the various analytical methods studied. The statistical analysis were performed in the Base SAS software (version 9.1.3, SAS Institute Inc., Cary, NC, USA).

The crops were tested for significant differences between growth systems, years, and locations (5 % significance level). The statistical model was fitted using the proc mixed procedure in the SAS/STAT software (version 9.2, SAS Institute Inc., Cary, NC, USA) as described in Manuscript I, II and III. Significant correlations between secondary metabolites was tested using a two-sided t-test *(224)*.

The intervention diets were tested for significant differences between growth system in each year by one-way ANOVA. Two-way ANOVA was used to test for significant differences between growth systems across growth year and between growth years across growth systems. A paired t-test was used to test for a significant difference between plasma status of carotenoids at baseline and the end of the dietary periods. A linear mixed model was used to test the effect of the intervention diets on plasma status with the following variables: diet, order of diets, period, group, subject, and baseline concentration. The statistical analyses of diets and human samples are described in detail in Manuscript III and were performed in the Base SAS software (version 9.1.3, SAS Institute Inc., Cary, NC, USA) with a 5 % significance level.

The application of multivariate data analysis *e.g.* principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) was investigated in order to reduce the complexity of the data, when considered relevant. The data was autoscaled and a full cross-validation was performed. The analyses were performed in the LatentiX data analytical software (version 1.00, Latent5, Copenhagen, Denmark).

5 Results and discussion

5.1 Analysis of secondary metabolites

Comparable sample preparation and validation of the chemical methods is of high importance, when evaluating the content of nutrients and secondary metabolites in food products, because differences can be induced in these last steps of the comparative studies. The necessary precautions taken in the sample preparation, when measuring secondary metabolites in crops, diets, and human samples are discussed below together with the chemical methods developed and validated. Trace elements and minerals were also studied in the OrgTrace project, and as a consequence some compromises had to be made in the common sampling and pre-treatment of crops, diets, and human samples in order to serve all interests.

5.1.1 Sample preparation

The importance of sampling, sample preservation, and preparation is often neglected in the analysis of secondary metabolites, even though they are the foundation for high quality and rugged chemical analysis *(225)*. The sample handling strategies (including sample collection, storage, and pre-treatment) are critical steps in the analysis *(226)* and it is very important to collect a representative sample *(225)*. Representative sampling of the crops was ensured by stepwise mass reduction as described in Petersen *et al*. *(227)*. It was tested by double sampling of random plots, which yielded an acceptable average relative standard deviation of 7 % (n = 24) *(Manuscript I, II, and III)*.

The pre-processing of crops can affect the content of secondary metabolites *(10)*, for instance, peeling and chopping can cause degradation of flavonoids in onions *(144)*, though an insignificant loss of quercetin-glucosides has also been observed after similar pre-processing procedures *(136)*. Furthermore, β-carotene can undergo *cis-trans* isomerism during pre-processing *(81)*. Nevertheless, the vegetables were peeled in this study, thus representing the eatable parts of the crops as they are ingested by the consumer. The crops were also chopped in order to facilitate the freeze-drying process and washed due to the analysis of trace elements and metals in the OrgTrace project. However, the washing was decreased to a minimum as secondary metabolites can leach into the water. The more lipid soluble compounds such as carotenoids are probably less affected by washing

than the more water soluble compounds such as flavonoids and phenolic acids, where leaching is more common *(88)*.

Crops and intervention diets were freeze-dried because it is considered an effective tool for preservation of secondary metabolites *(25, 81, 228, 229)* due to a low oxygen partial pressure and contact temperature (< 30°C) *(70)*. Freezing with liquid nitrogen is also considered effective for preservation *(221, 229)*, but freeze-drying is advantageous, when large sample sets need to be handled *(209)* due to the limited sample handling, and more samples can be freeze-dried at one time.

The storage conditions during pre-processing were thoroughly considered in order to avoid degradation of the secondary metabolites of interest. The exposure of the samples to light was decreased by storage in darkness as many secondary metabolites are sensitive to light, which can cause degradation *(66, 69, 111, 162, 201)* and also *cis-trans* isomerism of carotenoids *(66, 69)*. Oxygen also facilitates the degradation of secondary metabolites *(66, 69, 111, 160)*, so the crops and intervention diets were stored in an inert nitrogen atmosphere in order to preserve the secondary metabolites. The crops and intervention diets were stored at −18°C and the human samples at −80°C during pre-processing until chemical analysis because several secondary metabolites are sensitive to high temperatures *(66, 69, 111, 161, 201)* and low storage temperatures are recommended for better retention of secondary metabolites *(66, 69, 81, 129)*. The same precautions were generally taken during the following extractions and chemical analyses of secondary metabolites in crops, diets, and human samples with regard to light and oxygen exposure, as well as heat, in order to avoid unintended changes in the content of secondary metabolites.

5.1.2 Chemical analyses

5.1.2.1 Carotenoids

The chemical analysis of carotenoids in crops, intervention diets, and plasma samples was based on routine procedures at the National Food Institute, Technical University of Denmark. No optimization or further validation of the analytical method was performed in this study *(Manuscript III)*. The extraction of food and plasma samples was performed in accordance with normal procedures *(66)*. HPLC-UV was used for quantification of both types of samples and is the most convenient and reliable method used for routine analysis of carotenoids *(66)*. UV detection is

normally used due to the very high extinction coefficients of carotenoids at 400−500 nm and only few other natural compounds absorb at these wavelengths *(66, 230)*. The C30 column used for the chromatographic separation improves the separation of both polar and nonpolar carotenoids, and has also previously been used in food analyses of carotenoids *(25, 230)* together mixtures of MeOH, water, and MTBE as mobile phases *(66, 230)*. Butylated hydroxytoluene (BHT, 2,6-bis(1,1-dimethylethyl)-4-methylphenol) was added to solvents and functioned as an antioxidant in order to avoid loss of carotenoids during the chemical analyses *(25, 231)*.

5.1.2.2 Flavonoids and phenolic acids

Flavonoids are usually extracted with aqueous MeOH or ACN on *e.g.* water bath or ultrasonication *(221, 229)*. However, the application of new rapid and automated methods such as pressurised liquid extraction (PLE) and microwave-assisted extraction (MAE) has increased because they can be carried out in the absence of light and oxygen, and enables a reduced solvent consumption *(232)*. Furthermore, a reduction in extraction time can be obtained because the new methods are often carried out at elevated temperatures and/or pressures *(232)*. This is advantageous as long extraction times together with additional manual work might increase the risk of analyte degradation during the analysis *(111, 233)*.

A comparison of the two conventional and three new liquid extraction methods for analysis of flavonoids in onions was performed (Figure 13). In general, the conventional water bath extraction method yielded significantly lower extraction efficiencies than the other methods tested ($p < 0.02$), while the precisions were in the same range. PLE was the preferred extraction method because it can be highly automated, use only small amounts of solvents, provide the cleanest extracts, and allow extraction of the light- and oxygen-sensitive flavonoids in an inert atmosphere protected from light *(Paper I)*. PLE is very useful for routine analysis *(234)*, but its application in analyses of secondary metabolites from food samples has previously been limited, though it has been used for extraction of polyphenols from, for instance, apples *(235)*, barley *(124)*, and parsley *(236)*.

A one-step PLE method was optimised with regard to extraction temperature, sample weight, flush volume, and solvent type, and afterwards validated. It yielded a rapid, reliable and sensitive analytical method for quantification of flavonoids in onions with more than 98 % recovery of the extractable flavonoids. The method showed several advantages and possibilities in comparison with the other extraction methods tested. In particular, the simultaneous extraction and clean-up by in-

cell addition of C18-material to the extraction cells was a considerable improvement because the alternative solid-phase extraction (SPE) is very time- and solvent consuming. Furthermore, extra sample handling increases the risk of chemical degradation *(Paper I)*. The recovery was slightly improved (13−35 %, data not shown) with addition of C18-material, though only significantly higher for quercetin-7,4'-diglucoside (Q-7,4'-diglu; $p = 0.047$) and isorhamnetin-3,4'-diglycoside (I-3,4'-digly; $p = 0.027$). The precisions were also enhanced, especially of the more polar compounds, by in-cell addition of C18-material (Table 10), which is frequently used as sorbent for SPE of flavonoids *(229)*. The application of the PLE method could be extended to other food matrices and polyphenols, and was also applicable for extraction of phenolic acids in carrots and potatoes *(Paper I)*.

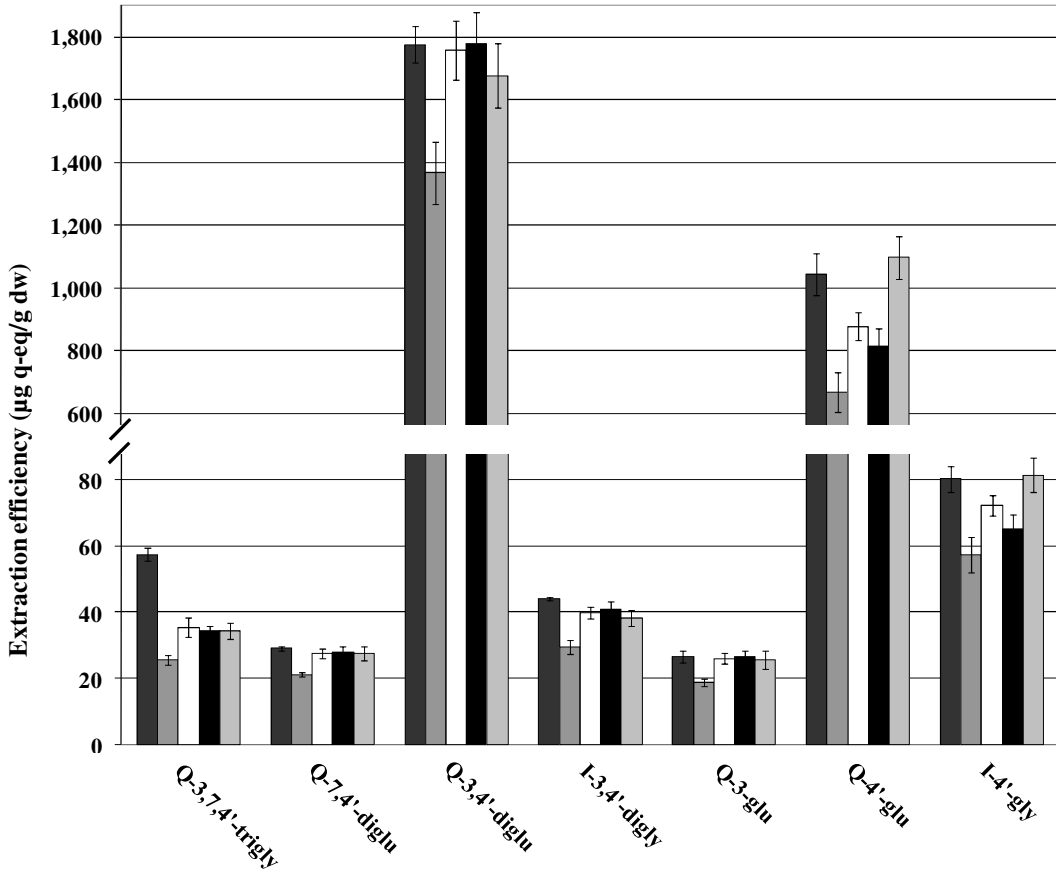


Figure 13. Extraction efficiency (µg quercetin-equivalents/g dry weight) of the methods tested: ultrasonication (■), water bath (▩), microwave-assisted extraction (□), ultrasonic liquid processor (■), and pressurised liquid extraction (▩).The error bars describe the standard deviation (n = 3). Q-3,7,4'-trigly: quercetin-3,7,4'-triglycoside, Q-7,4'-diglu: quercetin-7,4'-diglucoside, Q-3,4'-diglu: quercetin-3,4'-diglucoside, I-3,4'-diglycoside: isorhamnetin-3,4'-diglycoside, Q-3-glu: quercetin-3-glucoside, Q-4'-glu: quercetin-4'-glucoside, and I-4'-gly: isorhamnetin-4'-glycoside (modified from *Paper I*).

Table 10. Precision (relative standard deviation, %) in pressurised liquid extractions of flavonoids in onions with and without in-cell addition of C18-material to the extraction cells (n = 3) *(Paper I)*.

	RSD (%)	
Compound	**Addition of C18-material**	**No addition of C18-material**
Q-3,7,4'-trigly	5.8	25
Q-7,4'-diglu	4.3	7.7
Q-3,4'-diglu	3.8	7.0
I-3,4'-digly	3.8	9.0
Q-3-glu	9.9	7.3
Q-4'-glu	6.8	6.8
I-4'-gly	9.9	9.7

Q-3,7,4'-trigly: quercetin-3,7,4'-triglycoside, Q-7,4'-diglu: quercetin-7,4'-diglucoside, Q-3,4'-diglu: quercetin-3,4'-diglucoside, I-3,4'-diglycoside: isorhamnetin-3,4'-diglycoside, Q-3-glu: quercetin-3-glucoside, Q-4'-glu: quercetin-4'-glucoside, and I-4'-gly: isorhamnetin-4'-glycoside.

The previously applied method for analysis of flavonoids in intervention diets involved extraction with MeOH, defatting with heptan, and clean-up on SPE *(64)*. However, in this study a PLE method was optimised for extraction of flavonoids from intervention diets starting from the extraction method used for crops. Defatting is a very time-consuming step and has previously been included in PLE of corticosteroids in bovine liver *(237)*, but it resulted in fat droplets in the subsequent extracts with aqueous MeOH in the present study. The potential of fat retainers (*e.g.* activated silica or alumina) in comparison with C18-material for removal of fat and other interferences in food analyses was tested as they have previously been used in environmental analyses of fatty samples *(238-240)*. The best fat retainer in analysis of polyaromatic hydrocarbons in fish samples was activated silica *(238)*, but it did not yield significant improvements in extraction efficiency of flavonoids and phenolic acids in comparison with C18-material for removal of fat and interfering compounds ($p > 0.05$) (Figure 14). Furthermore, the extraction efficiency of Q-4'-glu in the first extract was significantly ($p < 0.0070$) higher with in-cell addition of only C18-material than the other tested methods (Figure 14), while no significant differences were observed in the extraction efficiency of the other flavonoids between the various in-cell additions tested (refer to Figure 14). Thus, the extraction of flavonoids and phenolic acids in intervention diets was based on the same PLE principle as in crops with in-cell addition of C18-material. However, the size of the extraction cells, sample weight, and amount of C18-material was optimised to compensate for the low content of flavonoids and phenolic acids in the intervention diets compared with the crops. Furthermore, the

flush volume was increased to 150 % leading to a higher percentage of extractable analytes in the first extract and it was also necessary to include an evaporation step *(Appendix I)*.

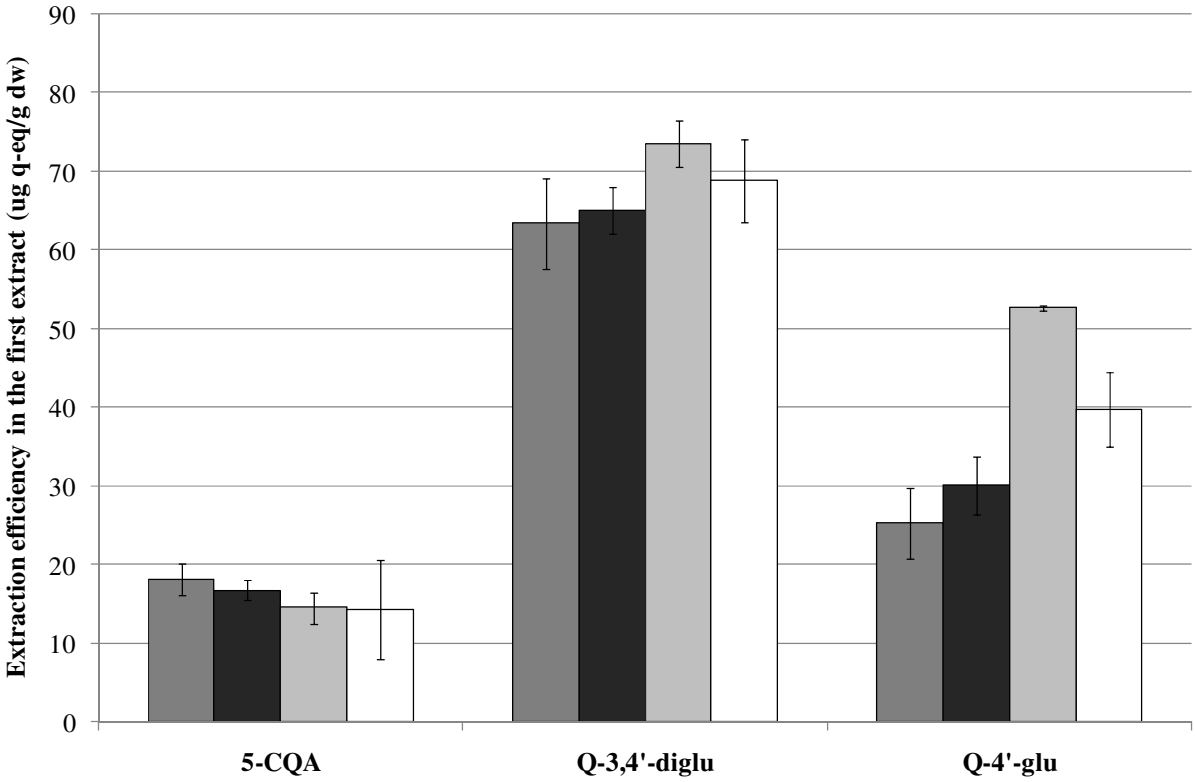


Figure 14. Extraction efficiency (µg quercetin-equivalents/g dry weight) of selected flavonoids and phenolic acids in the first extract with in-cell addition of the following: without addition of any material (▩), addition of activated silica (■), addition of C18-material (▩) and addition of activated silica and C18-material (□).The error bars describe the standard deviation (n = 3). 5-CQA: 5-*O*-caffeoylquinic acid, Q-3,4'-diglu: quercetin-3,4'-diglucoside, and Q-4'-glu: quercetin-4'-glucoside.

The PLE based methodology for analysis of flavonoids and phenolic acids in intervention diets was a considerable improvement compared to the previously applied method *(64)* involving more manual work and several time-consuming steps, which increase the risk of analyte degradation. The PLE method was sensitive, selective and precise, except for a low precision of quercetin due to a concentration below the limit of quantification (LOQ). The recovery of Q-7,4'-diglu and quercetin-3-glucoside (Q-3-glu) was 92 % and 85 %, respectively, and more than 98 % of the extractable flavonoids and phenolic acids were recovered *(Appendix I)*.

The chromatographic separation of flavonoids and phenolic acids was performed on a C18 column with mobile phases consisting of mixtures of water and methanol added formic acid, which are often used for chemical analysis of flavonoids *(229, 241)*. The flavonoids and phenolic acids were

quantified on HPLC with UV detection *(Paper I and Appendix I)*, which is the most applied quantification method together with MS detection *(242-244)*. Application of MS might yield higher method selectivity and sensitivity than UV quantification, but the advantage of UV is the lack of matrix effects, which are normally seen in MS analyses of plant materials *(245, 246)*. MS was used for identification of flavonoids *(Paper I)* and UV quantification was considered adequate for the present study.

5.1.2.3 Polyacetylenes

The traditional liquid extraction method for analysis of polyacetylenes in crops involve stirring *(198)* or ultrasonication *(211)* with ethyl acetate as the most frequently used solvent *(198, 201, 203, 209, 247)*, but a more modern technology using a Ultrasonic Liquid Processor (ULP) was applied in this study. A new and rapid analytical method was developed based on ULP methodology, which yielded significantly higher extraction efficiencies than the conventional stirring method ($p < 0.001$) (Figure 15). Furthermore, a considerable reduction in extraction time with ULP was achieved, which decreases the risk of degradation of the polyacetylenes due to their sensitive towards oxidation *(200)* as well heat and light *(201)*. PLE has also been applied for analysis of polyacetylenes *(209)* and is especially useful in routine and high-through-put analysis, but the ULP methodology was preferred due to the size of the sample set and the improvements with regard to efficiency and speed. The ULP method was optimised with regard to extraction time, sample weight, and extraction cycles. The optimised ULP method showed good sensitivity, selectivity, and precision with 93 % recovery of FaDOH and more than 95 % of the extractable polyacetylenes *(Manuscript I)*.

The content of polyacetylenes in intervention diets has not previously been determined, and an extraction method was developed based on the same principle as the extraction of crops. However, the concentration levels in the intervention diets were lower than in the crops, and consequently the sample weight and the volume of extraction solvent were increased. Furthermore, it was necessary to repeat the extraction three times and include an evaporation step. The method showed good selectivity, sensitivity, precision, and 129 % of FaDOH was recovered without time-consuming steps such as defatting with heptane or SPE *(Appendix II)*.

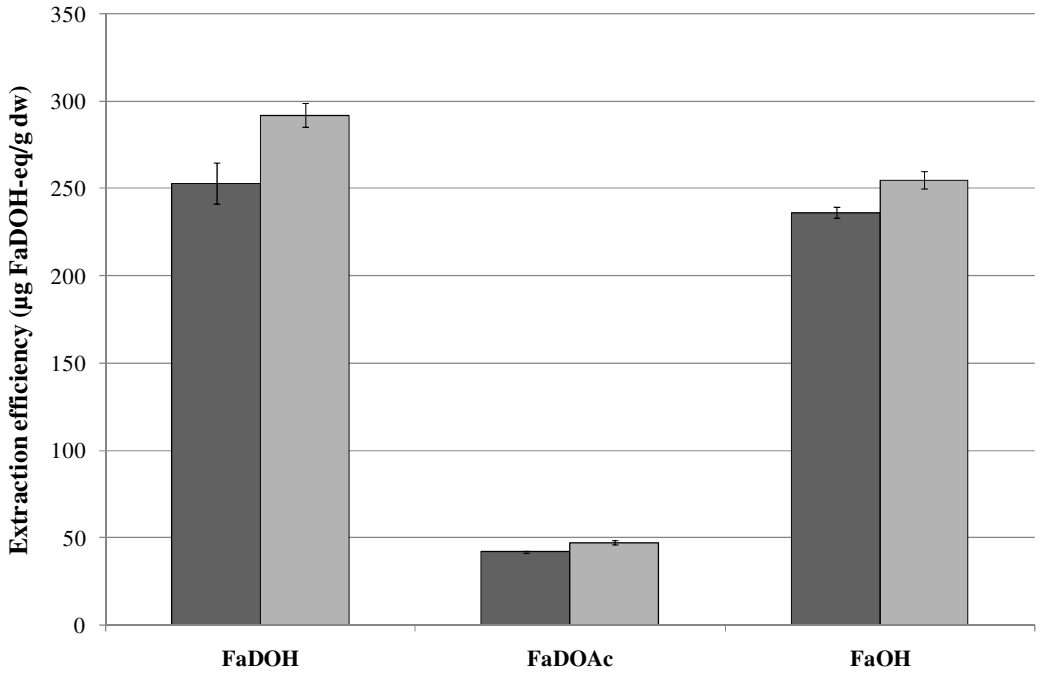


Figure 15. Extraction efficiency (FaDOH-equivalents/g dry weight) of the methods tested: extraction with stirring (■) and extraction with ultrasonic liquid processor (■).The error bars describe the standard deviation (n = 4). FaDOH: falcarindiol, FaDOAc: falcarindiol-3-acetate, and FaOH: falcarinol *(Manuscript I)*.

Polyacetylenes are normally quantified using HPLC-UV with a reversed phase column and gradient elution with MeOH or ACN and water *(28)*, which was also applied in this study. In this study, the run time of the chromatographic separation was considerably reduced (33 min) compared with run times of 45 to 95 min in previous studies *(198, 209, 211)*. The application of ultra performance liquid chromatography (UPLC) was expected to cause an even larger improvement in run time. However, poor resolution was obtained probably due to a different selectivity of the stationary phase applied as a column similar to the HPLC column was not commercially available. MS was used for identification of polyacetylenes *(Manuscript I)* and has previously also been applied for quantification of polyacetylenes yielding higher selectivity, but similar sensitivity *(209)*. Nevertheless, it was not considered necessary as the method selectivity and also sensitivity with UV quantification was satisfactory for the purpose of the method *(Manuscript I)*.

5.1.3 General conclusions

Representative sampling and comparable sample preparation was assured in this comparative study by, for instance, step-wise mass reduction during sample collection and freeze-drying of samples as

an effective tool for preservation of secondary metabolites in the crops and intervention diets. It was necessary to take precautions with regard to light and oxygen exposure as well as heat during the entire sample preparation as well as the chemical analyses of crops, intervention diets, and human samples due to the sensitivity of secondary metabolites towards these variables. Validated methods for analysis of carotenoids in crops, intervention diets, and plasma samples were used together with newly developed and validated methods for analysis of flavonoids, phenolic acids, and polyacetylenes in crops and intervention diets. Continuous quality assurance was performed by using in-house samples as reference material and random duplicate analyses.

The developed and optimised method for analysis of flavonoids and phenolic acids in crops and diets sets a new standard for this type of chemical analyses. The one-step PLE method showed considerable improvements and possibilities compared with conventional extraction methods *e.g.* simultaneous extraction and clean-up by in-cell addition of C18-material, which can be generally applied in future extractions using PLE. The ULP based methodology used in the analysis of polyacetylenes in crops and intervention diets is a novel technology, which can be used in future studies for rapid and effective extractions of secondary metabolites. In general, the applied analytical methods for analysis of secondary metabolites in crops, intervention diets, and plasma samples were sensitive, selective, and precise in combination with HPLC-UV quantification. MS was used for identification of the most abundant secondary metabolites (flavonoids, phenolic acids and polyacetylenes) and UV quantification was considered adequate for the purpose of the present study in comparison with MS quantification.

5.2 Secondary metabolites in vegetables

5.2.1 Carotenoids in carrots

The main carotenoids in carrots were α-carotene and β-carotene, but also small quantities of lutein were detected in agreement with previous studies of carrots *(66, 71, 74, 248)*. In year 1, the average concentrations (± standard deviation (sd), n = 9) in the three growth systems were 2.6 ± 0.4, 27 ± 3, and 90 ± 6 µg/g fresh weight (µg/g fw) of lutein, α-carotene, and β-carotene, respectively, in accordance with other studies of carrots *(25, 71, 72, 75-79)*. In year 2, the corresponding concentrations were 19 %, 7.4 % and 3.3 % higher (refer to Figure 16) (*Manuscript III*).

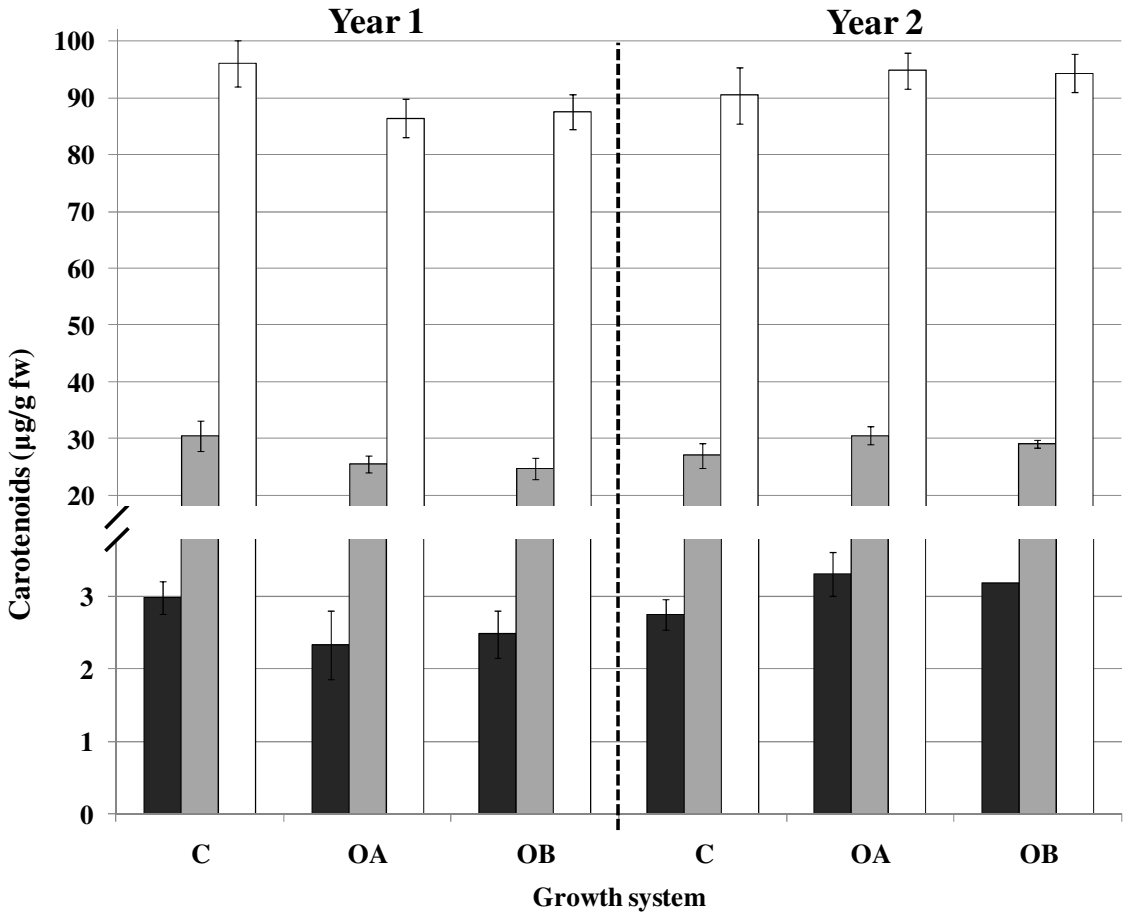


Figure 16. The concentrations of carotenoids in carrots (µg/g fresh weight) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (1 and 2). The error bars describe the standard deviation of replicates from the field (n = 3). ■: lutein, ■: α-carotene. and □: β-carotene *(Manuscript III)*.

However, no significant differences in the content of carotenoids in carrots across growth years were observed between the three growth systems ($p > 0.05$), despite differences in fertilization levels and strategies. The results were in agreement with the harvest yield and dry matter content in the carrots ($p > 0.05$). However, the nitrogen content at harvest was higher in the conventionally grown carrots than in the organically ones *(Manuscript III)*.

The results were supported by previous studies of carotenoids in tomatoes *(86, 249)* and carrots *(250)*, but in contrast, a positive correlation between the levels of nitrogen fertilization and carotenoids in carrots has also been observed *(41, 82)*. Insignificant effects of growth system (organic versus conventional) on the content of carotenoids in carrots was also seen in another cultivation study *(43)*, while a significantly higher content of β-carotene in organically compared to conventionally grown carrots was observed in previous farm studies *(40, 41)*. However, the

significance of the growth system is difficult to predict in farm studies due to different growing and soil conditions.

A significant impact of growth year on the content of carotenoids in carrots has previously been shown *(85, 250)*. However, no significant year-to-year variation across growth systems was observed in this study ($p > 0.05$) *(Manuscript III)*, even though higher temperatures and more precipitation occurred in year 1 compared with year 2.

Furthermore, a significant positive correlation ($p < 0.05$) between α-carotene and lutein ($R^2 = 0.55$) as well as α-carotene and β-carotene ($R^2 = 0.58$) was observed. The correlations illustrate the connections between the carotenoids in their biosynthesis in plants (Figure 3). Thus, if a high level of one of the carotenoids in carrots is observed, then a high production of the other carotenoids can also be expected probably due to a general stimulation of the biosynthetic pathways of carotenoids.

5.2.2 Flavonoids in onions

The most abundant flavonoids in onions were quercetin-3,4'-diglucoside (Q-3,4'-diglu) and Q-4'-glu. Traces of quercetin-3,7,4'-triglycoside (Q-3,7,4'-trigly), Q-7,4'-diglu, Q-3-glu, I-3,4'-digly, and isorhamnetin-4'-glycoside (I-4'-gly) were also detected (Figure 17), but Q-7,4'-diglu only in concentrations below LOQ *(Manuscript II)*. Quercetin and kaempferol aglycones are the main contributors to the total content of flavonols in onions (Table 3), but neither the aglycone nor glycosides of kaempferol were identified. Nevertheless, the flavonoid profile found in onions was similar to the profile previously found in red *(251)* and white onions *(140)*. In year 1, the average concentrations (± sd, n = 9) in the three growth systems were 262 ± 38 and 208 ± 41 µg quercetin-equivalents/g fresh weight (µg q-eq/g fw) of Q-3,4'-diglu and Q-4'-glu, respectively, equal to 530 ± 77 µg Q-3,4'-diglu/g fw and 330 ± 65 µg Q-4'-diglu/g fw (refer to Figure 17). In year 2, the corresponding average concentrations were 9 % and 8 % lower *(Manuscript II),* and the concentration levels were generally in agreement with previously found levels in onions *(120, 132)*.

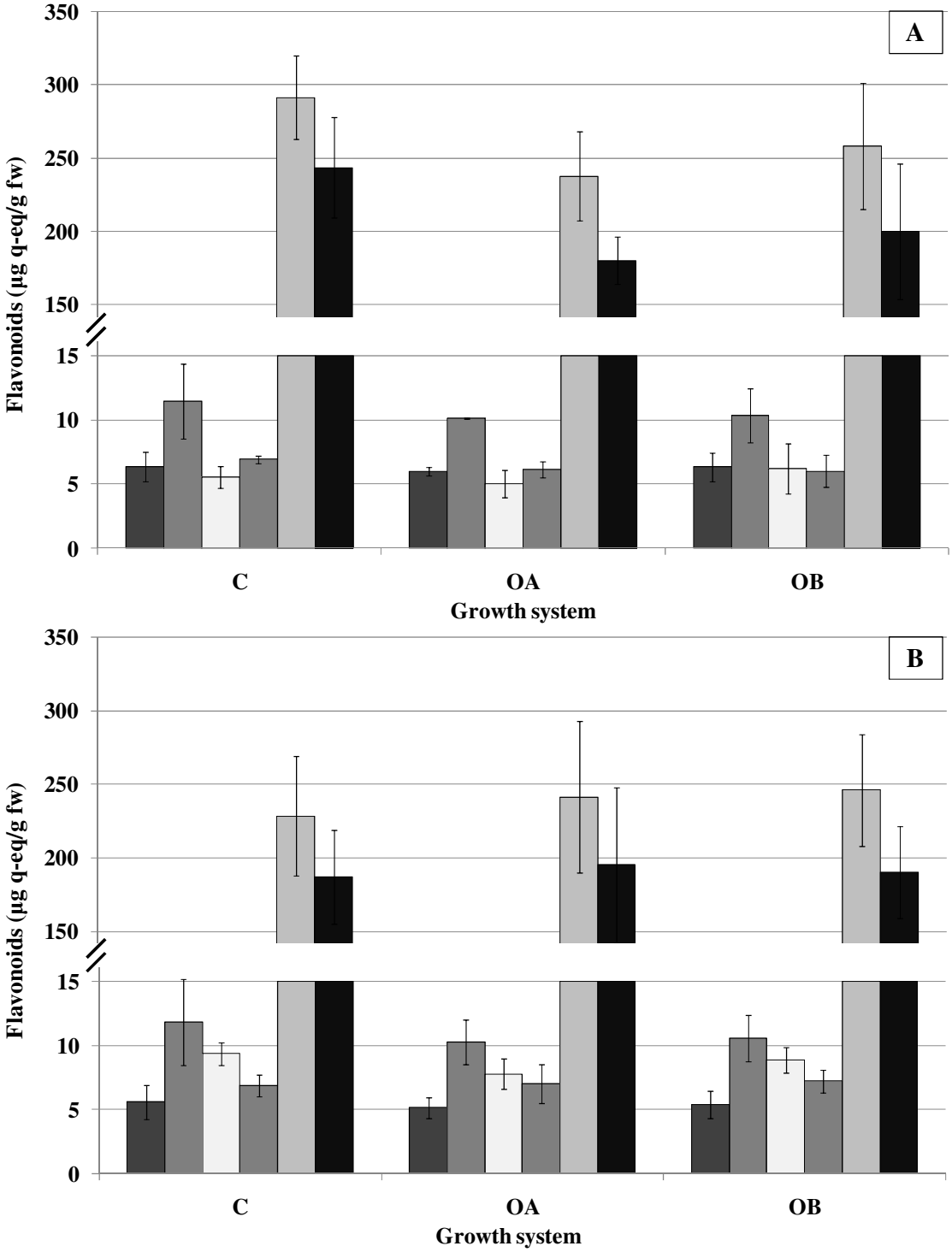


Figure 17. The average concentrations of flavonoids in onions (µg quercetin-equivalents/g fresh weight) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (A: year 1 and B: year 2). The error bars describe the standard deviation of replicates from the field (n = 3). ■: isorhamnetin-3,4'-diglycoside, ■: isorhamnetin-4'-glycoside, □: quercetin-3,7,4'-triglycoside, ■: quercetin-3-glucoside, □: quercetin-3,4'-diglucoside, and ■: quercetin-4'-glucoside *(Manuscript II)*.

No significant differences in the content of flavonoids in onions between the three growth systems across growth years were observed ($p > 0.05$) *(Manuscript II)*. Multivariate data analysis did not reveal any separation of the growth systems either, when all samples and quantified flavonoids were included in a PCA or PLS-DA model (data not shown). The results were in agreement with a previous cultivation study of organically and conventionally grown onions *(44)*. In contrast, higher contents of flavonoids in organic compared to conventional onions were found in a previous farm study *(42)*, but as mentioned previously the impact of the growth system is difficult to determine in such studies. However, conventional plants can also be exposed to stressed conditions due to application of sub lethal doses of synthetic pesticides and for that reason conventional plants are not automatically subjected to lower stress levels than organically grown plants *(252)*.

The levels of flavonoids and nitrogen in the onions at harvest were comparable between the three growth systems, despite differences in fertilization strategies and levels *(Manuscript II)*. The results are similar to previous fertilization studies of onions, where the concentration of flavonoids was unaffected by both the source (organic or inorganic) and level of nitrogen fertilization *(44, 132)*. In contrast, the harvest yield of the conventionally grown onions was significantly higher than the organically grown onions ($p < 0.001$), which indicated that the fertilization of the organic crops during the growth season was below optimum *(Manuscript II)*. Nevertheless, no significant differences in the content of flavonoids at harvest were observed ($p > 0.05$), even though increased accumulation of flavonoids can occur as a consequence of nitrogen and phosphorous depletion *(253-255)*.

The growth year can affect the content of flavonoids in onions considerably *(44, 131, 132)* and multivariate data analysis of all quantified flavonoids indicated a separation of the two growth years (Figure 18). Nevertheless, no significant differences were observed across the growth systems ($p < 0.05$), except a significantly higher concentration of Q-3,7,4'-trigly in year 2 ($p = 0.046$), despite the higher temperatures and more precipitation in the first than in the second growth year *(Manuscript II)*, and the sensitivity of the biosynthesis of flavonoids in plants to stressed conditions such as temperature and water availability *(108)*.

Significant positive correlations between the following flavonoids in onions were observed ($p < 0.05$): Q-7,4'-diglu and Q-4'-glu ($R^2 = 0.67$), Q-3,4'-diglu and Q-4'-glu ($R^2 = 0.87$), and I-3,4'-diglu and I-4'-glu ($R^2 = 0.64$). The correlations substantiate the connections of flavonoids in their biosynthesis (Figure 6) and a similar positive correlation has previously been observed for quercetin

and quercetin-3-rutinoside *(256)*. The results indicate that a stimulation of the biosynthesis of flavonoids (Figure 6) cause an increased accumulation of several flavonoids due to their biosynthetic relations in plants as was also seen for carotenoids.

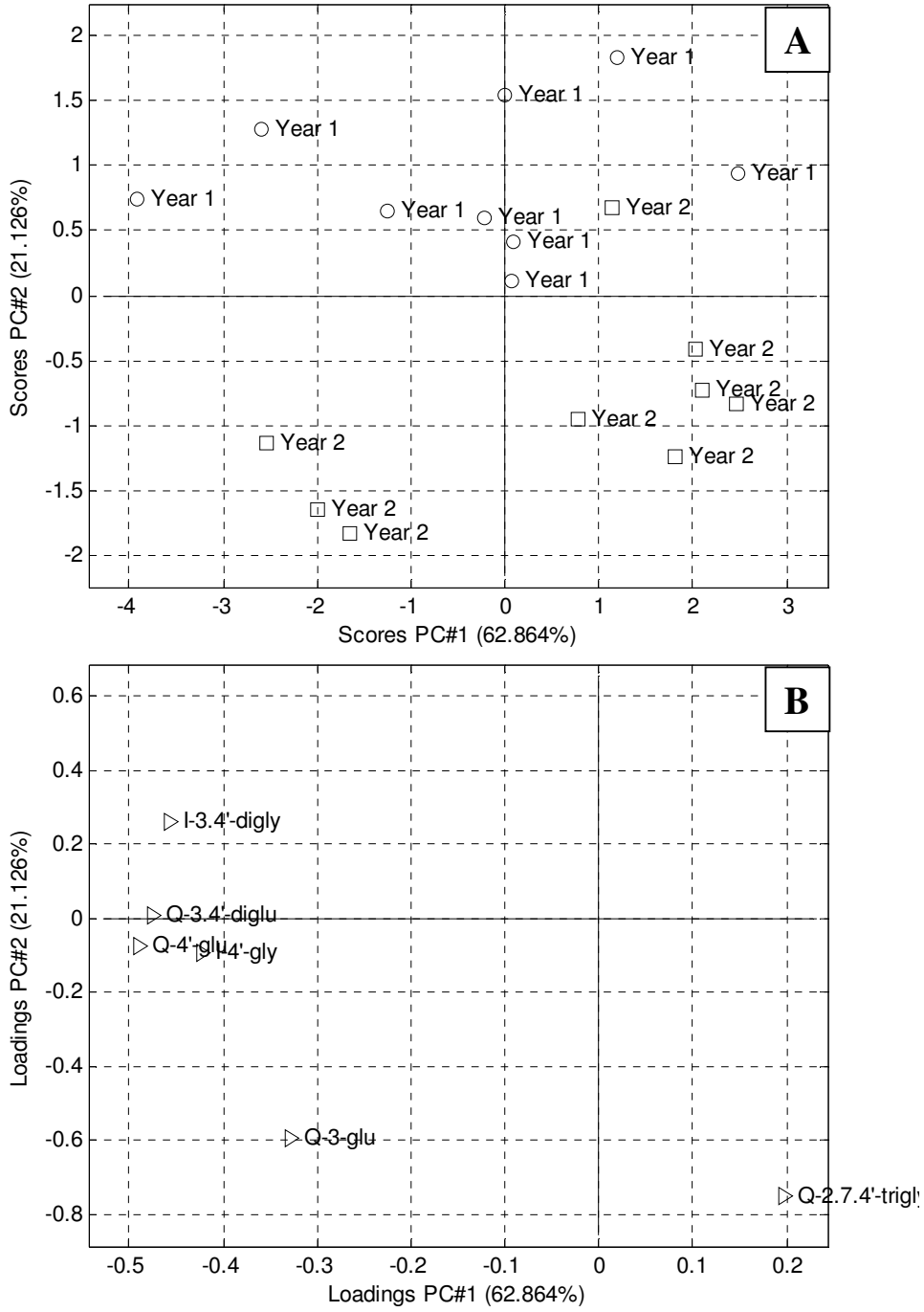


Figure 18. Score plot (A) and loading plot (B) from Principal Component Analysis of flavonoids in onions (quercetin-3,7,4'-triglycoside (Q-3,7,4'-trigly), quercetin-3,4'-diglucoside (Q-3,4'-diglu), isorhamnetin-3,4'-diglycoside (I-3,4'-digly), quercetin-3-glucoside (Q-3-glu), quercetin-4'-glucoside (Q-4'-glu), and isorhamnetin-4'-glycoside (I-4'-gly)) and samples from two growth years (○: year 1 and □: year 2)

5.2.3 Phenolic acids in carrots and potatoes

The most abundant phenolic acid in carrots and potatoes was 5-CQA in agreement with previous studies *(171, 176, 181, 257)*. No other phenolic acids or polyphenols were identified in the carrots, while smaller quantities of 4-*O*-caffeoylquinic acid (4-CQA) and traces of CA were also detected in the potatoes, but the concentrations of CA were below LOQ *(Manuscript II)*.

In year 1, the average concentration (± sd, n = 9) of 5-CQA in the three growth systems was 15 ± 5 µg q-eq/g fw in carrots equal to 55 ± 18 µg 5-CQA/g fw, while it was 28 % lower in year 2 (refer to Figure 19) *(Manuscript II)*. The concentrations were in agreement with previous reported levels in carrots *(171, 176)*. The concentrations of 5-CQA were slightly higher in potatoes, but in accordance with *(170)* or slightly higher *(181)* than the concentrations found in previous studies. In year 1, the average concentrations (± sd, n = 6) of 5-CQA in the three growth systems were 22 ± 2, 20 ± 3, and 28 ± 2 µg q-eq/g fw in potatoes at the three locations Foulum, Jyndevad, and Flakkebjerg, respectively, which is equal to 80 ± 7, 72 ± 10, and 104 ± 5 µg 5-CQA/g fw. In year 2, the corresponding average concentrations were 22 % lower at the Foulum location and 17 % higher at the two other locations (refer to Figure 20) (*Manuscript II*). The differences in the content of 5-CQA between the two growth years at the three locations might be related to the growing and weather conditions at the three individual locations in Denmark.

No significant differences in the content of 5-CQA in carrots between the three growth systems were observed across growth years ($p > 0.05$), although a significantly higher content of 5-CQA was found in the growth system OA in comparison with the other growth systems in year 1 ($p = 0.0072$). The results were supported by insignificant differences in the yield and dry matter content of carrots at harvest ($p > 0.05$). No significant correlation between the content of 5-CQA and nitrogen at harvest was observed ($p > 0.05$) and the content of nitrogen at harvest was significantly higher in the conventionally compared to the organically grown carrots ($p < 0.001$) *(Manuscript II)*.

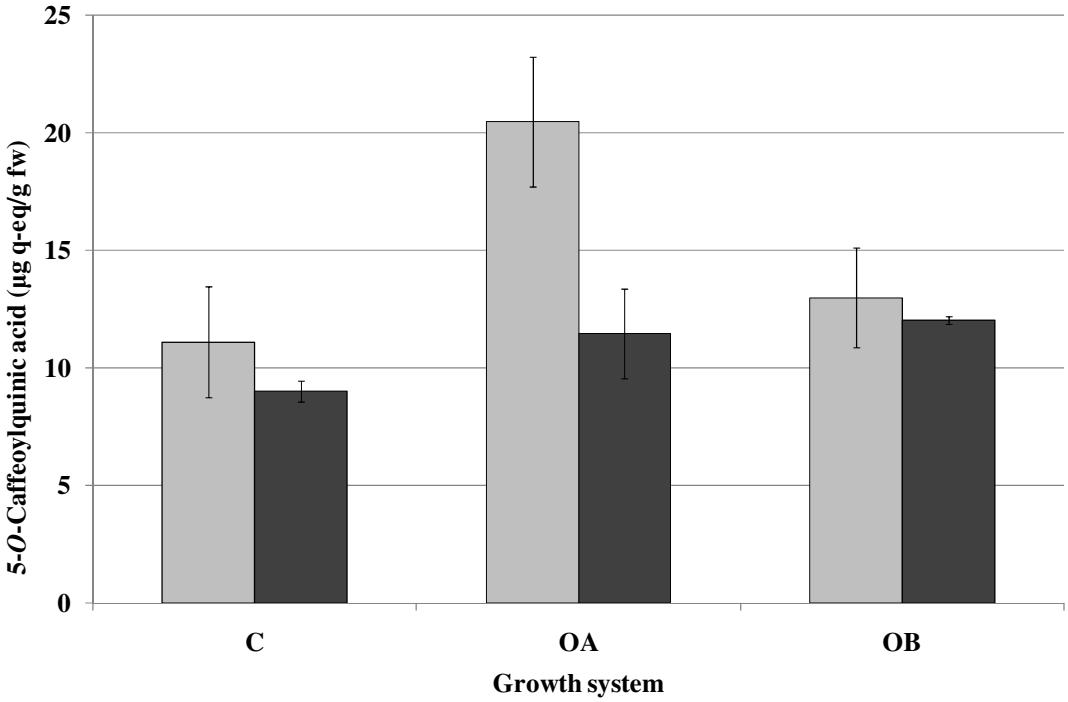


Figure 19. The average concentrations of 5-*O*-caffeoylquinic acid (5-CQA) in carrots (µg quercetin-equivalents/g fresh weight) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crop) and in two growth years (□: year 1 and ■: year 2). The error bars describe the standard deviation of replicates from the field (n = 3) *(Manuscript II)*.

No significant differences in the content of 4-CQA in potatoes between the three growth systems across growth years and locations were observed ($p > 0.05$). However, a significantly higher content of 5-CQA was found in the organically growth system OB compared with the two other growth systems (OB > C and OA, $p = 0.03$) *(Manuscript II)*. This is in agreement with a previous study of phenolic acids in potatoes, but the study was carried out as a farm study with the limitations mentioned earlier regarding these type of studies *(48)*. The harvest yield was significantly higher in the conventionally grown potatoes (C > OA > OB, $p < 0.0001$) and associated with the nitrogen fertilization levels *(Manuscript II)*, which also previously had a significant impact on the content of phenolic acids in tomatoes *(86)*. However, cultivation with different nitrogen levels did not induce an increased accumulation of 5-CQA in another study of potatoes *(181)*, even though nitrogen depletion has caused an increased accumulation of secondary metabolites derived from the phenylpropanoid pathway *e.g.* 5-CQA in tobacco plants *(255)*. Instead, the concentration differences might be associated with variation in potassium fertilization level because low potassium fertilization levels can cause accumulation of polyphenols in potatoes *(258, 259)*.

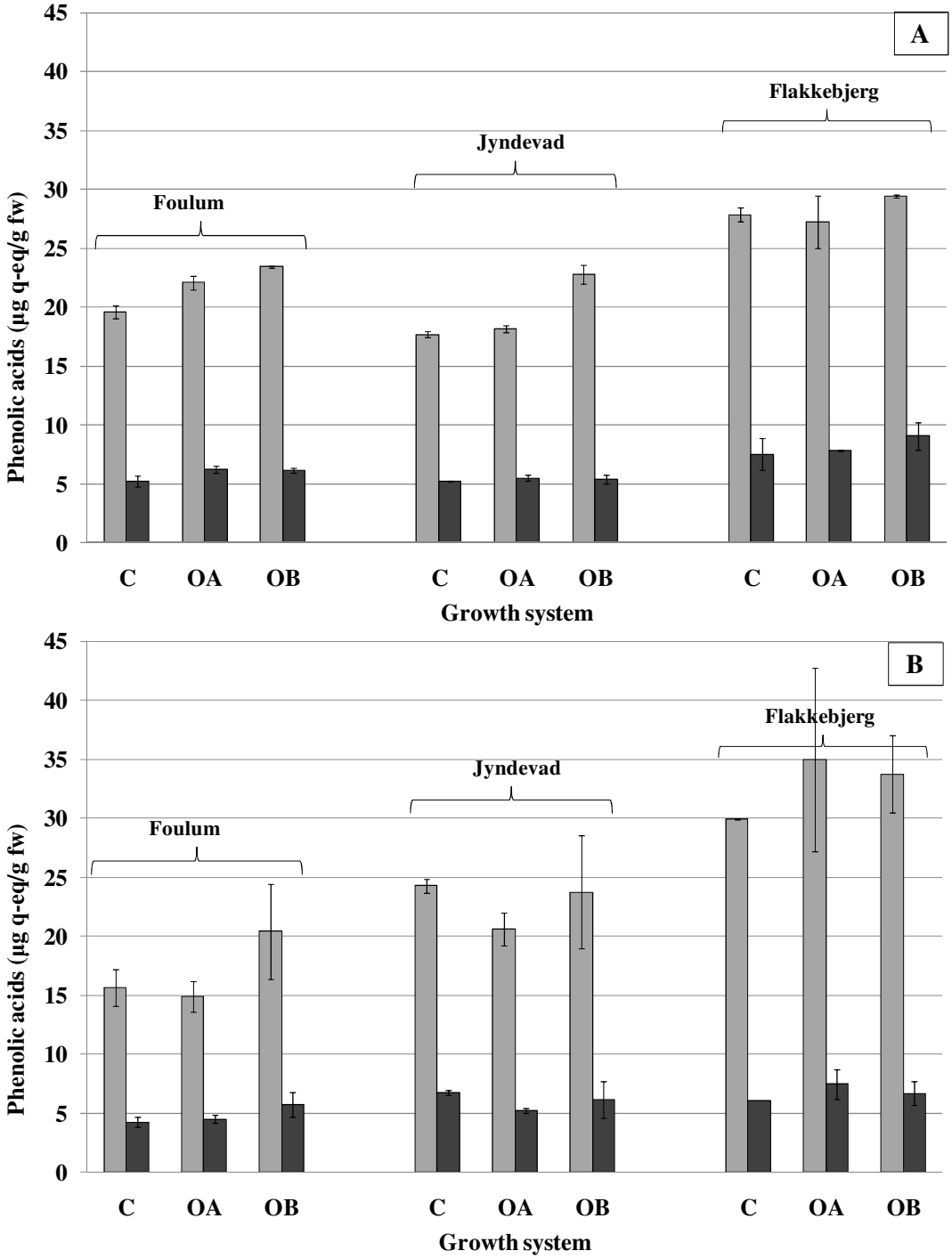


Figure 20. The average concentrations of phenolic acids in potatoes (µg quercetin-equivalents/g fresh weight) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (A: year 1 and B: year 2) at three different locations (Foulum, Jyndevad, and Flakkebjerg). The error bars describe the standard deviation of replicates from the field (n = 2). ▫ : 5-*O*-caffeoylquinic acid (5-CQA) and ▪ : 4-*O*-caffeoylquinic acid (4-CQA) *(Manuscript II)*.

The content of phenolic acids varied between growth years in previous studies of vegetables *(48, 54)*, but no significant year-to-year differences across growth systems and locations were observed

in carrots and potatoes in this study ($p > 0.05$) in spite of differences in temperature and humidity between the two growth years *(Manuscript II)*. Increased accumulation of secondary metabolites derived from the phenylpropanoid pathway has been observed at extremely low temperatures (5 and 10 ^{o}C) *(254)*, but such low temperatures are not normally reached during the growth season in Denmark. Furthermore, no significant differences in the content of phenolic acids in potatoes between the three locations across growth systems were observed ($p > 0.05$) *(Manuscript II)* in contrast to farm study of phenolic acids in potatoes performed at locations in the Czech Republic *(48)*.

Furthermore, a significant correlation ($p < 0.05$) between 4-CQA and 5-CQA in potatoes was observed ($R^2 = 0.61$, data not shown) and is possibly related to the biosynthetic pathways of phenolic acids in plants (Figure 8) similarly to the carotenoids and flavonoids in the cultivated crops.

5.2.4 Polyacetylenes in carrots

The most abundant polyacetylene in peeled carrots was FaOH in a previous study *(198)*, but FaDOH was the major polyacetylenes in carrots in this study. The distribution of polyacetylenes in the present study is in agreement with other studies *(20, 199, 212)*, even though FaDOH and FaDOAc are primarily found in the carrot peel *(171, 198, 203)*. In year 1, the average concentrations (± sd, n = 9) of polyacetylenes in the three growth systems were 26 ± 4.0, 3.6 ± 0.4, and 11 ± 2.3 µg FaDOH-eq/g fresh weight of FaDOH, FaDOAc, and FaOH, respectively, and the level of FaDOH was in agreement with previously reported concentrations in carrots *(20, 171, 209, 211, 212)*. In year 2, the corresponding concentrations were 15 %, 12 % and 3 % lower (refer to Figure 21) *(Manuscript I)*.

The content of polyacetylenes did not differ significantly between growth systems across growth years ($p > 0.05$), despite differences in fertilization strategies and levels. The results were verified by no significant differences observed in yields and dry matter contents at harvest ($p > 0.05$). It could be related to a generally high fertility of the soil reducing the impacts of variation in fertilization levels, even though a higher content of nitrogen in the conventionally grown compared with the organically grown carrots at harvest was observed *(Manuscript I)*. Furthermore, it cannot automatically be assumed that higher stress levels in organically grown crops than in conventionally grown lead to higher levels of secondary metabolites because crops grown in conventional

agriculture can also be exposed to high stress levels due to application of sub lethal doses of synthetic pesticides as mentioned earlier *(252)*.

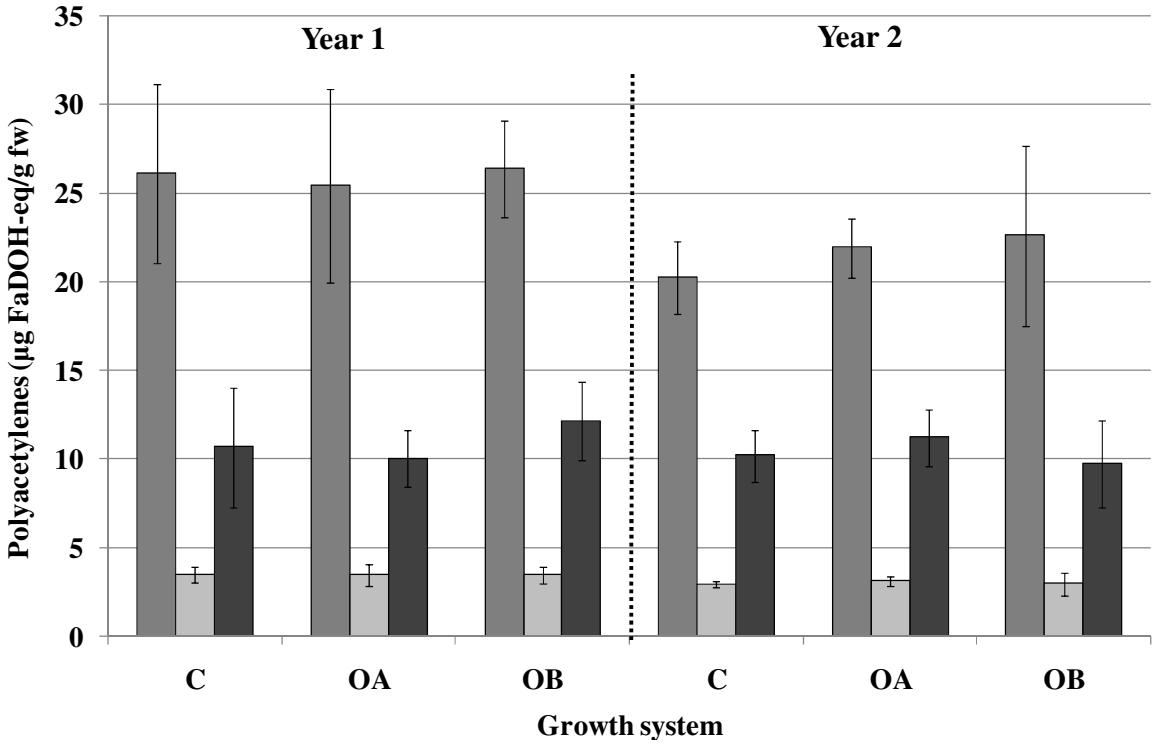


Figure 21. The average concentrations of polyacetylenes in carrots (ug FaDOH-equivalents/g fresh weight) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (1 and 2). The error bars describe the standard deviation of replicates from the field (n = 3). ■: falcarindiol, □: falcarindiol-3-acetate, and ■: falcarinol (modified from *Manuscript I*).

A significant year-to-year variation was observed for FaDOAc ($p = 0.003$), while no significant differences were observed for FaOH ($p > 0.05$). In addition, the results indicated a year-to-year variation in the content of FaDOH with lower concentrations in year 2 ($p = 0.07$). In general, the differences in weather conditions between the grown years might have induced increased accumulation of polyacetylenes, because the water availability has previously been shown to affect the content of polyacetylenes *(199)*.

A significant correlation ($p < 0.05$) between FaOH and FaDOAc ($R^2 = 0.51$, data not shown) was observed and is probably related to the biosynthetic pathways of polyacetylenes, where FaDOAc is formed from FaOH with FaDOH as an intermediate. However, no significant correlations between the other polyacetylenes were observed ($p > 0.05$), despite the associations in the biosynthesis of

polyacetylenes in plants (Figure 10). The correlation is in agreement with the previously observed correlation for selected carotenoids, flavonoids, and phenolic acids.

The application of multivariate data analysis was investigated, but no separation of the carrot samples according to growth system or year was possible in a PCA or PLS-DA model including all quantified carotenoids, phenolic acids, and polyacetylenes. Furthermore, no significant correlations between the three groups of secondary metabolites in carrots were observed ($p > 0.05$) in agreement with previous observations of carotenoids and polyacetylenes in carrots *(20)*.

5.2.5 General conclusions

The major secondary metabolite in carrots was β-carotene, but they also contained other carotenoids (lutein and α-carotene) as well as 5-CQA and polyacetylenes (FaDOH, FaDOAc and FaOH). The most abundant compound in potatoes was 5-CQA and they also contained 4-CQA and CA. Seven flavonoids were found in onions, the major compounds being Q-3,4'-diglu and Q-4'-glu.

No systematic differences in the content of selected carotenoids, flavonoids, phenolic acids, and polyacetylenes in vegetables between the three growth systems across growth years were observed ($p > 0.05$), despite differences in fertilization strategies and levels. Although, the organically grown potatoes with cover crops as nutrient supply ($p = 0.03$) showed a significantly higher content of 5-CQA. Cultivation studies are considered the most accurate and valid form of comparative studies and the crops were grown, harvested, and stored under comparable and well-defined conditions. Nevertheless, the growth system (organic versus conventional) did not generally have a significant impact on the ability of carrots, onions, and potatoes to synthesise selected secondary metabolites ($p > 0.05$). The application of multivariate data analysis, including several secondary metabolites in onions and carrots, did not reveal any distinction between the content of secondary metabolites according to growth systems either.

One of the weaknesses of cultivation studies might be that the results only apply to the specific location investigated, but the location did not have a significant impact on the content of phenolic acids in potatoes ($p > 0.05$). Thus, the trends observed for the content of phenolic acids in potatoes does not appear only to be relevant for the specific locations. In general, no systematic year-to-year variation was observed in the content of carotenoids, flavonoids, phenolic acids, and polyacetylenes in vegetables across growth years ($p > 0.05$), except for Q-3,7,4'-trigly in onions ($p = 0.0046$) and FaDOAc in carrots ($p = 0.003$).

5.3 Secondary metabolites in intervention diets

5.3.1 Carotenoids

The distribution of carotenoids in the intervention diets was similar to the distribution in the carrots. The most abundant compound in the intervention diets was β-carotene (Figure 22). In year 1, the average concentrations (average of menu 1 and 2 ± sd, n = 6) in the intervention diets were 1.8 ± 0.3, 4.5 ± 0.7, and 15 ± 2 mg/10MJ of lutein, α-carotene, and β-carotene, respectively. In year 2, the corresponding average concentration levels were 10 % lower for lutein, and 2 % and 4 % higher for α-carotene, and β-carotene, respectively (refer to Figure 22). Zeaxanthin, β-cryptoxanthin and lycopene were not detected in the intervention diets in accordance with the carotenoids detected in carrots *(Manuscript III)*.

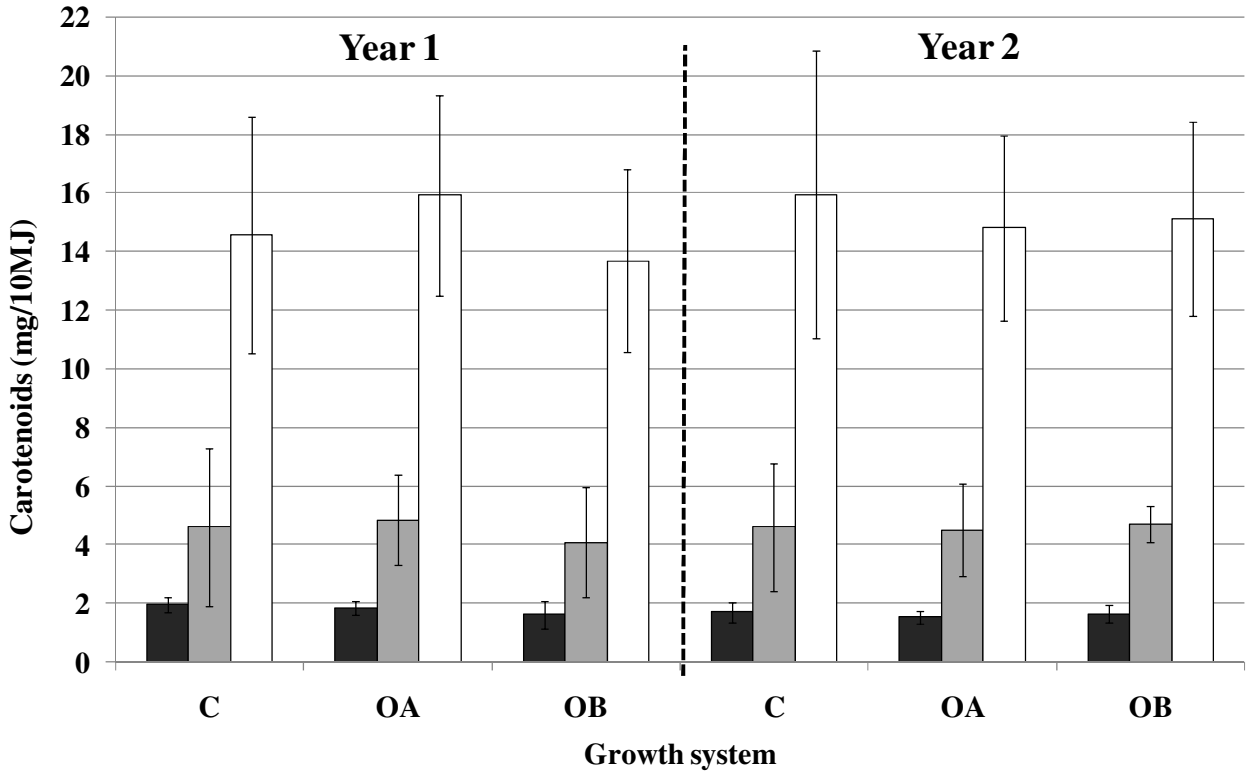


Figure 22. The concentrations of carotenoids in the intervention diets (mg/10MJ, average of menu 1 and 2) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (1 and 2). The error bars describe the standard deviation of the replicates from the field (n = 2). ■: lutein, ▒: α-carotene, and □: β-carotene.

The concentration of β-carotene determined by chemical analysis was similar to the 17 mg β-carotene/10 MJ calculated using the Dankost 2000 dietary assessment software (Danish Catering Center, Herlev, Denmark) *(Manuscript III)* and considerable higher than the estimated average daily

intake of β-carotene (3.7 mg/10 MJ) for Danish men *(260)*. The total intake of carotenoids (sum of lutein, α-carotene, and β-carotene) was 9.5 mg/day in year 1 and 5 % higher in year 2. Thus, the intervention diets were rich in carotenoids compared to a habitual Danish diet, where the total intake of carotenoids in Denmark has been estimated to 4.8 mg/day *(71)*.

The concentrations of carotenoids in the intervention diets were not significantly different between the three growth systems across growth year ($p > 0.05$) and the concentration levels of carotenoids did not vary significantly between growth years across growth system ($p > 0.05$). The results were similar to the results obtained on carotenoids in carrots, where no significant differences between the growth systems or growth years were detected *(Manuscript III)*.

The concentrations of carotenoids can be affected both negatively and positively by food processing procedures *e.g.* steaming and boiling *(25, 66, 87)*, but the preparation of the intervention diets only had a small impact on the concentration of α-carotene and β-carotene. The levels of these two carotenoids were slightly lower (5 % on average) than the expected concentration calculated from the intake and content in carrots (see calculation example in Appendix III), which was considered the main dietary source of carotenoids. Butter was another possible source of β-carotene (5.0 mg/kg), but the concentration and consumption were minor in comparison with the carrots. The levels of lutein were quite low (< 2 mg/10 MJ), but in contrast to α-carotene and β-carotene, the determined concentration of lutein was higher (260 % on average) than expected and the relative percentage of lutein (8.1 %) was also higher than in the cultivated carrots. This could be explained by contribution from the cultivated faba beans and rape seeds as chemical analysis revealed small quantities of lutein in these crops. However, the total concentration of carotenoids (sum of lutein, α-carotene, and β-carotene) was similar to the expected concentration indicating that the loss of some carotenoids was compensated by an increase in the concentration of other carotenoids. Thus, it can be concluded that carrots could be considered the main dietary source of carotenoids, while the other crops used for the intervention diets did not contain major amounts of carotenoids.

5.3.2 Flavonoids

The major flavonoids in the intervention diets were Q-3,4'-diglu and Q-4'-glu similarly to the distribution in the onions, but also smaller quantities of Q-7,4-diglu, Q-3-glu, I-3,4'-digly and I-4'-gly were found (Figure 23). In contrast to the onions, Q-3,7,4'-trigly was not detected in the intervention diets and quercetin was only detected at concentrations close to LOD (0.1 mg/10 MJ,

Appendix I). In year 1, the average concentrations (average of menu 1 and 2 ± sd, n = 6) of Q-3,4'-diglu and Q-4'-glu in the intervention diets were 11 ± 1 and 7.7 ± 0.7 mg q-eq/10 MJ, respectively, which corresponds to 23 ± 3 mg Q-3,4'-diglu/10 MJ and 12 ± 1 mg Q-4'-glu/10 MJ. In year 2, the average concentrations of Q-3,4'-diglu and Q-4'-glu were 19 % and 1 % lower, respectively (refer to Figure 23).

The total average concentration of flavonols (sum of I-3,4'-digly, I-4'-gly, Q-7,4-diglu, Q-3,4'-diglu, Q-4'-glu, and Q-3-glu) was 22 mg q-eq/10 MJ in year 1 and 12 % lower in year 2, which corresponds to an average total intake of 30 mg q-eq/day in year 1 using the average energy level of the subjects seen in Table 7. The total average intake of flavonols in the present study was higher than the estimated daily intake of 20 mg flavonols and flavananones for Danish men *(146)* probably due to the high consumption of vegetables in comparison with a regular diet. The intake was also higher than the estimated intake in other European countries *(116, 145)*, but the intake can vary greatly between countries *(120)*. The estimation of intake in the present study was based on the most abundant flavonoids in the intervention diets, but other non-identified flavonoids might still be present and maybe contribute to the total intake.

The concentrations of flavonoids in the intervention diets did not differ significantly between the three growth systems across growth years ($p > 0.05$), despite the different fertilization strategies and levels applied. The only significant difference was observed in the concentration of Q-3,4'-diglu, which was significantly higher in the growth system OA than in the OB system in year 2 ($p < 0.03$). Discrimination of the intervention diets based on growth system was not possible using multivariate data analysis either. Hence, no systematic differences between the intervention diets were observed in agreement with the flavonoids in onions. In contrast, a significant difference in the content of quercetin was observed between organically and conventionally produced diets in a previous human intervention study *(64)*. However, different crop cultivars were used in the farm study and the amounts of food products and the crops were also different from the present study.

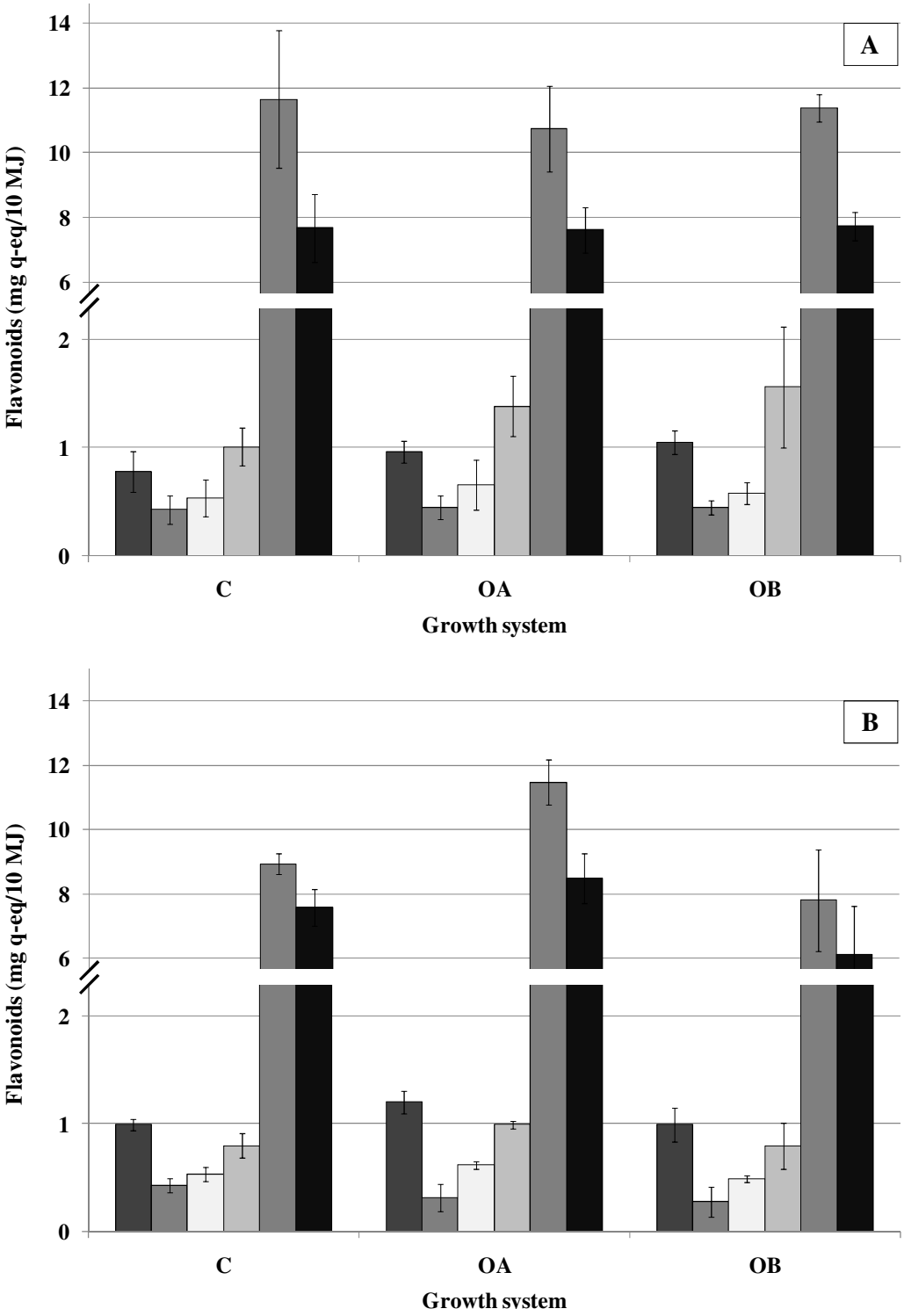


Figure 23. The concentrations of flavonoids in the intervention diets (mg quercetin-equivalents/10MJ, average of menu 1 and 2) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (A: year 1 and B: year 2). The error bars describe the standard deviation of the replicates from the field (n = 2). ■: isorhamnetin-3,4'-digly, ■: isorhamnetin-4'-glycoside, □: quercetin-7,4'-diglucoside, ■: quercetin-3-glucoside, ■: quercetin-3,4'-diglucoside, and ■: quercetin-4'-glucoside.

The concentration of the flavonoids in the intervention diets varied significantly between the growth years across growth systems ($p < 0.05$), except for Q-7,4'-diglu and Q-4'-glu ($p > 0.05$), and were generally lower in year 2. It is in accordance with the significant year-to-year effect observed in the dry matter content in the intervention diets *(Manuscript III)*, but in contrast to the results of flavonoids in onions and carotenoids in the intervention diets. The year-to-year variation might be related to the fact that higher temperatures and more precipation was seen year 1, which might cause higher stress levels in the plants and thereby increase the production of flavonoids, but it is uncertain why similar year-to-year variation was not observed in the cultivated onions used to prepare the intervention diets.

Onions were considered the main source of flavonoids in the present intervention study, but other crops *e.g.* carrots and white cabbage might also contribute to the total intake of flavonoids (Table 3). However, none of the identified flavonoids in onions were detected in these vegetables and neither were other abundant flavonoids. Furthermore, meat balls contain onions and thus can contribute to the total intake of flavonoids. However, the quantities of flavonoids in meat balls were small in comparison with the onions (< 2 % of the content in onions), and the daily consumption of meat balls was approximately half of that of onions on weight basis.

An average loss of 27 % was observed in the total content of flavonoids (sum of I-3,4'-digly, I-4'-gly, Q-7,4-diglu, Q-3,4'-diglu, Q-4'-glu and Q-3-glu) in the three growth systems and the two growth years (see calculation example in Appendix III), which was probably due to storage and pre-processing procedures (*e.g.* peeling, chopping, boiling, and roasting) during preparation of the intervention diets and has also been observed in previous studies *(135, 140-144)*. The average concentrations of Q-3,4'-diglu, Q-4'-glu, and I-4'-gly in the intervention diets were lower than expected assuming that onions were the main dietary source of flavonoids, and Q-3,7,4'-trigly was not even detected in the intervention diets. The loss of flavonoids can possibly be explained by their sensitivity to oxidation as well as heat and light *(110, 111)*, leading to degradation of glycosides to other glycosides containing fewer glycosidic substituents or aglycones *(114)*. Consequently, it could be expected to find quercetin and isorhamnetin in the intervention diets. However, isorhamnetin was not detected and quercetin was only detected in concentrations LOQ. On the contrary, the average concentrations of Q-7,4'-diglu, I-3,4'-digly, and Q-3-glu in the intervention diets were higher than expected, which was presumably a result of the degradation of other flavonoids in the food products, for instance, degradation of Q-3,7,4'-trigly to Q-7,4'-diglu, and Q-3,4'-diglu to Q-3-glu.

5.3.3 Phenolic acids

The only phenolic acid found in the intervention diets was 5-CQA, while neither 4-CQA nor CA were detected in contrast to the carrots and potatoes. In year 1, the average concentration (average of menu 1 and 2 ± sd, n = 6) of 5-CQA in the intervention diets was 2.2 ± 0.4 mg q-eq/10 MJ, which is equal to 7.8 ± 2 mg 5-CQA/10 MJ. In year 2, the average concentration was 11 % lower (refer to Figure 24).

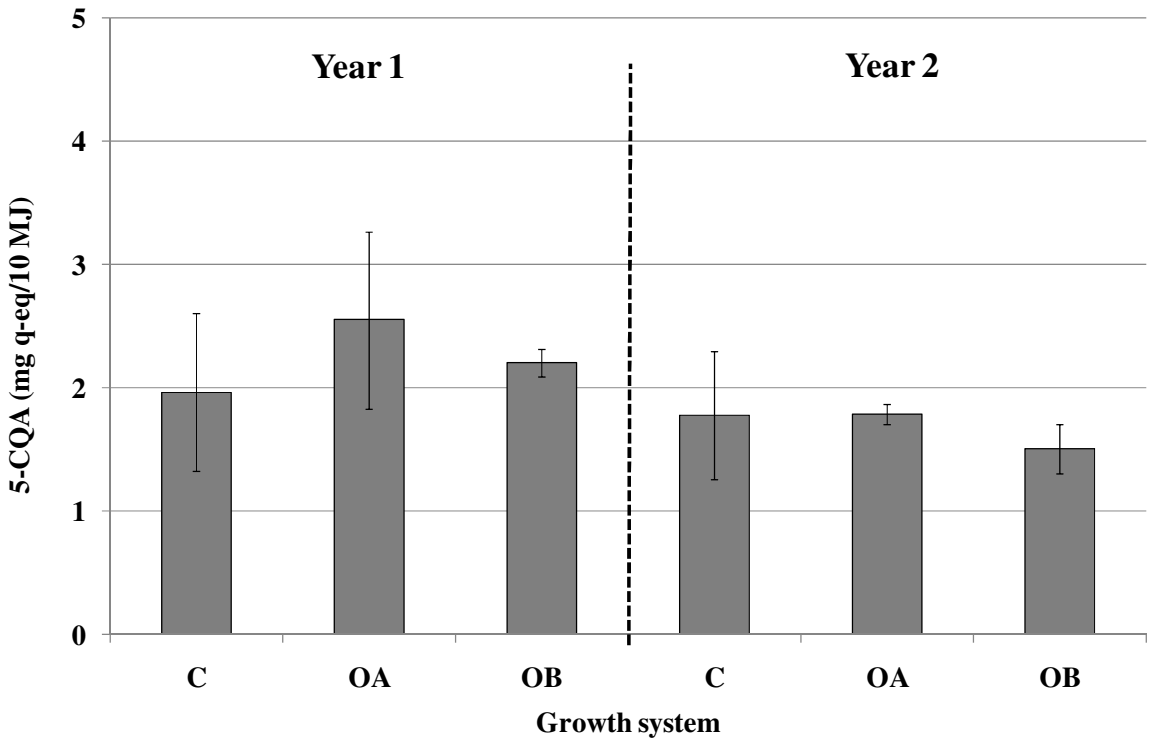


Figure 24. The concentrations of 5-*O*-caffeoylquinic acid (5-CQA) in the intervention diets (mg quercetin-equivalents/10MJ, average of menu 1 and 2) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (1 and 2). The error bars describe the standard deviation of the replicates from the field (n = 2).

The concentration of 5-CQA in the intervention diets was not significantly different between the three growth systems across the two growth years ($p > 0.05$), even though the content of 5-CQA was significantly higher in potatoes from the growth system OB than from the two other growth systems. However, this variation was diminished in the intervention diets, where also the carrots contributed to the total content of 5-CQA, and no significant difference between the growth systems were observed in the carrots. The concentration of 5-CQA was not significantly different between the two growth years ($p > 0.05$) similarly to the results found in the potatoes and carrots, but in

contrast to the content of flavonoids in the intervention diets, where a significant year-to-year variation was observed.

The average total intake of 5-CQA was 11 mg/day in year 1 and 9 % lower in year 2 based on the average energy level of the subjects (Table 7). The intake was considerably lower than the estimated daily intake of hydroxycinnamic acids (211 mg; 206 mg CA) in a German study *(155)*, despite a high consumption of potatoes and carrots in comparison with a regular diet, but other non-identified phenolic acids might also contribute to the total intake of hydroxycinnamic acids. However, coffee was the main contributor to the total daily intake of caffeic acid (190 mg CA) in the German study meaning that the other dietary sources only contributed with 16 mg CA/day, which is comparable to the content of 5-CQA in the intervention diets in the present study.

Carrots and potatoes were the main dietary sources of 5-CQA in the intervention diets in comparison with the other crops included in the human intervention study (Table 4) and in agreement with previous statements about the main vegetable sources of phenolic acids *(170, 171)*. However, the subjects were allowed to drink coffee, which could also have contributed to the total intake of phenolic acids due to the high content of chlorogenic acids in coffee *(158)*.

The average concentration of 5-CQA in the intervention diets was lower (73 %) than expected assuming that potatoes and carrots were the main sources of phenolic acids in the intervention diets (see calculation example in Appendix III). The loss during storage and processing of the intervention diets can be explained by their sensitivity towards oxidation *(160)*, heat *(161)*, and light *(162)*. Previous studies have observed similar negative effects on the content of phenolic acids in potatoes and carrots due to various cooking processes *(161, 175, 183)*. Phenolic acids appear to be more susceptible to storage and processing than, for instance, carotenoids *(88)*, where only a minor loss due to processing procedures was observed in the present study. Chlorogenic acids can be hydrolysed to caffeic and quinic acids during food processing *(87)*, but CA was not detected in the intervention diets.

5.3.4 Polyacetylenes

The major polyacetylenes in the intervention diets was FaOH followed by FaDOH and FaDOAc (Figure 25) in contrast to the distribution in carrots, where FaDOH was the most abundant compound. In year 1, the average concentrations (average of menu 1 and 2 ± sd, n = 6) in the

intervention diets were 2.8 ± 0.7, 1.5 ± 0.4, and 4.7 ± 0.9 mg FaDOH-eq/10 MJ of FaDOH, FaDOAc, and FaOH, respectively (refer to Figure 25). In year 2, the corresponding average concentrations were 15 %, 32 %, and 25 % lower than in year 1.

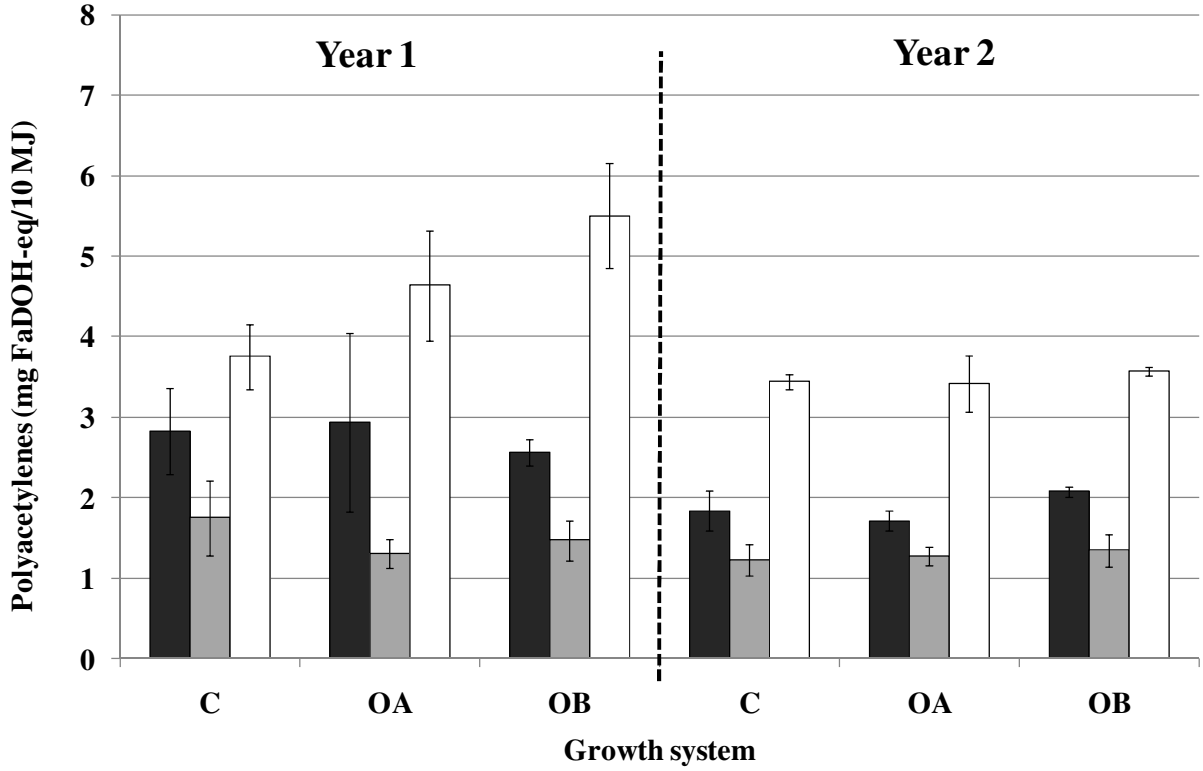


Figure 25. The average concentrations of polyacetylenes in the intervention diets (mg falcarindiol-equivalents/10MJ, average of menu 1 and 2) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (1 and 2). The error bars describe the standard deviation of the replicates from the field (n = 2). ■: falcarindiol, ▫: falcarindiol-3-acetate, and □: falcarinol.

No significant differences in the content of polyacetylenes in the carrots were observed between growth systems and the same general pattern was visible in the intervention diets. In general, the concentrations of polyacetylenes in the intervention diets were not significantly different between the three growth systems across the growth years ($p > 0.05$), except a significant difference in the content of FaOH between the growth system OB and C across growth years ($p = 0.032$). In addition, the concentration of FaDOH was significantly higher in the growth system OB than in the OA system in year 2 ($p = 0.012$). In agreement with the content of flavonoids in the intervention diets, the concentration of polyacetylenes was significantly higher across growth systems in year 1 in comparison with year 2 ($p < 0.05$) probably due to the different weather conditions in the two growth years. However, a significant year-to-year variation was only observed in the content of

FaDOAc in the carrots, while the other two compounds did not vary significantly between the growth years.

The total intake of polyacetylenes (sum of FaDOH, FaDOAc, and FaOH) was 12 mg FaDOH-eq/day in year 1 and 26 % lower in year 2 based on the average energy level of the subjects (Table 7). The total average content of polyacetylenes in the intervention diets was slightly higher (20 %) than expected assuming that carrots were the main dietary source (see calculation example in Appendix III). Thus, a contribution from other crops to the content of polyacetylenes in the intervention diets could be considered. However, none of the other crops in the present study were expected to contribute to the total intake of polyacetylenes because they are especially abundant in species of the plant families *Apiaceae* and *Araliaceae (28, 195)*, which were not included in the present study, except for carrots.

The average concentration of FaDOH in the intervention diets was lower than expected assuming that carrots were the main dietary source, while the average concentrations of FaDOAc and FaOH were higher than expected. The negative impact of storage and processing on the concentration of FaDOH could be related to the susceptibility of polyacetylenes, especially FaDOH, towards oxidation *(200)*, heat, and light *(28, 201)*. A previous study also showed a decreased content of polyacetylenes in carrots after food processing such as blanching and boiling *(201)*. The concentrations of FaDOAc and FaOH increased after preparation of the intervention diets in the present study and the increase in the concentration of FaOH is in agreement with a previous study of blanched carrots *(20)*. The increased production of FaOH and FaDOAc could be due to dehydroxylation and acetylation of FaDOH, respectively.

It was not possible to discriminate the intervention diets produced from the three different growth systems based on all the quantified secondary metabolites by applying multivariate data analysis such as PCA and PLS-DA models. On the other hand, a PCA model indicated a possible separation of the intervention diets based on the growth years with higher levels of secondary metabolites in year 1 (Figure 26), despite no significant year-to-year variation in the content of secondary metabolites in the individual crops. However, a significant year-to-year variation was observed for some of the secondary metabolites in the intervention diets *e.g.* flavonoids and polyacetylenes. In general, higher temperatures and more rainfall was seen in year 1 than in year 2, which might have induced a higher production of secondary metabolites due to their susceptibility to stress *e.g.* plant

available water and temperature. Nevertheless, it is uncertain why a year-to-year variation was indicated in the intervention diets, when it was not visible in the crops.

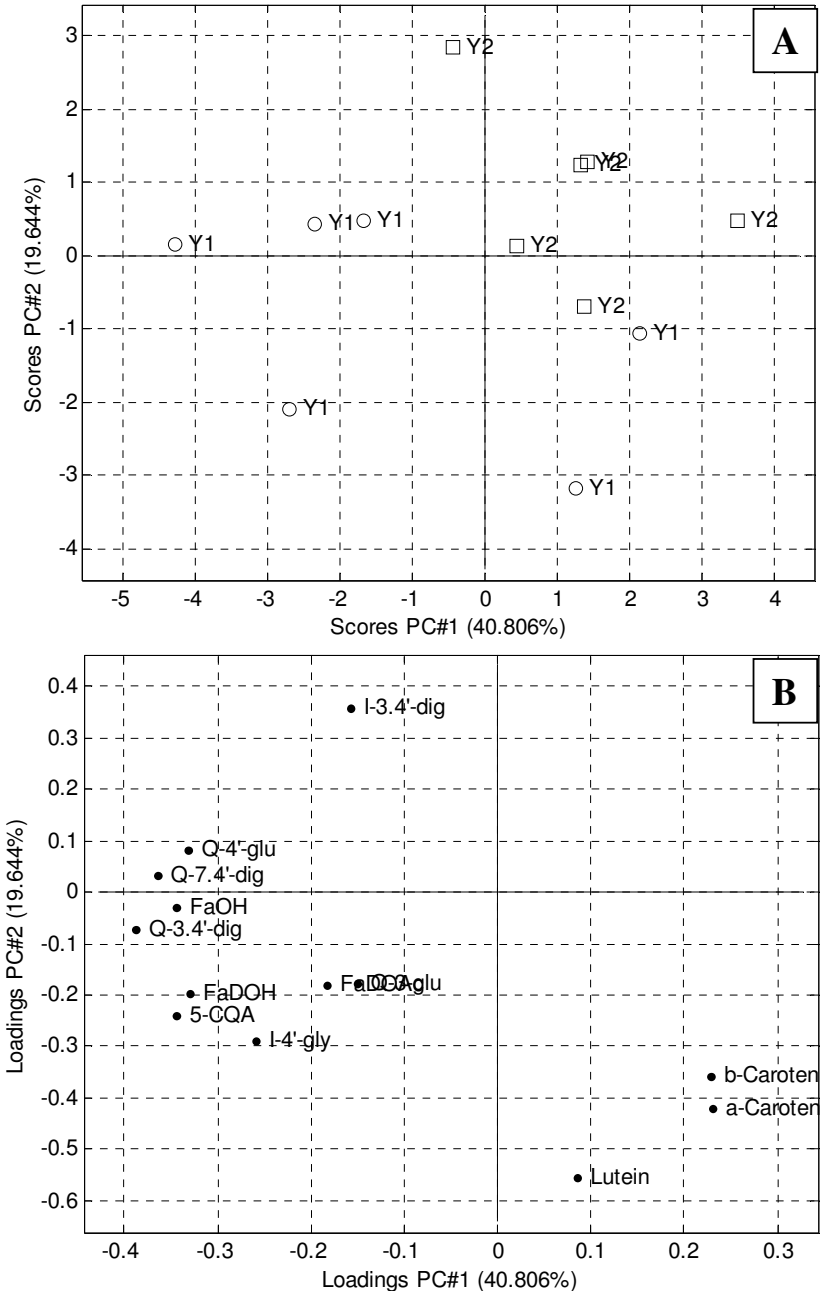


Figure 26. Score plot (A) and loading plot (B) from Principal Component Analysis of carotenoids, flavonoids, phenolic acids and polyacetylenes in the intervention diets and samples from two growth years (○: year 1 (Y1) and □: year 2 (Y2)).

5.3.5 General conclusions

The most abundant compound in the intervention diets was Q-3,4'-diglu together with β-carotene and Q-4'-glu. Other carotenoids and flavonoids were also found as well as 5-CQA. The major polyacetylene in the intervention diets was FaOH in contrast to the carrots, where FaDOH was the most abundant polyacetylene.

In general, no systematic differences were observed between the intervention diets prepared of cultivated crops from the three growth systems, even though a few significant differences were observed in the content of the individual secondary metabolites. Hence, the growth system did not have a significant impact on the content of the selected secondary metabolites in the intervention diets in agreement with the results obtained on the cultivated crops. The content of secondary metabolites was generally lower in year 2 and multivariate data analysis indicated a possible grouping of the intervention diets based on growth year. However, a significant year-to-year variation was only observed for some of the individual flavonoids and polyacetylenes in the intervention diets.

The storage and processing of the cultivated crops for preparation of the intervention diets had a considerable impact on the content of most of the secondary metabolites probably due to their sensitivity towards oxygen, light, and heat. The effects were both positive and negative with regard to concentration and the more water-soluble compounds (*e.g.* flavonoids and phenolic acids) appeared to be more susceptible towards storage and processing than, for instance, the more lipid-soluble carotenoids.

5.4 Secondary metabolites in human samples and their bioavailability

5.4.1 Carotenoids

The most abundant carotenoid in plasma samples from baseline and after the intervention was β-carotene (Table 11). Furthermore, α-carotene and lutein were found together with zeaxanthin, β-cryptoxanthin, and lycopene, which are all among the predominant carotenoids normally found in human plasma *(25, 75).* The focus in this project was on lutein, α-carotene, and β-carotene in plasma as zeaxanthin, β-cryptoxanthin, and lycopene were not detectable in the intervention diets *(Manuscript III)*.

The relative bioavailability of carotenoids is often determined as the increase in plasma concentration *(93, 96)* and the intervention had a significant impact on the plasma status ($p < 0.001$). It increased after the intervention *(Manuscript III)* in agreement with previous intervention studies with diets rich in carotenoids *(62, 63, 261, 262)*. The plasma status varied considerably between the subjects (Table 11), which could be due to, for instance, the physiological and nutritional status of the subjects *(25)*, and their ability to absorb carotenoids *(263)*.

Table 11. Plasma status of carotenoids (average ± standard deviation, n = 18; µg/mL) at end time after consumption of diets produced from cultivated crops from three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (1 and 2). Plasma statuses with different letters within a row are significantly different ($p < 0.05$) after correction of possible variation in baseline concentrations and between periods *(Manuscript III)*.

		Plasma status (µg/mL)		
		Growth system		
Year	**Carotenoid**	**C**	**OA**	**OB**
1	Lutein	0.23 ± 0.09 ^{A}	0.19 ± 0.09 ^{B}	0.19 ± 0.07 ^{B}
1	α-carotene	0.26 ± 0.09	0.23 ± 0.09	0.26 ± 0.08
1	β-carotene [a]	0.69 ± 0.3 ^{A} 0.40 ± 0.09 ^{C}	0.49 ± 0.2 ^{B}	0.55 ± 0.2 ^{B}
2	Lutein	0.18 ± 0.05 ^{A}	0.16 ± 0.05 ^{C}	0.17 ± 0.05 ^{B}
2	α-carotene	0.26 ± 0.09	0.26 ± 0.07	0.26 ± 0.09
2	β-carotene	0.62 ± 0.4	0.65 ± 0.4	0.63 ± 0.4

[a]: the plasma status of β-carotene in growth system C was significantly different between the two replicates ($p = 0.01$). Thus, the average (± standard deviation, n = 9) of each replicate is shown.

The plasma status of lutein differed significantly between growth systems across growth years ($p < 0.001$). It was significantly higher after the intervention with the conventional diet in comparison with the diets based on organically produced crops in both growth years ($p < 0.002$, Table 11). No other significant differences between growth systems across growth years ($p < 0.001$) were observed after correction for possible period effects and differences in baseline levels. No significant carry-over effects were observed *i.e.* the effect of the diet from the previous period was insignificant on the plasma status at the following period ($p > 0.05$). However, the plasma status of

β-carotene in subjects given the conventionally based diet was also significantly different from the organically based diets in year 1 ($p < 0.007$), but the same trend was not observed in year 2 (Table 11) *(Manuscript III)*. The composition of the food matrix normally affects the bioavailability of carotenoids *(93, 94)* and differences in the food matrix between the diets could be expected due to the different fertilization strategies and levels. Nevertheless, the growth system did not have any systematic effects on the plasma status of carotenoids after the intervention, except for lutein *(Manuscript III)*, similarly to previous human intervention studies of carotenoids with supplementation of organic and conventional carrots *(62)* and tomato puree *(63)*. The results were in agreement with the insignificant differences observed in the content of carotenoids in carrots and intervention diets, even though an insignificant difference in the crops or intervention diets does not necessarily imply a non-significant difference in the bioavailability of carotenoids *(264)*.

In contrast to previous studies *(265, 266)*, no significant correlation between dietary intake and plasma status of carotenoids was observed, which indicated that the plasma status of carotenoids was not solely determined by the dietary intake, but other parameters might have an impact, for instance, the physiological status of the subjects, and their ability to absorb carotenoids. An increase in plasma status after intervention with diets rich in carotenoids has also previously been observed, but no major impact on human health parameters *e.g.* immune parameters and markers of oxidative stress were observed in those studies *(62, 261)*, while health parameters were not investigated in this study. Nevertheless, it seems unlikely that the few observed significant differences due to different growth systems will have considerable human health impacts due to the small concentration differences observed *(7)*.

5.4.2 Flavonoids

Urinary flavonoids have been suggested as useful biomarkers for fruit and vegetable intake *(267, 268)*, even though only 1–4 % is normally excreted in the urine with an intact flavonoid backbone due to the extensive metabolisation and conjugation in the small intestine *(120, 147)*. The urinary excretion of flavonoids could therefore be helpful when investigating possible health effects of flavonoids *(267)*. Q'-4-glu had a higher abundance in the intervention diets than, for instance, I-4'-gl (Figure 23), but the extent of urine excretion is markedly affected by minor structural differences and the urinary excretion of I-4'-glu was considerably higher in comparison with Q-4'-glu in a

previous intervention study with onion consumption. This could be due to preferential absorption of I-4'-glu or a post-absorption conversion of Q-4'-glu to I-4'-glu via 3'-*O*-methylation *(269)*.

The deglycosylation of the flavonoids during preparation of the intervention diets was limited, so the flavonoids had to be hydrolysed by intestinal enzymes or the colonic microflora before they could be absorbed *(26, 147)*. The absorption of the most abundant group of compounds in the intervention diets, quercetin glucosides, has previously been estimated to 20–50 % in humans *(120)*.

The health effects of secondary metabolites depend on the amounts ingested and their bioavailability *(26)*, but the intake of flavonoids was insignificantly different between the conventionally and organically based intervention diets (Figure 23). In general, the bioavailability of polyphenols varies greatly between the individual compounds *(26)* and it is affected by minor structural differences as mentioned earlier with the urinary excretion *(269)*. The flavonoids identified in the intervention diets were mainly glucosidic conjugates, which together with aglycones are expected to have a greater bioavailability than other glycosides *(26, 120, 270)*.

The content of flavonoids in onions and intervention diets was not significantly affected by the growth system, but it cannot automatically be assumed that the human bioavailability will not be affected by the growth system either as mentioned earlier with carotenoids *(264)*. The bioavailability of flavonoids could possibly be affected by the different fertilization strategies and levels applied in the three growth systems, and as a result differences in the health effects of the organic compared to the conventional food products might be induced. However, the plant matrix has been proposed to have minor effects on the bioavailability of flavonoids *(271)*, although the bioavailability was higher from onions than apples *(272)*, which might be due to the different flavonoids in the different food products. To my knowledge, only one study has so far compared the impact of different agricultural production systems on the bioavailability or related parameters in humans *(64)*. They found that the urinary excretion of quercetin and kaempferol was higher after intake of a diet based on organically than conventionally produced foods, but it was a market-orientated supply study *i.e.* differences in cultivars, geographical locations, and physiological age etc. could also have induced the observed variation. Similarly, a significant increase in the total antioxidant capacity in human plasma was observed after two weeks consumption of a Mediterranean organic diet in comparison with a conventional diet based on products from local green grocers *(273)*.

5.4.3 Phenolic acids

The only phenolic acid quantified in the intervention diets was 5-CQA, which is rapidly absorbed *(16)* and extensively metabolised *(184)*. The excretion of 5-CQA is mainly in the conjugated form *(168, 186)* and the human urinary excretion of intact 5-CQA has been estimated to 1.7 % *(184)*, which is in a similar range to flavonoids *(120, 147)*.

The absorption of phenolic acids varies between the individual compounds *(187, 188)* and esterification reduces the absorption *(16)*. Thus, the absorption of 5-CQA is considerably lower than CA and has been estimated to 35 % and 95 %, respectively, in human ileostomy patients *(187)*. CA was not detectable in the intervention diets, so the degradation of 5-CQA to quinic acids and CA was not observed, but it would have increased the absorption of the phenolic acids in the subjects in the present study.

Similar to other secondary metabolites, the health effects of phenolic acids depend on the amounts consumed and their bioavailability *(26)*, but the intake did not vary significantly between the intervention diets based on the organically and conventionally produced crops (Figure 24). However, the bioavailability of phenolic acids could possibly still differ between the conventional and organic growth systems, despite insignificant differences in the intervention diets *(264)*. The bioavailability of phenolic acids is normally lower than for flavonoids *(157)*, but animal studies have shown that phenolic acids are bioavailable *(185, 188)*. To my knowledge, no previous studies have investigated the effect of growth system on the bioavailability of phenolic acids, but it might have an impact as expected with flavonoids *(188)*. However, the health effects would also be affected by the extensive metabolisation of phenolic acids as the metabolites generally seem to have a lower antioxidant activity *(184)*.

5.4.4 Polyacetylenes

FaOH was the most abundant compound in the intervention diets. It is also the most bioactive of the three polyacetylenes (FaDOH, FaDOAc, and FaOH) *(196, 211, 219)*, but synergistic effects of FaOH and FaDOH have been observed on cancer cells, which improves their bioactivity *(219)*.

A recent human intervention study with supplementation of carrot juice showed that the maximum plasma concentration levels could be expected to have health-promoting effects *(213)*, although polyacetylenes have previously been considered undesirable in food products *(214, 215)*. In the

present study, the average daily intake of FaOH in year 1 was 6.3 mg FaDOH-eq. The intake was most likely comparable to the concentration levels of FaOH in the carrot juice given to the subjects in the above mentioned human intervention study *(213)*, even though the actual concentration of FaOH could not be determined due to unavailability of commercial standards. Thus, FaOH is also expected to be rapidly absorbed and biologically available in the present study, and probably also present at concentration levels expected to have beneficial health effects. The bioavailability of FaDOH and FaDOAc is unknown and further studies are needed in this area of research.

The health effects of polyacetylenes are most likely dependent on the intake and bioavailability as seen for other secondary metabolites *(26)*. It is presently difficult to evaluate the impact of growth system on the health effects of polyacetylenes due to the limited knowledge about their bioavailability, even though no significant differences were observed in the content of polyacetylenes in the organically and conventionally based intervention diets (Figure 25).

5.4.5 General conclusions

The health effects of secondary metabolites are dependent on the ingested amounts and the bioavailability of the compounds ingested. In general, the intake was not significantly different between the three intervention diets, but it does not necessarily imply insignificant differences in the bioavailability. The bioavailability might be affected by variation in food matrices due to different fertilization strategies and levels applied in the organic and conventional growth systems.

However, the growth system (organic versus conventional) did not have any systematic impact on the plasma status of carotenoids, except a significantly higher plasma concentration of lutein after the conventional in comparison with the organic intervention diets. The small concentration differences of lutein are unlikely to have considerable health effects and the results were in agreement with the insignificant differences detected in the carrots and intervention diets. Nevertheless, the plasma status of carotenoids generally increased significantly after the human intervention with diets rich in carotenoids.

The absorption of flavonoids and phenolic acids is generally low and the bioavailability varies between compounds. In flavonoids, the sugar moiety is the major determinant of the bioavailability, while esterification reduces the bioavailability of phenolic acids. The knowledge about the bioavailability of polyacetylenes is limited, but a previous intervention study showed that the

compounds are bioavailable and can occur in plasma at concentrations, which are expected to have health-promoting effects. At the moment, it is difficult to evaluate the impact of growth system on the health effects of flavonoids, phenolic acids and polyacetylenes in organic and conventional produced diets due to few previous intervention studies and limited knowledge about the bioavailability of many secondary metabolites.

6 Conclusions

Conventional and new extraction methods were compared for chemical analysis of flavonoids, phenolic acids, and polyacetylenes in crops and intervention diets leading to new rapid and effective methods. The validated methods set new standards for chemical analysis of secondary metabolites. The one-step PLE method for analysis of flavonoids and phenolic acids showed considerable improvements and possibilities, especially the simultaneous extraction and clean-up by in-cell addition of C18-material, which can be applied in future PLE studies. Furthermore, the extraction of polyacetylenes was based on a novel technology, ULP, and combined with the optimised HPLC method, which showed a considerable reduction in run time. ULP is a very rapid and effective methodology compared with the conventional method and can be applied with great advantage in future similar studies of polyacetylenes or other secondary metabolites.

The major carotenoids in the carrots, intervention diets, and plasma samples were β-carotene, α-carotene, and lutein, while the main phenolic acid in the carrots, potatoes, and intervention diets was 5-CQA. Furthermore, the following three polyacetylenes were identified in carrots and the intervention diets: FaDOH, FaDOAc, and FaOH. The onions contained seven different isorhamnetin- and quercetin-glycosides with Q-3,4'-diglu and Q-4'-glu being the major compounds, and a comparable flavonoid profile was found in the intervention diets.

In general, no systematic differences in the content of carotenoids, flavonoids, phenolic acids, and polyacetylenes in the crops and intervention diets between the growth systems across growth years were found, though a few significant differences were observed for individual secondary metabolites in crops or intervention diets. Hence, overall the agricultural production system (organic versus conventional) did not have a significant impact on the ability of certain crops to synthesise selected secondary metabolites or on the content of secondary metabolites in the intervention diets. The growth year was important for the levels of selected flavonoids and polyacetylenes in the crops and intervention diets with a higher content in the first growth year with higher temperatures and more precipitation than in the second. However, the same year-to-year variation was not observed for all secondary metabolites studied and it is difficult to make a general statement about the importance of the weather conditions for the content of secondary metabolites in food products.

The study indicated that the quantities reaching our plate are not necessary the same as the ones found in the individual food products. Storage and processing procedures during preparation of the intervention diets had a considerable impact on most of the secondary metabolites probably due to their sensitivity towards oxygen, heat, and light exposure. The concentrations were both negatively and positively affected, but the more water-soluble compounds such as flavonoids and phenolic acids appeared to be more susceptible to storage and processing than, for instance, the lipid-soluble carotenoids.

The health effects of organic and conventional food products depend on the amounts ingested and the bioavailability of the compounds. The intake of secondary metabolites did not vary significantly between the conventional and organic diets, but the bioavailability could be affected due to different fertilization strategies and levels applied and thereby induce differences in the food matrix. However, the growth system did not cause any systematic differences in the plasma status of carotenoids, except for lutein, after consumption of diets based on organically and conventionally food products. Nevertheless, the small concentration differences observed in plasma status seem unlikely to be of significance for human health, although other health parameters (*e.g.* immune response and markers of oxidative stress) were not tested in this study. The most abundant flavonoids, phenolic acids, and polyacetylenes in the intervention diets seem to be bioavailable and might have health effects. However, it was not possible to conclude whether the growth system was of significance for the bioavailability of these secondary metabolites based on the literature search.

The study was performed under well-controlled growing conditions and sampling procedures, and as a cultivation study, which is considered the most valid type of comparative study. Nevertheless, the content of presumable health-promoting secondary metabolites was not significantly different between organic and conventional food products and the hypothesis pointed out in the objectives ought to be rejected. Hence, the perception about organic food products being healthier than conventional was not supported and can probably not be attributed to the secondary metabolites included in the present study, although further research regarding the impact of growth system on the bioavailability of flavonoids, phenolic acids, and polyacetylenes is needed. The study has contributed to the knowledge about organic food products indicating that the selected secondary metabolites (carotenoids, flavonoids, phenolic acids and polyacetylenes) are not of major importance for the expected greater health effects of organic food products compared to conventional ones. The results are of interest to both the consumers and to the scientists working in the area of organic agriculture.

7 Perspectives

This study showed that the content of potential health-promoting secondary metabolites in food products and diets was generally not significantly affected by the agricultural production system (organic versus conventional) and neither was the plasma status of carotenoids. Nevertheless, it is necessary to assess the effect of growth system on the bioavailability of flavonoids, phenolic acids, and polyacetylenes because a non-significant difference in the crops and intervention diets does not automatically imply non-significant differences in the bioavailability *(264)*. However, few studies have previously investigated the impact of growth system on the bioavailability of both primary and secondary metabolites, but it is essential for future research in order to evaluate possible health effects of organic food products *(7, 8)*. Furthermore, it is necessary to gain further knowledge about the expected health effects of secondary metabolites and additional *in vivo* studies are needed. The knowledge about the mechanisms of secondary metabolites in the human body is still not clear and more research is needed to study the impact of secondary metabolites on human health and diseases.

The impact of growth system on several other chemical compounds such as minerals, trace elements, vitamins, and phytate in crops is also studied in the OrgTrace project. It will be interesting to see whether interactions between these different groups of chemical compounds are present and if the crops can be grouped according to the agricultural production system by including all chemical compounds and apply multivariate data analysis. In the OrgTrace project, selected chemical compounds were studied, but other secondary metabolites of possible interest to human health could also be investigated, for instance, glucosinolates and proanthocyanidins, which are abundant in white cabbage and cereals, respectively. Furthermore, an impact of growth system might be significant in other food products, *e.g.* fruits and berries, in contrast to the crops included in this study (vegetables, beans, and cereals). The content of secondary metabolites has previously been shown to vary between cultivars *(80, 134, 209)*. The concentrations might be higher in cultivars with high resistance towards, for instance, pathogen infection and pest attack, compared with susceptible cultivars due to the function of secondary metabolites as defence compounds in plants *(30)*. Thus, this could be a possible area of future research, which would be beneficial from both a health and an agricultural perspective.

The targeted approach applied in this study was driven by knowledge about possible health effects of the selected secondary metabolites, but another possibility would be a non-targeted approach applying metabolomics. Current studies of secondary metabolites focus on specific compounds and

their health effects, but high-throughput analytical technologies (HPLC/GC and NMR/MS) in combination with multivariate data analysis and hierarchical cluster analysis make it possible to do simultaneous analysis of numerous metabolites of *(274)*. A metabolomic fingerprinting approach can be used to discriminate samples based on growth systems *(274, 275)* followed by identification of the discriminating markers and evaluation of their importance for human health *(274)*.

The health benefits of organic food products might be associated with the content of nutrients and secondary metabolites *(5)*, but generally it is not scientifically well-documented *(11, 38)* and not in the present study either. However, the health benefits are also associated with the lower levels of pesticide residues in organic food products compared with conventional *(5)*. Pesticide residues are not present or in lower levels in organically grown crops than in conventionally ones *(7, 11, 276, 277)*, but the levels in the conventionally grown crops are normally below the maximum limits allowed *(11, 276)* and are not expected to possess a health risk to the consumers *(12)*. However, the health risks are normally tested on individual pesticides *(277)* and there is growing concern about the cocktail effects of multiple pesticide residues on human health. Hence, it is essential to investigate the possible health impacts of cocktail effects of pesticide residues in concentrations below the maximum limits allowed to gain further knowledge about whether the conventional food products are unhealthier than organic.

The impact of growth system on the content of secondary metabolites was insignificant in the present study, but larger differences in the fertilization levels might have induced differences between the growth systems. However, the agricultural production systems applied in this study reflect common farmer practise in Danish crop production and increased levels of fertilization are also allowed in organic agriculture with the new EU regulation, which came into force in 2009 (http://pdir.fvm.dk). Thus, the allowed fertilization levels in organic agriculture come closer to the levels normally applied in conventional agriculture and for that reason it seems unlikely that considerable differences in the content of secondary metabolites between the growth systems will be induced. Hence, the growth system might be of minor importance for the content of secondary metabolites in food products and it might be more important to consume a balanced diet rich in fruits and vegetables *(38)*. Thus, future studies should also focus on the general human health effects of fruits and vegetables in order to gain further knowledge about the human metabolism of secondary metabolites and the mechanisms behind their potential health effects.

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9 Appendix

9.1 Appendix I. Chemical method for analysis of flavonoids and phenolic acids in intervention diets and method validation.

9.1.1 Reagents and chemicals

Methanol (MeOH, HPLC-grade; Rathburn Chemicals Ltd., Walkerburn, Scotland) and Milli-Q water (18 MΩ; Millipore, Billerica, MA, US) were used for standards and eluents. Dimethyl sulfoxide (DMSO, >99%) and formic acid (98−100 %) were purchased from Merck (Darmstadt, Germany). The following standards were purchased: quercetin (Q) and isorhamnetin (I) from Extrasynthése (Genay Cedex, France), quercetin-3,4'-diglucoside (Q-3,4'-diglu) from Polyphenols (France), quercetin-7,4'-diglucoside (Q-7,4'-diglu) from Apin (Abingdon, United Kingdom), quercetin-4'-glucoside (Q-4'-glu) from Plantech (Berkshire, United Kingdom); and quercetin-3-glucoside (Q-3-glu, purity >90%), 5-*O*-caffeoylquinic acid (5-CQA, purity >95%), and caffeic acid (CA, purity >99%) from Sigma-Aldrich (Steinheim, Germany).

Individual standard stock solutions were prepared by dissolving the solid compounds in DMSO (100 µg/mL). Working solutions were prepared by ten-times dilutions of the stock solutions with MeOH. Stock solutions were stored at −80°C (<1 year), while the working solutions were prepared shortly before analyses and kept at −20°C until analyses.

9.1.2 Samples

The freeze-dried samples from the two-year human intervention study including two organic and one conventional intervention diet with two replicates of each were analysed for the content of flavonoids and phenolic acids. A detailed description of the human intervention study and the intervention diets can be seen in Manuscript III.

9.1.3 Analytical method

The chemical analysis involved pressurised liquid extraction and quantification by HPLC-UV with a similar principle as described in Paper I. One glass fiber filter (90 mm, Advantec) was placed at the bottom of the 66 mL extraction cells followed by 2.5 g C18-material (Sepra E-C18, 50 µm;

Phenomenex, Allerød, Denmark) mixed with 10 g glowed, chemically inert Ottawa sand (particle size 20-30 mesh; Fisher Chemicals, Fairlawn, NJ, US), and another glass fiber filter. The sample material (8 g) was mixed with Ottawa sand (32 g) and transferred to the extraction cell, which was filled up with Ottawa sand, and a glass fiber filter was placed at the top of the cell. The samples were extracted with 65 % aqueous MeOH using an ASE-200 (Dionex, Sunnyvale, CA, US) and the same extraction parameters as described in Paper I were used. The extracts were collected in vials and evaporated to almost dryness *in vacuo* at 30 ^{o}C on a rotary evaporator. The residues were redissolved in 65 % aqueous MeOH (5 mL) and filtered through a 0.20 µm filter before chemical analysis.

A Waters 2695 Alliance Separations Module was used for the chemical analysis of flavonoids in combination with a Waters 2996 Photodiode Array Detector (PDA) and a Waters 2487 Dual λ Absorbance Detector (Waters, Milford, MA, US) as described in Paper I. However, the gradient was adjusted to the following (1 mL/min): 5% B for 1 min, a linear gradient to 50% B for 34 min, a linear gradient to 100% B for 10 min, isocratic elution for 5 min, followed by a 1-min ramp back to 5% B, and re-equilibration for 7 min giving a total run-time of 58 min. The injection volume was adjusted to 50 µL.

The chemical structure of the flavonoids and phenolic acids was elucidated by MS and MS/MS analysis and a comparison of retention times, UV, and MS data with commercially available standards (Q-3,4'-diglu, Q-7,4'-diglu, Q-4'-glu, Q-3-glu, Q, 5-CQA, and CA) as described in Paper I.

9.1.4 Validation of analytical method

The linear range of measurement and sensitivity were determined for Q-7,4'-diglu, Q-3,4'-diglu, Q-4'-glu, Q-3-glu, and Q by varying the injection volumes of the standards (10 µg/mL), which provided comparable results to serial dilution of standards *(Paper I)*. The selectivity was evaluated by extraction of blank samples (Ottawa sand) because comparable diet samples without flavonoids or phenolic acids were not available. The precision was determined by five replicate analyses. The limit of detection (LOD) and quantification (LOQ) were determined as three and ten times the standard deviation. The precision samples were used for determination of LOD and LOQ and diluted if necessary to obtain a concentration close to the expected LOQ. The trueness of the method was determined by recovery experiments using an in-house diet sample as reference

material because no certified reference samples or samples without or with a low concentration of the relevant flavonoids or phenolic acids were available. The recovery was determined by extraction of five reference samples with and without addition standard addition. An internal standard was not included in the analysis because it was not possible to find a suitable reference standard, which had similar chemical and physical properties, did not co-elute with the compounds of interest, and was not present in the matrix.

The linear range of measurement of flavonoids and phenolic acids (Q-7,4'-diglu, Q-3,4-diglu, Q-4'-glu, Q-3-glu, Q, 5-CQA, and CA) was 0.5−10 μg/mL corresponding to 0.3−6.3 μg/g dry weight (R^2 > 0.998), except for Q-7,4'-diglu (1.5−10 μg/mL) (Table IA). The sensitivity was $2.2–7.0\times10^4$ AU/(μg/mL) *(Paper I)*. LODs and LOQs ranged from 0.2 to 1.0 and 0.8 to 3.3 μg quercetin-equivalents/g dry weight (μg q-eq/g dw), respectively (Table IA). The selectivity was good as no interfering or co-eluting compounds were detected in the blank samples. The precisions were in an acceptable range for the purpose of the method (relative standard deviation (RSD): 5.5−11%), except for Q because the concentration was close to LOD (Table IA). Recovery experiments were only possible for Q-7,4'-diglu and Q-3-glu due to the available amounts of commercial standards in comparison with the high content of flavonoids and phenolic acids in the reference material. The recovery was satisfactory (Q-7,4'-diglu: 92 % and Q-3-glu: 85 %, Table IA) and more than 98 % of the extractable compounds were recovered within two consecutive extraction cycles.

Quality assurance was performed by including the in-house diet sample as reference material in each series of analyses yielding an average RSD of 8.2 % for Q-7,4'-diglu, Q-3,4-diglu, Q-4'-glu, Q-3-glu, and 5-CQA (n = 4). The samples were analysed in duplicates (incl. weighing and extraction) in two different series of analysis, which yielded an average RSD of 7.6 % for the same flavonoids and phenolic acids as above (n = 12).

Table IA. The linear range of measurement, limit of detection (LOD, n = 5) and quantification (LOQ, n = 5), the precision (relative standard deviation (RSD), %, n = 5) and the recovery (%) of the analysis of flavonoids and phenolic acids in the intervention diets. The concentrations are expressed in µg quercetin-equivalents/g dry weight (µg q-eq/g dw).

Compound	**Linear range of measurement (µg q-eq/g dw)**	**LOD** [b] **(µg q-eq/g dw)**	**LOQ** [b] **(µg q-eq/g dw)**	**Precision** [b] **(RSD, %)**	**Recovery (%)**
Q-3,7,4'-trigly	- [a]	- [c]	- [c]	- [c]	- [d]
Q-7,4'-diglu	0.9─6.3	0.3	0.9	11	92
Q-3,4'-diglu	0.3─6.3	0.9	3.1	7.7	- [d]
I-3,4'-digly	- [a]	- [c]	- [c]	- [c]	- [d]
Q-3-glu	0.3─6.3	0.4	1.3	5.6	85
Q-4'-glu	0.3─6.3	0.6	2.1	5.5	- [d]
I-4'-gly	- [a]	- [c]	- [c]	- [c]	- [d]
Q	0.3─6.3	0.2	0.8	24	- [d]
5-CQA	0.3─6.3	1.0	3.3	11	- [d]
CA	0.3─6.3	- [c]	- [c]	- [c]	- [d]

Q-3,7,4'-trigly: quercetin-3,7,4'-triglycoside, Q-7,4'-diglu: quercetin-7,4'-diglucoside, Q-3,4'-diglu: quercetin-3,4'-diglucoside, I-3,4'-digly: isorhamnetin-3,4'-diglycoside, Q-3-gly: quercetin-3-glucoside, Q-4'-glu: quercetin-4'-glucoside, I-4'-gly: isorhamnetin-4'-glycoside, Q: quercetin, 5-CQA: 5-*O*-caffeoylquinic acid, and CA: caffeic acid.

[a] Linearity not determined due to unavailability of commercial standards.

[b] Concentrations in the samples used for determination of LOD, LOQ and precision: Q-7,4'-diglu: 0.8, Q-3,4'-diglu: 4.0, Q-3-glu: 2.3, Q-4'-glu: 2.6, 5-CQA: 3.0 and Q: 0.3 ug q-eq/g dw.

[c] Not determined

[d] Recovery not determined due to lack of sufficient amounts of commercially available standards.

9.2 Appendix II. Chemical method for analysis of polyacetylenes acids in intervention diets and method validation.

9.2.1 Reagents and chemicals

Methanol (MeOH), ethylacetate (EtOAc) and acetonitrile (ACN) were purchased from Rathburn Chemicals Ltd. (HPLC-grade, Walkerburn, Scotland). Dimethyl sulfoxide (DMSO, >99 %) and formic acid (98-100 %) were purchased from Merck (Darmstadt, Germany). Milli-Q water (18 MΩ, Millipore, Billerica, MA, US) was used for eluents.

Falcarindiol (FaDOH, 97 % purity) was purchased from Atomax Chemicals Co., Ltd. (Shenzhen, China) and a standard stock solution of FaDOH was prepared by dissolving the oily compound in MeOH (1 mg/mL). Working solutions of FaDOH (10 µg/mL) were prepared by diluting the stock solutions with MeOH. Stock solutions of FaDOH were stored (<1 year) at −80 ^{o}C, while the working solutions were prepared shortly before analyses and kept at −20 ^{o}C until analyses.

9.2.2 Samples

The freeze-dried samples from the two-year human intervention study including two organic and one conventional intervention diet with two replicates of each were analysed for the content of polyacetylenes. A detailed description of the human intervention study and the intervention diets can be seen in Manuscript III.

9.2.3 Analytical method

The chemical analysis involved liquid extraction using an Ultrasonic Liquid Processor as previously applied for analysis of polyacetylenes in carrots *(Manuscript I)*. The freeze-dried material (4 g) was extracted three times with 30 mL of EtOAc at room temperature for 60 s using an ultrasonic liquid processor operated at 10 Watt (Microson XL 2000, Misonix, Newtown, CT, US). The extracts were centrifuged (5 min, 3000 *g*, 20 ^{o}C) and 25 mL of each supernatant was collected. The supernatants were combined and evaporated to dryness *in vacuo* at 30 ^{o}C on a rotary evaporator. The residue was redissolved in MeOH (5 mL) and filtered through a 0.45 and a 0.20 µm filter before chemical analysis.

A Waters 2695 Alliance Separations Module in combination with a Waters 2996 Photodiode Array Detector (PDA) and a Waters 2487 Dual λ Absorbance Detector (Waters, Milford, MA, US) was used for the chromatographic analysis of polyacetylenes as described in Manuscript I, but with the following adjustments of the gradient program: 70 % B for 5 min, a linear gradient to 86 % B for 13 min, a linear gradient to 100 % B for 2 min, isocratic elution for 10 min, followed by a 2-min ramp back to 70 % B, and re-equilibration for 5 min giving a total run-time of 37 min. The injection volume was adjusted to 30 µL. The polyacetylenes were identified using MS as described in Manuscript I.

9.2.4 Validation of analytical method

The linear range of measurement and the sensitivity were determined for FaDOH by varying the injection volumes of the standard, which provided comparable results to serial dilution of standards. The selectivity was evaluated by extraction of blank samples (*i.e.* extractions without addition of diet material) because comparable diet samples without polyacetylenes were not available. The precision was determined by four replicate analyses. The limit of detection (LOD) and quantification (LOQ) were determined as three and ten times the standard deviation. The precision samples were used for determination of LOD and LOQ. The trueness of the method was determined by recovery experiments using an in-house diet sample as reference material because no certified reference samples or samples without or with a low concentration of the polyacetylenes were available. The recovery was determined by extraction of four reference samples with and without addition of the standard FaDOH, which was the only commercially available standard. An internal standard was not used in the analysis because it was not possible to find a suitable reference standard, which had similar chemical and physical properties, did not co-elute with the compounds of interest, and was not present in the matrix.

The linear range of measurement for FaDOH was 0.3─58 µg/mL, which corresponds to 1.0─109 µg FaDOH/g dry weight (µg FaDOH/g dw) ($R^2 > 0.999$) (Table IIA) *(Manuscript I)*. The linear range of measurement of FaDOAc and FaOH were in the same range as FaDOH, when tested by varying the injection volume of a sample, but exact concentration levels could not be obtained due to lack of commercially available standards. The sensitivity of FaDOH was 8.6×10^4 AU/(µg/mL). The selectivity was good as no major interfering or co-eluting compounds around the peaks of interest were detected in blank samples. The LODs were 0.5, 0.2, and 1.3 µg FaDOH-eq/g dw and the

LOQs were 1.7, 1.0, and 4.2 µg FaDOH-eq/g dw of FaDOH, FaDOAc, and FaOH, respectively (Table IIA). The precisions were in an acceptable range for the purpose of the present study (relative standard deviation (RSD): 3.2─6.6 %, Table IIA). The recovery of FaDOH was 129 % (Table IIA) with three extraction cycles, which was acceptable, although in the upper acceptable range.

Quality assurance was performed by including an in-house sample as reference material (a mix of freeze-dried diets from year 2) in each series of analyses, which yielded an average RSD of 7.5 % for FaDOH, FaDOAc and FaOH (n = 4). The samples were analysed in duplicates (incl. weighing and extraction) in two different series of analysis yielding an average RSD of 5.8 % for FaDOH, FaDOAc and FaOH (n = 12).

Table IIA. The linear range of measurement, limit of detection (LOD, n = 4) and quantification (LOQ, n = 4), precision (expressed as the relative standard deviation, RSD, n = 4) and the recovery (%, n = 4) of the analysis of polyacetylenes in intervention diets. Concentrations are expressed in µg falcarindiol-equivalents/g dry weight (µg FaDOH-eq/g dw).

	Linear range of measurement (µg FaDOH-eq/ g dw)	**LOD [a] (µg FaDOH-eq/g dw)**	**LOQ [a] (µg FaDOH-eq/g dw)**	**Precision (RSD, %) [a]**	**Recovery (%)**
FaDOH	1.0-109	0.5	1.7	4.2	129
FaDOAc	1.0-109	0.2	1.0	3.2	- [b]
FaOH	1.0-109	1.3	4.2	6.6	- [b]

FaDOH: falcarindiol, FaDOAc: falcarindiol-3-acetate, and FaOH: falcarinol.

[a] Concentrations in samples used for determination of LOD, LOQ and precision: FaDOH: 4.0, FaDOAc: 2.5, and FaOH: 6.4 µg FaDOH/g dw.

[b] Recovery not determined due to unavailability commercial standards.

9.3 Appendix III. Calculation of the expected concentration of secondary metabolites in the intervention diets.

Falcarindiol (FaDOH) from the conventional growth system (year 1) is used as an example.

Average concentration of FaDOH in the carrots = 26 µg/g fresh weight

Average daily content of carrots in the intervention diets = 173 g fresh weight/10 MJ

Expected content of FaDOH in the intervention diets

= 26 µg/g fresh weight × 173 g fresh weight/10 MJ = 4.5 mg/10 MJ

Content of FaDOH in the intervention diets determined by chemical analysis = 2.8 mg/10 MJ

The difference between the expected content of FaDOH and the content determined by chemical analysis = (2.8 mg/10 MJ – 4.5 mg/10 MJ) / 4.5 mg/10 MJ = – 38 %

Hence, the content of FaDOH was 38 % lower than expected. The same calculations were carried out for the two other growth systems and two growth years, and the average gain or loss was calculated. Similar calculations were performed for the other secondary metabolites.

10 Publications

Paper I

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Pressurised liquid extraction of flavonoids in onions. Method development and validation

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ABSTRACT

A rapid and reliable analytical method for quantification of flavonoids in onions was developed and validated. Five extraction methods were tested on freeze-dried onions and subsequently high performance liquid chromatography (HPLC) with UV detection was used for quantification of seven flavonoids.

The extraction efficiencies were lowest for the conventional water bath extraction compared to pressurized liquid extraction (PLE), ultrasonication, ultrasonic liquid processor, and microwave extraction, which yielded similar efficiencies. The reproducibility was in the same range (RSD: 1–11%) for all tested extraction methods. However, PLE was the preferred extraction method because the method can be highly automated, use only small amounts of solvents, provide the cleanest extracts, and allow the extraction of light and oxygen-sensitive flavonoids to be carried out in an inert atmosphere protected from light.

The method parameters: extraction temperature, sample weight, flush volume and solvent type were optimised, and a clean-up step was integrated in the PLE procedure by in-cell addition of C18-material to the extraction cells, which slightly improved the recovery and reproducibility of the method. The one-step PLE method showed good selectivity, precision (RSDs = 3.1–11%) and recovery of the extractable flavonoids (98–99%). The method also appeared to be a multi-method, i.e. generally applicable to, e.g. phenolic acids in potatoes and carrots.

1. Introduction

Flavonoids belong to the group of polyphenols and are secondary metabolites [1], which play an important role in the normal growth, development and defence of plants [2]. Polyphenols in general have antioxidant properties and are proposed to have beneficial health effects such as decreasing the risk of heart diseases and protection against cancer [3–5].

Onions are a rich source of flavonoids [6,7], which generally occur widely in the plant kingdom [3], and onions are one of the main sources of polyphenols in daily food intake [8–10]. The content of polyphenols varies in different plant parts [11,12] and between varieties due to genetic factors [13–15], environmental and cultural factors [1], as well as time of harvest, processing and storage [16].

The main flavonoids in onions are quercetin mono- and diglucosides [17,18], which account for up to 80% of the total content of flavonols, but kaempferol [17] and isorhamnetin derivates [18,19] as well as myricetin [2] have also been found (see chemical structures in Fig. 1).

Conventional liquid extraction methods, e.g. water bath or ultrasonication, with aqueous methanol or acetonitrile have been widely used as simple and easy methods for analysis of flavonoids [20,21]. Extraction of flavonoid aglycones has most often been done by acid hydrolysis [2,22,23], but harsh extraction conditions (e.g. acid or heating) should be avoided if intact flavonoid-glycosides are desired [21]. An increased risk of degradation is often seen with conventional extraction methods owing to long extraction times, where samples are exposed to light and oxygen, which facilitate the degradation of flavonoids [24,25].

More rapid and automated methods have recently been used, e.g. supercritical fluid extraction (SFE), pressurized liquid extraction (PLE) or microwave-assisted extraction (MAE). They are advantageous compared to conventional methods because they can be carried out in the absence of light and protected from oxygen, cope with the demand for a reduction in organic solvent consumption and improve the extraction time due to the possibility of working at elevated temperatures and/or pressures in inert atmospheres [26].

SFE has been used for extraction of polyphenols in grapes [27]. However, SFE of polyphenols requires a high percentage of organic modifiers, which is a disadvantage as it decreases the method selectivity [25]. PLE is advantageous because high temperatures and pressures can be applied, which accelerate the extraction [25], but its application in food analyses has been limited although it

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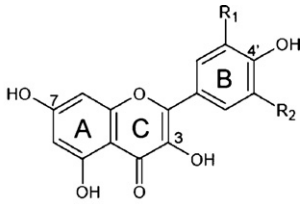
	Abbreviation	R_1	R_2
Quercetin [a]	Q	OH	H
Kaempferol [b]	K	H	H
Isorhamnetin [c]	I	OCH_3	H
Myricetin [d]	M	OH	OH



[a] 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one
[b] 3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one
[c] 3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)chromen-4-one
[d] 3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)chromen-4-one.

Fig. 1. Chemical structures of selected flavonols. The full chemical names are listed in the footnotes.

has been used to extract polyphenols from various food products [28,29]. MAE has been used for extraction of polyphenols from food products and plants and has the advantage of being rapid with a low solvent-consumption [32]. Another promising technique is Ultrasonic Liquid Processing (ULP), which has so far not been used for extraction of polyphenols from plants. The principle of ULP is the same as sonication, but shorter extraction times are expected due to a more intense cavitational action and greater disruption of the sample [33].

The objective of our study was to develop a rapid, sensitive and robust analytical method, which sets a new standard for extraction of flavonoids in onions. The recommended method of choice should be based on a comparison of the efficiency, reproducibility and sensitivity of conventional methods (water bath and ultrasonication) with more modern extraction methods (PLE, MAE and ULP). The best extraction method will be optimised with regards to relevant extraction parameters and validated.

2. Materials and methods

2.1. Reagents and chemicals

Methanol (HPLC-grade, Rathburn Chemicals Ltd., Scotland) and Milli-Q water (18 MΩ, Millipore, USA) were used for standards and eluents. Dimethyl sulfoxide (DMSO, >99%) and formic acid (98–100%) were purchased from Merck (Germany). The following standards were purchased: quercetin (Q) and isorhamnetin (I) from Extrasynthése (France), quercetin-3,4′-diglucoside (Q-3,4′-diglu) from Polyphenols (France), quercetin-7,4′-diglucoside (Q-7,4′-diglu) from Apin (United Kingdom), quercetin-4′-glucoside (Q-4′-glu) from Plantech (United Kingdom), and quercetin-3-glucoside (Q-3-glu, purity >90%), chlorogenic acid (ChlAcid, purity >95%) and caffeic acid (CafAcid, purity >99%) from Sigma–Aldrich (Germany).

Individual standard stock solutions were prepared by dissolving the solid compounds in DMSO (100 μg/mL). Working solutions were prepared by 10-times dilutions of the stock solutions with methanol. Stock solutions were stored at −80 °C (<1 year), while the working solutions were prepared shortly before analyses and kept at −20 °C until analyses.

2.2. Samples

The onion sample (*Allium cepa* var. *zittauer*, 40–60 mm, Soeris I/S) used for comparison of extraction methods and PLE method optimisation was organic and purchased in a local supermarket. The onion (*Allium cepa* var. *hytech*), carrot (*Daucus carota* var. *bolero*), potato (*Solanum tuberosum* var. *sava*) and white cabbage (*Brassica oleracea* var. *impala*) samples used for method validation were internal reference samples. Only the bulb of the onion sample was used for analysis comprising the eatable part by removing the top, bottom and outer leaves. The stalk and outer leaves of the white cabbage heads were also removed. The top and bottom of the carrots were discarded and both carrots and potatoes peeled. The samples were freeze-dried at 1×10^{-3} bar for approximately 2 days and afterwards crushed and homogenised in a blender. The samples were stored at −20 °C in a nitrogen atmosphere until analysis. In general, the samples were protected from light and oxygen during the entire sample preparation.

2.3. Extraction methods

Throughout the entire method comparison 60% aqueous methanol was used as extraction solvent because it has previously been used successfully in our conventional water bath method (data not shown).

2.3.1. Ultrasonication

The sample material (0.1 g) was mixed with 5 mL solvent and sonicated at room temperature for 60 min (Branson 5200 Ultrasonic Cleaner, 120 W) following the procedure suggested by Lin and Harnley [34].

2.3.2. Water bath

The sample material (0.5 g) was extracted with 50 mL solvent at 30 °C for 60 min in a shaking-water bath (Julabo SW 23). The extracts were then filtered through a filter paper (Whatman1) and evaporated close to dryness *in vacuo* at 30 °C on a rotary evaporator. The residue was redissolved in 5 mL solvent.

2.3.3. Microwave-assisted extraction

The sample material (0.1 g) was transferred to a glass tube and 5 mL solvent was added. The extraction was carried out in the tubes on a Microwave Synthesizer (Initiator, Biotage, 15 watt) at 60 °C for 2 min.

2.3.4. Ultrasonic liquid processor

The sample material (0.1 g) was transferred to a glass tube and 5 mL solvent was added. The microprobe was immersed into the tube and the extraction was performed at room temperature for 30 s using an ultrasonic liquid processor (Microson XL 2000, Misonix, 10 W).

2.3.5. Pressurised liquid extraction

The extractions were carried out using an ASE-200 (Dionex). The sample material (0.1 g) was mixed with 3 g of glowed and chemically inert Ottawa sand (particle size 20–30 mesh, Fisher Chemicals) and added to 5 mL extraction cells. Two glass fiber filters (90 mm, Advantec) were placed at the bottom and one at the top of the extraction cell, which was filled with Ottawa sand. The extraction parameters were: temperature: 40 °C, pressure: 1500 psi, pre-heating time: 1 min, static extraction time: 5 min, heating: 5 min, static cycles: 2, flush volume: 100% of cell volume, purge time with N_2: 60 s. The extracts were collected in vials and

the volume adjusted to 20 mL with extraction solvent in volumetric flasks.

All extracts were filtered through a 0.20 μm filter (Sartorius Minisart) before chemical analysis.

2.4. Quantification of flavonoids by HPLC–UV

A Waters 2695 Alliance Separation Module was used for the chemical analysis of flavonoids in combination with a Waters 2996 Photodiode Array Detector (PDA) and a Waters 2487 Dual λ Absorbance Detector. Empower 2 was used for instrument control and data acquisition. The chromatographic separation was carried out at a flow rate of 1.0 mL/min at 30 °C with an injection volume of 20 μL (temperature of autosampler: 5 °C). The column was a Phenomenex Prodigy RP-C18 column (4.6 mm × 250 mm, 5 μm). The A-eluent was a mixture of 0.1% formic acid in Milli-Q water (v/v) and methanol (90:10, v/v) and the B-eluent was 100% methanol. The gradient program was as follows: 5% B for 1 min, a linear gradient to 50% B for 34 min, a linear gradient to 100% B for 5 min, isocratic elution for 4 min, followed by a 1-min ramp back to 5% B and re-equilibration for 6 min giving a total run-time of 51 min. The PDA collected data from 190–500 nm (sampling rate: 1.0 spectra/s and resolution: 1.2 nm). The Dual λ Absorbance Detector was used for quantification and set at 254 nm (sampling rate: 1.0/s), which is in the range of the first UV-maximum of flavonoids (240–285 nm) [21]. The flavonoids were quantified relative to quercetin and it is common practise in analyses of bioactive compounds to quantify relative to one or more selected reference compounds [20].

2.5. Identification of flavonoids by HPLC–MS and MS/MS

Structure elucidation of flavonoids in onions was based on accurate mass measurements, isotopic pattern fit of measurement compared to theoretical (i-FIT values, Elemental Composition version 4.0 in MassLynx software version 4.1, Waters, USA) and MS/MS fragmentation patterns. An ultra performance liquid chromatograph (UPLC) interfaced to a quadropole time of flight tandem mass spectrometer (qTOF) was used for exact mass determinations and an HPLC interfaced to a triple quadropole mass spectrometer (HPLC–QqQ) was used to study fragmentation patterns. Afterwards, the identities were confirmed with available and affordable standards by comparison of retention times, UV and MS data.

2.5.1. UPLC–qTOF

The same mobile phases as those used for the HPLC–UV analyses were used for the UPLC (Waters) analyses. The column was a Waters Acquity BEH-C18 column (2.1 mm × 100 mm, 1.7 μm), which was retained at 30 °C. The HPLC gradient was scaled according to the column dimensions and gradient flow rate applied in UPLC in order to obtain a UPLC gradient with the same number of column volumes and linear velocity. The gradient program was as follows (flow rate of 0.2 mL/min): 5% B until 0.4 min, a linear gradient to 50% B until 14.6 min, a linear gradient to 100% B until 15.4 min, isocratic elution until 17.5 min, a 0.4 min ramp back to 5% B and re-equilibration for 2.1 min giving a total run-time of 20 min. A sample volume of 8 μL was injected using the partial loop with needle overfill mode. The qTOF (Waters/Micromass qTOF Ultima Global) used electrospray ionisation in negative ion mode. The operating conditions were: ion source temperature: 120 °C, desolvation gas (N_2): 350 °C, 600 L/h, cone gas: 50 L/h, capillary voltage: 3 kV, scan time: 1.0 scan/s and inter-scan delay time: 0.1 s. MassLynx software version 4.1 was used for instrument control and data acquisition and leucine enkephaline (554.2615 g/mol) was used as lock-spray mass (reference standard). External mass calibration (*m*/*z* 150–1000) was carried out before the analyses using a sodium formate solution containing 10 mM sodium hydroxide in isopropanol/0.2% formic acid (1:1, v/v).

2.5.2. HPLC–QqQ

A Waters 2699 Alliance Separations Module combined with a PDA (Waters 2996) and a QqQ (Waters Micromass Quattro LC) mass spectrometer with an electrospray interface was used for analyses. The same chromatographic conditions as in the quantitative analyses (see section above) were used. The initial scanning for flavonoids in onions included both negative and positive full scan (single MS) mode at different cone voltages (±10, 30, 50 and 70 V; *m*/*z* 100–850). MS/MS analyses were carried out at different collision energies (±10, 30 and 50 V) with a cone voltage of 30 V. The operating conditions were: ion source temperature: 120 °C, desolvation gas (N_2): 300 °C, 580 L/h, nebulizer gas: 90 L/h, capillary voltage: 3 kV, scan time: 1.0 scan/s and inter-scan delay time: 0.10 s. MassLynx software version 4.1 was used for instrument control and data acquisition.

2.6. Optimisation and validation of PLE method

The following PLE parameters were optimised: temperature (40, 75 and 100 °C), sample weight (0.1, 0.3 and 0.5 g), flush volume (100,

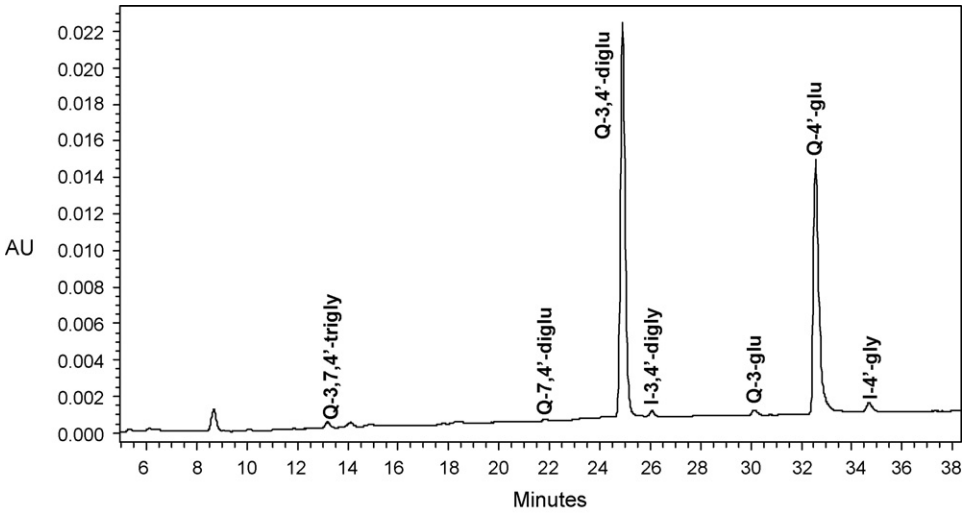


Fig. 2. Chromatogram of an onion extract (HPLC–UV, 254 nm).

Table 1
Flavonoids present in the onion extracts. The MS/MS–MS fragmentation pattern is described in the structure and the low *m*/*z* values are derived from multiple fragmentations.

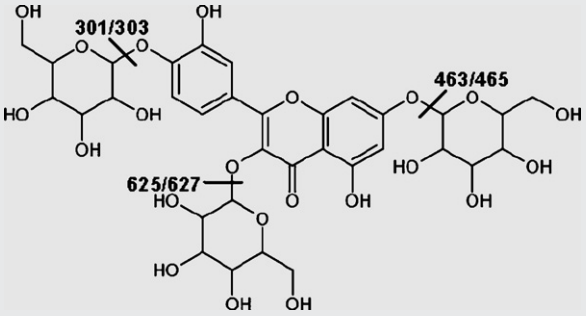
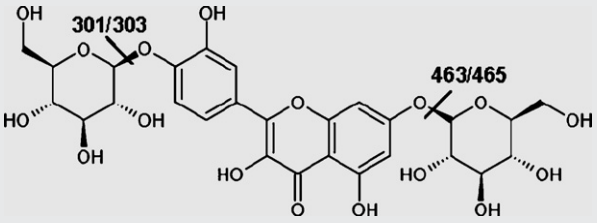
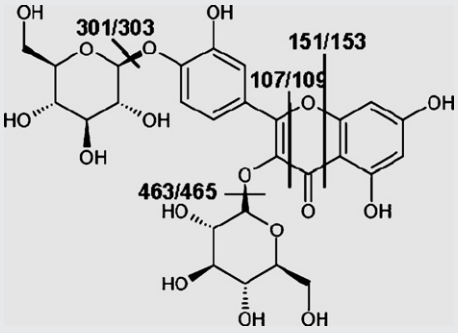
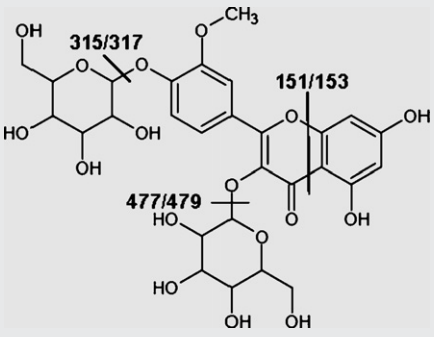
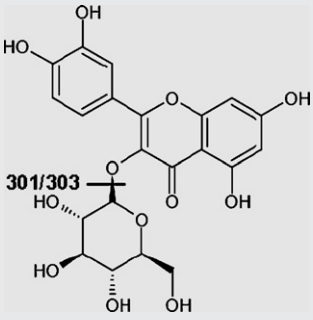
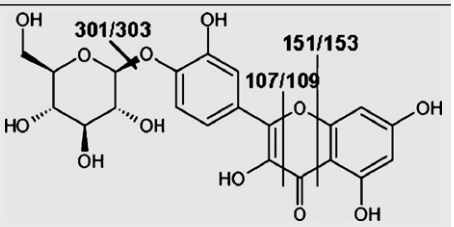
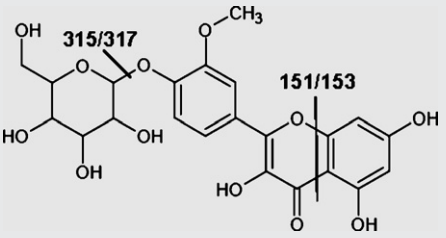
Compound	Measured mass (amu)	Formula	Δm (ppm)	i-FIT[b]	t_R[c] (min)	UV_{max} (nm)	Structure
Q-3,7,4′-trigly	788.27	$C_{33}H_{40}O_{22}$	3.4	0.6	13.2	253 266sh 345	
Q-7,4′-diglu[a]	626.22	$C_{27}H_{30}O_{17}$	−0.5	0.9	21.8	253 270sh 362	
Q-3,4′-diglu[a]	626.22	$C_{27}H_{30}O_{17}$	−0.5	15.5	24.9	255 265 345	
I-3,4′-digly	640.23	$C_{28}H_{32}O_{17}$	−0.1	2.8	26.1	252 266 345	
Q-3-glu[a]	464.17	$C_{21}H_{20}O_{12}$	−5.9	0.8	30.1	256 266sh 355	

Table 1 (*Continued*)

Compound	Measured mass (amu)	Formula	Δ*m* (ppm)	i-FIT[b]	t_R[c] (min)	UV_{max} (nm)	Structure
Q-4′-glu[a]	464.17	$C_{21}H_{20}O_{12}$	−5.9	1.7	32.6	253 267sh 363	
I-4′-gly	478.18	$C_{22}H_{22}O_{12}$	−0.6	13.3	34.7	254 269sh 364	

sh: shoulder.

[a] Identity confirmed with standards.

[b] Isotopic pattern fit of measurement compared to theoretical.

[c] Retention time.

125 and 150%) and solvent type (30, 40, 50, 60, 70, 80 and 90% aqueous methanol). It was also tested whether a clean-up step by in-cell addition of 0.5 g C18-material (Sepra E-C18, 50 μm, Phenomenex, Denmark) to the extraction cells would improve the method selectivity and reproducibility. Each sample was extracted three times during the optimisation experiments.

The PLE method was validated. The selectivity was determined from blank samples containing Ottawa sand and the reproducibility by two replicate analyses in four series. The limit of detection (LOD) and quantification (LOQ) was determined as three and ten times the standard deviation. Diluted reproducibility samples with a concentration close to the expected LOQ were used for determination of LOD and LOQ. The trueness of the method was determined by recovery experiments using the internal onion reference sample because no samples were available without or with a low concentration of the relevant flavonoids. The recovery was determined by extraction of five onion samples with and without addition of flavonoid standards. Linearity, sensitivity and range of measurements were determined for Q-7,4′-diglu, Q-3,4′-diglu, Q-4′-glu, Q-3-glu and Q (10 μg/mL) by varying the injection volumes of the standards, which provided comparable results to serial dilution of standards. We did not use an internal standard in the analysis because it was not possible to find a reference standard, which had similar chemical and physical properties, did not co-elute with the compounds of interest and was not present in the matrix.

3. Results and discussion

3.1. Identification of flavonoids (HPLC–MS and MS/MS)

An HPLC chromatogram confirmed that onions are a rich source of quercetin compounds (Fig. 2). The two most abundant compounds were Q-3,4′-diglu and Q-4′-glu in agreement with previous studies [12,18,35], but Q-3,7,4′-trigly, Q-7,4′-diglu and Q-3-glu were also present. Two isorhamnetin compounds, I-3,4′-digly and I-4′-gly were also identified in the onions. The flavonoid profile was similar to that found in southern Italian red onions [18] and white onions [12]. Neither aglycone quercetin (retention time (t_R): 37.1 min) nor isorhamnetin (t_R: 40.5 min) were detected in the onion extract indicating that hydrolysis of glycosides did not take place during sample preparation and analysis [35].

Two absorption maxima were observed in the UV spectra of the identified flavonoids at 345–363 nm (band I) and 253–256 nm (band II) (Table 1) associated with the absorption owing to the cinnamoyl B-ring and benzoyl A-ring, respectively [18]. The UV spectra were in agreement with previously obtained spectra of similar compounds [18,34] and confirmed the characteristic maxima of flavonols [36].

The glucosidic compounds showed loss of glucosyl moieties (162 amu per glucose moiety) in the mass spectral data leading to the aglycone fragment of quercetin (*m*/*z* 301/303 in ESI−/ESI+) or isorhamnetin (*m*/*z* 315/317) as earlier observed with similar compounds [12,18]. We observed fragments at *m*/*z* 151/153, which are typical for flavonoids and results of a retro-Diels–Alder fragmentation [21,37,38]. The fragments at *m*/*z* 107/109 are also characteristic, especially in negative mode [21].

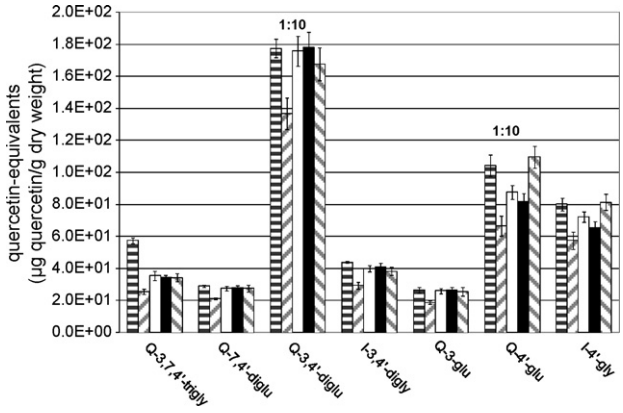


Fig. 3. Efficiency (expressed in quercetin equivalents) of the tested extraction methods. The concentrations of Q-3,4′-diglu and Q-4′-glu have been reduced by a factor of 10 to improve the visualisation of the remaining five less abundant flavonoids. (▤) Ultrasonication, (▨) water bath, (□) microwave-assisted extraction, (■) ultrasonic liquid processor, (▧) pressurised liquid extraction. The error bars describe the standard deviation ($n = 3$).

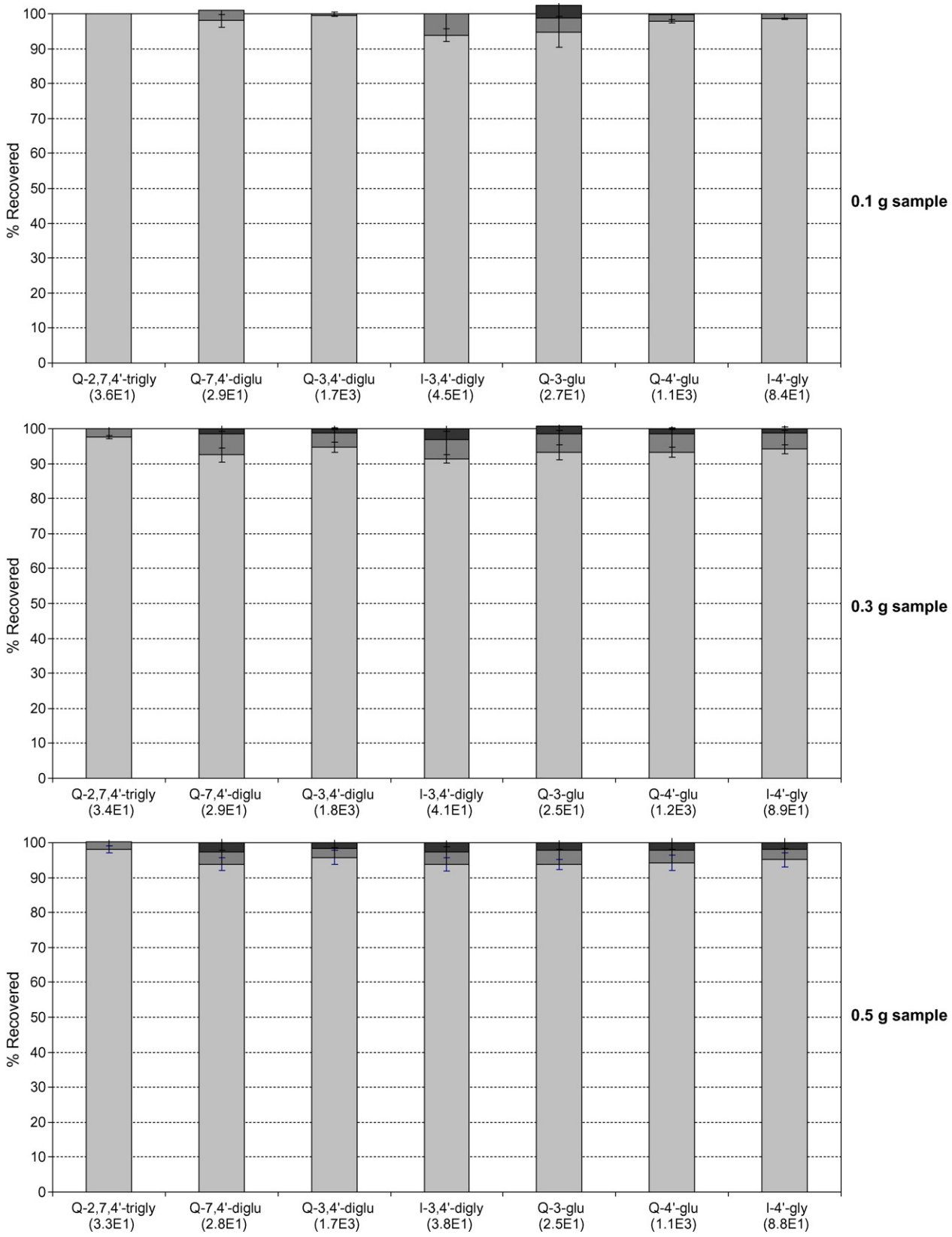


Fig. 4. Effect of sample weight on recovery in the three collected extracts (pressurised liquid extraction). (▒) 1st extract; (▓) 2nd extract and (■) 3rd extract. The total recovery in all three extracts (μg quercetin-equivalents/g dry weight) is shown in parenthesis below each compound. The error bars describe the standard deviation ($n = 3$).

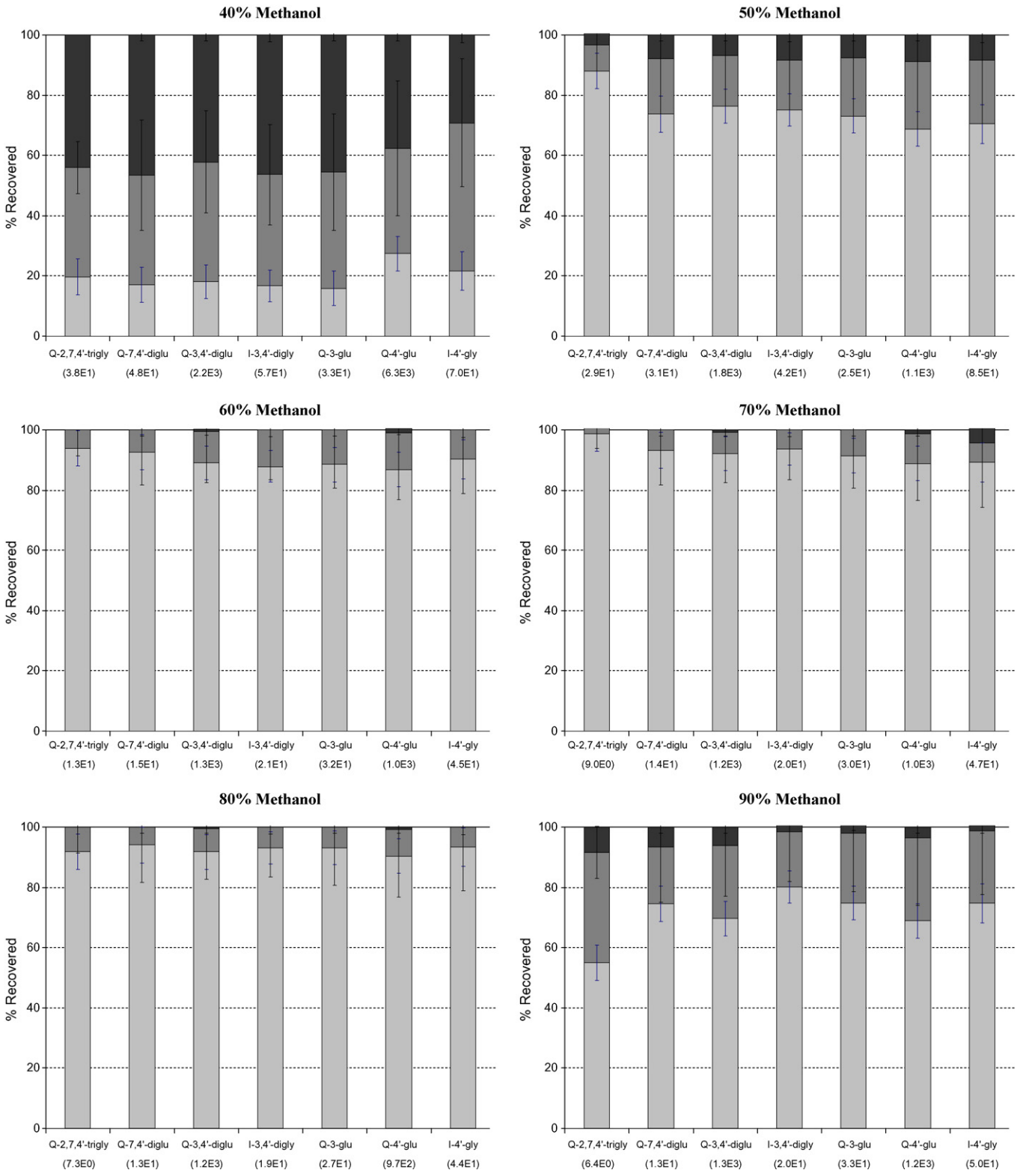


Fig. 5. Effect of solvent type on recovery in the three collected extracts (pressurised liquid extraction). (▒) 1st extract; (▓) 2nd extract; (■) 3rd extract. The total recovery in all three extracts (μg quercetin-equivalents/g dry weight) is shown in parenthesis below each compound. The error bars describe the standard deviation ($n = 3$).

The identified isorhamnetin glycosides might contain glucose as the sugar moiety, but also other sugars like galactose and mannose are possible (loss of 162 amu). However, glucose is the most frequently found sugar moiety in flavonoids [21], but it was not possible to determine the exact identity of the sugars and the linkage positions for the attached sugars. The loss of a methyl radical $[M\pm H\text{-}15]^{\bullet+/-}$ is often seen for methoxylated flavonols such as isorhamnetin [21,39], but was not observed in our experiments. Nevertheless, the identification is supported by previous studies, where both I-3,4′-digly and I-4′-gly have been identified in red [18] and white onions [12], respectively, with similar relative retention time and in other studies of onions [12,15].

3.2. Comparison of extraction methods

The efficiencies of MAE and ULP were comparable and at the same level as ultrasonication and PLE (Fig. 3) despite an expected strong disruption of the tissue structure with MAE and ULP due to the powerful shock waves radiating throughout the sample. Lower extraction efficiency was observed for the water bath extraction method. However, lower and thus better LODs were obtained with the water bath extraction method owing to the required preconcentration step of this method. An increase in sample weight and flush volume in PLE is expected to improve the LODs, which were otherwise the highest among the methods. The reproducibilities of the extraction methods were in a similar range (relative standard deviation (RSD): 1–11%, $n = 3$), which is generally considered acceptable for this type of analyses.

Ultrasonication is a fast and easy method, but difficulties were observed during the filtration of extracts as with MAE and ULP, so an additional sample preparation step (e.g. centrifugation) was needed. In contrast, PLE yielded clean extracts, which could be filtered directly after extraction. We also observed an increase in sample temperature during ultrasonication, which is a disadvantage as heating should be avoided during extraction [21]. High temperatures accelerate the oxidation of flavonoids [25] though no effects on recovery were observed in this study.

Higher efficiencies of MAE in comparison with conventional extraction methods have previously been obtained in extraction studies of anthocyanins from raspberries [30] and isoflavonoids and saponins from Chinese medicinal plants [31]. However, we did not observe such superior efficiencies, maybe because some cell structures were already destroyed during the freeze-drying process, which might also explain the non-superior efficiencies obtained with ULP.

An advantage of PLE is that it is a highly automated extraction process carried out in an inert atmosphere and protected from light. High extraction temperatures in PLE should yield improvements relative to time and solvent usage [40]. Flavonoids are temperature-sensitive [25], but the extraction can be carried out at high temperatures in PLE because it is performed in an inert atmosphere, which decreases the risk of chemical degradation [25]. The efficiencies of PLE were similar or higher than those found for the other tested methods. The solvent consumption (20 mL) was also decreased by its application in comparison with the conventional water bath method (50 mL), whereas the solvent consumption was lower for the other methods (5 mL). Altogether, we recommend PLE as the method of choice for extraction of flavonoids from onions because it shows several advantages and possibilities, e.g. simultaneous extraction and clean-up, which can otherwise be very time-consuming.

3.3. Optimization of PLE method

Extraction temperatures of 40, 75 and 100 °C were tested, but no significant differences in the efficiencies were observed. So, we decided to carry out the extractions at 40 °C in agreement with the temperatures used in previous PLE studies [28,41] and because of the known temperature-sensitivity of polyphenols [25].

Decreases in the LODs were observed as the sample weight increased and the total efficiencies after three extractions at all tested sample weights (0.1, 0.3 and 0.5 g) were comparable. The recoveries in the first extract were always greater than 91%, but increased with sample weight for the second and third extract (Fig. 4). The RSDs were acceptable (0.1–6.2%), but a slight tendency towards lower RSDs with higher sample weights was observed. So, 0.5 g is the recommended sample weight due to the improved LODs as well as acceptable recovery (98–99% of the total extracted with two extraction cycles) and reproducibility. Larger sample weights have been used in previous studies in combination with larger extraction cells [25,29,42], but larger sample weights caused clogging of our system due to the fine freeze-dried powder used.

Neither the total efficiency nor the efficiency in each extract was affected by the flush volumes (viz. 100, 125 and 150%) tested in this study (see Figure S1 in supporting information). A further increase in flush volume would have been interesting; however, the ASE-200 system does not allow flush volumes above 150%. A 100% flush volume is in accordance with flush volumes used by others [43,44] and was preferred because lower extract volumes were obtained at lower flush volumes, which leads to an improvement in the LODs.

The total extraction efficiencies were almost constant using a solvent composition of methanol between 50 and 90%, while slightly higher efficiencies were obtained at lower percentages of methanol. However, the results showed lower RSDs at methanol percentages below 50 as well as above 80% and the largest percentages were recovered in the second and third extracts, while only 1% of the total extracted flavonoids were recovered in the third extract with 60–80% methanol, except I-4′-gly with 70% methanol (Fig. 5). The decreased reproducibility when using solvents with high percentages of water has also previously been observed in another PLE study of polyphenols in apples [42]. An interfering compound was observed in the chromatogram of the second extract with 70–90% methanol (data not shown) and it more or less co-eluted with Q-3,4′-glu. Hence, the optimum solvent type appeared to be 60% methanol, but 65% methanol was afterwards tested. The recommended extraction solvent was 65% methanol owing to the obtained recovery as well as no co-eluting compounds in the HPLC analyses were observed (results not shown).

A clean-up step was included in the extraction by in-cell addition of C18-material, which has often been used as sorbent for solid-phase extraction (SPE) of flavonoids [21]. The total recoveries were improved with 13–35% in the samples added C18-material (data not shown). The RSDs were slightly higher (0–19%) for the majority of compounds in the samples without addition of C18-material, especially the more polar compounds (Table 2). This might be because of reduced interferences and/or increased selectivity. However, the HPLC–UV chromatograms did not reveal any immediate differences with respect to interference or noise. The simultaneous extraction and clean-up is a considerable improvement because the alternative SPE can be very time and solvent consuming and additional sample handling increases the risk of chemical degradation, though it has previously been used for clean-up of onion-extracts [19,22].

Our results show that two extraction cycles were sufficient and the third extract only contained 1–2% of the total extracted flavonoids in the three extracts. The optimum number of extraction cycles is in accordance with previous used extraction cycles (1–4) in PLE studies of polyphenols from plant materials [29,41,42] and so is the recovery, which in other similar PLE studies ranged from 81 to 100% of the total extractable polyphenols [25,41,42].

Table 2
Precision in extractions with and without addition of C18-material to the extraction cells ($n = 3$).

Compound	RSD (%)	
	Addition of C18-material	No addition of C18-material
Q-2,7,4′-triglu	5.8	25
Q-7,4′-digly	4.3	7.7
Q-3,4′-digly	3.8	7.0
I-3,4′-diglu	3.8	9.0
Q-3-gly	9.9	7.3
Q-4′-gly	6.8	6.8
I-4′-glu	9.9	9.7

Table 3
Limit of detection (LOD) and quantification (LOQ) as well as the precision (expressed as the relative standard deviation, RSD) of the analysis of polyphenols in vegetables ($n = 8$).

Compound	Vegetable	LOD (μg q-eq/g dw)	LOQ (μg q-eq/g dw)	Precision (RSD, %)	Concentration (μg q-eq/g dw)
Q-2,7,4′-trigly	Onion	3.0	20	11	4.6×10^1
Q-7,4′-diglu	Onion	1.4	60	10	2.5×10^1
Q-3,4′-diglu	Onion	0.90	20	3.1	2.7×10^3
I-3,4′-digly	Onion	1.5	20	8.6	4.5×10^1
Q-3-glu	Onion	2.0	20	4.9	4.6×10^1
Q-4′-glu	Onion	1.1	20	5.7	2.0×10^3
I-4′-gly	Onion	4.8	20	9.6	9.1×10^1
Chorogenic acid	Potato	0.4	20	1.8	1.2×10^2
Chorogenic acid	Carrot	1.5	20	7.3	1.3×10^2
Caffeic acid	Potato	2.7	20	–[a]	<LOQ

The average concentration of the compounds in the vegetables used for precision measurements can be seen in the last column. The LOD, LOQ and concentrations are expressed in μg quercetin-equivalents/g dry weight (μg q-eq/g dw).

[a] Precision was not determined because the concentration was below LOQ.

3.4. Method validation

The selectivity of the method was studied by extraction of blank samples (Ottawa sand) because onion samples without flavonoids were not available. The selectivity was good as interfering compounds were neither immediately detected in blank samples nor in the alternative analysis by LC–MS with electrospray ionization in negative ionization mode. The linearities of the standard curves of selected flavonoids (Q-7,4′-diglu, Q-3,4-diglu, Q-4′-glu, Q-3-glu and Q) were in the range of 0.5–10 μg/mL (equal to 20–400 μg/g dry weight), except for Q-7,4′-diglu (linearity between 1.5 and 10 μg/mL), and regression coefficients (R^2) were above 0.998. The sensitivity (measured in area/(μg/mL)) ranged from 2.2×10^4 to 7.0×10^4.

LODs ranged from 0.9 to 4.8 μg quercetin-equivalents/g dw (μg q-eq/g dw) and LOQs were equal to the lower limit of the linear range (20 μg q-eq/g dw), except for Q-7,4′-diglu (i.e. LOQ of 60 μg q-eq/g dw) (Table 3). The precisions were in an acceptable range for our purpose (RSD: 3.1–11%, Table 3).

Recovery experiments were used to estimate the trueness of the method, but the experiment was only of relevance for Q-7,4′-diglu and Q-3-glu due to insufficient amounts of pure standards in comparison with the high content of flavonoids in the reference sample. The recovery of Q-7,4′-diglu and Q-3-glu were 127% and 98%, respectively., which we consider acceptable though it is in the higher range for Q-7,4′-diglu. Furthermore, as previously stated the PLE method prior to optimization yielded satisfactory recoveries compared to the other tested extraction methods (Fig. 3) and 98–99% of the extracted flavonoids were recovered within two consecutive extraction cycles.

Our method was also applicable to other matrices and groups of polyphenols, e.g. extraction of phenolic acids from potatoes and carrots. The reproducibility (RSD: 1.3–7.3%, Table 3), linearity (0.5–10 μg/mL equal to 20–400 μg/g dw, $R^2 > 0.998$) and sensitivity ($2.4–4.6 \times 10^4$ area/(μg/mL)) of phenolic acids were in the similar range of flavonoids in onions. The LODs of chlorogenic acid and caffeic acid were between 0.4 and 2.7 μg q-eq/g dw, while LOQs of 20 μg q-eq/g dw were obtained (Table 3). Extraction of white cabbage was also possible, but the compound identification is ongoing.

4. Conclusion

Five extraction methods (water bath extraction, pressurized liquid extraction (PLE), ultrasonication, ultrasonic liquid processor and microwave extraction) were tested and PLE was the preferred extraction method. The PLE method sets a new standard for analyses of flavonoids from onions and showed considerable improvements, especially the simultaneous extraction and clean-up by in-cell addition of a stationary phase (in this case C18-material) improved the extraction efficiency and reproducibility. However, the integration of extraction and clean-up into one combined analytical step can also be advantageous in future development of PLE methods for rapid and reliable extraction and clean-up of bioactive compounds from various food products. The PLE method yielded similar efficiency and reproducibility in comparison with the other modern extraction methods (ULP and MAE), but it is advantageous because it can be highly automated, small amounts of solvents are used, the cleanest extracts are obtained and the extraction of the light and oxygen-sensitive flavonoids is carried out in an inert atmosphere protected from light. The optimised and validated method is rapid, reliable, sensitive and its application can be extended to other food matrices and polyphenols, e.g. phenolic acids in potatoes and carrots.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2009.06.073.

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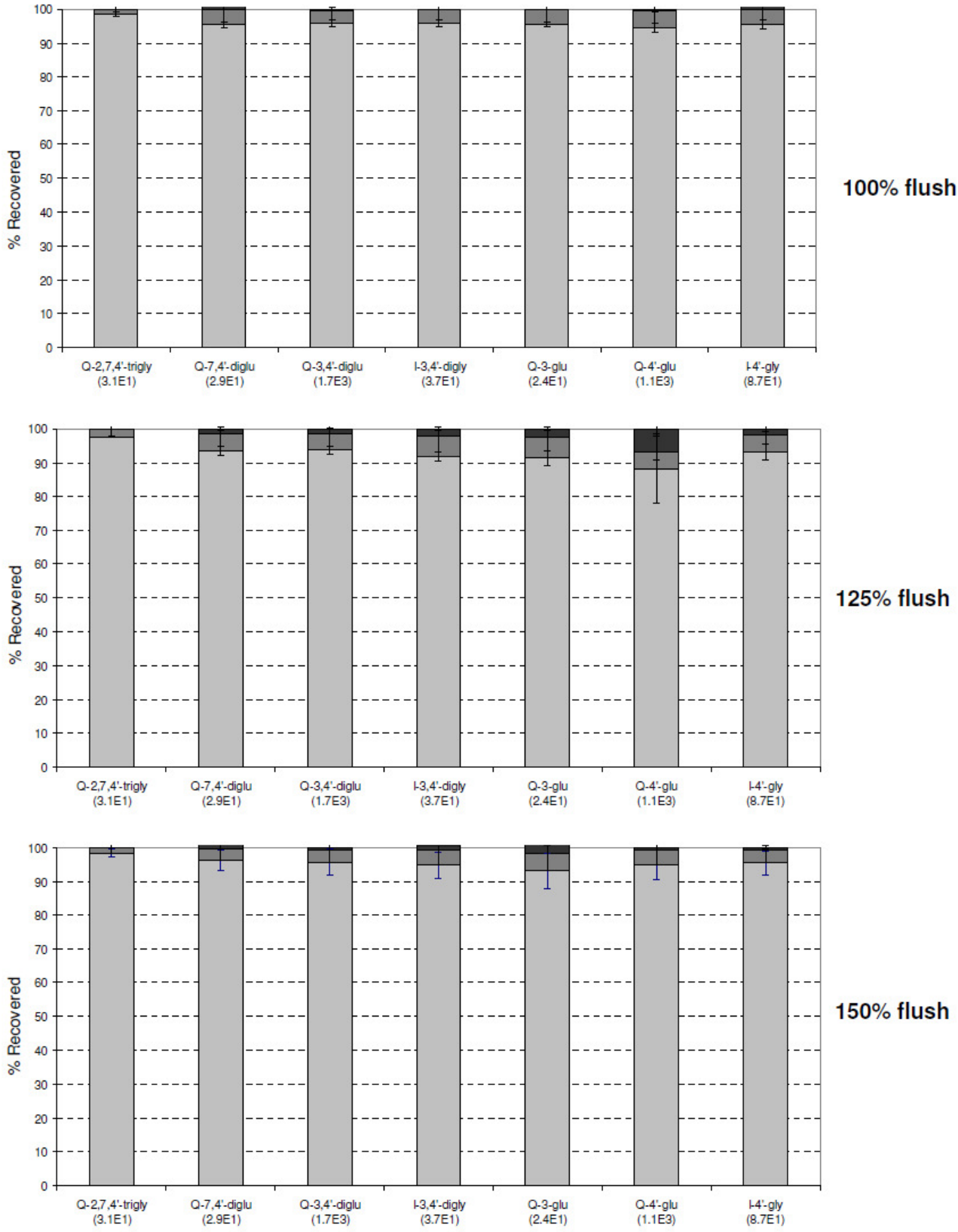
Figure S1. Effect of flush volume on recovery in the three collected extracts. ■ : 1st extract ■ : 2nd extract and ■ : 3rd extract. The total recovery in all three extracts (μg quercetin-equivalents/g dry weight) is shown in parenthesis below each compound. The error bars describe the standard deviation (n =3).



Corrections to paper I

- Figure 4, figure 5, table 2, and table 3: Q-2,7,4’-trigly should be replaced by Q-3,7,4’-trigly.
- Table 2: Q-7,4’-digly, Q-3,4’-digly, I-3,4’-diglu, Q-3-gly, Q-4’-gly, and I-4’-glu should be replaced by Q-7,4’-diglu, Q-3,4’-diglu, I-3,4’-digly, Q-3-glu, Q-4’-glu, and I-4’-gly, respectively.

Manuscript I

Comparing the content of polyacetylenes in organically and conventionally grown carrot roots using a new and fast ultrasonic liquid extraction method

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Abstract

A rapid and sensitive analytical method for quantification of potentially health-promoting polyacetylenes in carrot roots was developed. The traditional extraction method (stirring) was compared to a new Ultrasonic Liquid Processor (ULP) based methodology using HPLC-UV and MS for identification and quantification of three polyacetylenes. ULP was superior because a considerable reduction in extraction time and improved extractions efficiencies were obtained. Following optimization, the ULP method showed good selectivity, precision (RSDs: 2.3─3.6 %) and recovery (93 % of falcarindiol) of the polyacetylenes. The applicability of the method was documented by comparative analyses of carrots grown organically or conventionally in a two-year field trial study. The average concentrations of falcarindiol, falcarindiol-3-acetate, and falcarinol in year one were 222, 30, and 94 µg falcarindiol-equivalents/g dry weight, respectively, and 3–15 % lower in year two. The concentrations were not significantly influenced by growth system, but a significant year-to-year variation was observed for falcarindiol-3-acetate.

Keywords

Carrots (*Daucus carota*), conventional and organic growth systems, HPLC-UV and MS, method development and validation, polyacetylenes, Ultrasonic Liquid Processor (ULP)

Introduction

Epidemiological studies have shown that a high intake of fruits and vegetables improves the protective effects against cancer *(1-3)* and cardiovascular diseases *(4)*. Carotenoids are believed to play an important role for the health promoting properties of *e.g.* carrots *(5)*, though the protective effect of carotenoids on certain types of cancer can be attenuated or reversed by smoking *(6,7)*. In addition, it has been suggested that other less abundant bioactive compounds such as the aliphatic polyacetylenes might contribute significantly to the potential positive effects of carrot consumption *(8)*.

Polyacetylenes are toxic to certain fungi *(9)* and other pathogens *(10)*, have anti-inflammatory effects in macrophage and endothelial cells *(11)*, and can cause strong allergenic reactions *(12)*. They have protective effects against various cancer cells *(13, 14)* including colorectal cancer in rats *(15)*, but the response appear to be dose-dependent *(8, 16)*. It has also been shown that falcarinol (FaOH) is readily bioavailable to humans. After consumption of carrot juice containing 4-12 mg FaOH, concentration levels ranging from 1-2.5 ng/mL plasma was found, which is above the postulated threshold levels observed *in vitro (17)*.

More than 1400 different polyacetylenes are found in plants and they are believed to be synthesized from unsaturated fatty acids. The aliphatic C_{17}-polyacetylenes are widely distributed in *e.g.* carrots, celery and parsley *(18)*, all belonging to the *Apiacea* family. Carrots are the major human dietary source of polyacetylenes *(15)* and the chemical structures of the main compounds found in carrots are shown in Figure 1. The concentration of polyacetylenes in plants is normally in the range from 0.1 to 10 g/kg fresh weight depending on the individual species *(19)*. The content in carrots depends on factors such as cultivar *(5, 20, 21)*, physiological age, geographical origin *(5)*, and climatic conditions *e.g.* plant available water content of soils *(22)* as well as storage and processing procedures *(23)*. The amount of plant available nitrogen might also be of importance as seen for many other secondary metabolites such as carotenoids *(24)* and flavonoids *(25)*. The spatial distribution of individual polyacetylenes in a carrot root differs. FaOH appears to be uniformly distributed, whereas falcarindiol (FaDOH) and falcarindiol-3-acetate (FaDOAc) are primarily found in the peel *(9, 21, 26)*.

Polyacetylenes in carrots have previously been extracted from fresh or dried plant material with ethyl acetate (EtOAc) as the most frequently used extraction solvent *(9, 20, 23, 26, 27)*. Extraction of lyophilized plant material has been shown to yield similar results in comparison with fresh plant

material *(20)*. The traditional liquid extraction methods include stirring *(26)* and ultrasonication *(14)*, but pressurized liquid extraction has also recently been used *(20)*. The risk of degradation is increased with long extraction times due to the instability of polyacetylenes, especially FaDOH which is sensitive to oxidation and/or enzymatic degradation *(28)* as well as intolerant to heat and light exposure *(23)*. Ultrasonic Liquid Processing (ULP) is a promising technique, which has not previously been used for extraction of polyacetylenes from carrots. The advantage of ULP is that shorter extraction times are expected due to a more intense cavitational action and greater disruption of the sample *(29)*.

The chemical analysis of polyacetylenes is usually performed on a high-performance liquid chromatographic system (HPLC) equipped with a reversed-phase column using gradient elution with methanol (MeOH) or acetonitrile (ACN) and water, but gas chromatography has also been used. UV detection is used for quantification because polyacetylenes yields characteristic UV spectra due to their conjugated triple bonds, but their extinction coefficients are generally low at UV-maxima due to the low number of conjugated unsaturated bonds. Hence, the quantification is often performed at 205 nm, where the sensitivity is ten times higher than at their characteristic UV-maxima *(18)*. Quantification by mass spectrometry (MS) has also been applied *(20, 30)* and is especially relevant when measuring small concentration levels in *e.g.* blood plasma due to its high sensitivity *(18)*.

The objectives of the current study were to identify polyacetylenes in carrot roots by application of HPLC-MS and to develop a faster, more sensitive, and robust analytical method based on ULP extraction and HPLC-UV quantification. The developed ULP method was optimised and compared with the traditional analytical protocol. The applicability of the method was demonstrated by comparative analysis of organically and conventionally grown carrots in order to study the effect of growth system on the concentration of polyacetylenes in carrot roots. Organic carrots are produced and consumed in large quantities in Denmark and possible health benefits of organic foods in general are of major interest to *e.g.* consumers and producers.

Materials and methods

Reagents and chemicals

Methanol (MeOH), ethyl acetate (EtOAc) and acetonitrile (ACN) were purchased from Rathburn Chemicals Ltd. (HPLC-grade, Walkerburn, Scotland). Dimethyl sulfoxide (DMSO, >99 %) and formic acid (98−100 %) were purchased from Merck (Darmstadt, Germany). Milli-Q water (18 MΩ, Millipore, Bedford, USA) was used for sample preparation and eluents.

Falcarindiol (FaDOH, yellowish oil, 97 % purity) was purchased from Atomax Chemicals Co., Ltd. (Shenzhen, China) and a standard stock solution was prepared by dissolving the compound in MeOH (1 mg/mL). Working solutions of FaDOH were prepared by diluting the stock solutions with MeOH (20 µg/mL). Stock solutions of FaDOH were stored (<1 year) at −80 ^{o}C, while the working solutions were prepared shortly before analyses and kept at −20 ^{o}C until analyses.

Samples for method development, optimization and validation

A conventionally grown carrot sample (*Daucus carota* cv. *bolero*, 50-250 g fresh weight, Lammefjorden, Faarevejle, Denmark) was purchased at a local supermarket and used for comparison of extraction methods, ULP method optimisation and validation. An in-house carrot sample was used as reference material for method validation together with randomly selected carrot samples from the field trial experiment (described below). The samples were washed in Milli-Q water, peeled, cut into 0.5 cm thick slices and freeze dried at 0.1 kPa for 2 days (Beta 1-8, Christ, Osterode am Harz, Germany). Afterwards, the samples were crushed, homogenised and stored at −20 ^{o}C in an inert nitrogen atmosphere until analysis. In general, the samples were protected from light and oxygen during the entire sample preparation by wrapping in aluminium foil and storing in a nitrogen atmosphere.

Samples from field trial experiment

The applicability of the method was documented by comparative analyses of organically and conventionally grown carrot roots from a two-year field trial study undertaken in 2007 (year one) and 2008 (year two). The carrots (*Daucus carota* cv. *bolero*) were grown in the VegQure rotation experiment (http://www.vegqure.elr.dk/uk/) located at the Aarslev field trial station, Funen, Denmark (10^{o}27'E, 55^{o}18'N). A sandy loam soil (15% clay, 27% silt, and 55% sand) with pH 6.2

(measured in 0.01 M $CaCl_2$) was used. Plant available P, K and Mg was 2.6, 12.4 and 4.3 mg per 100 g soil, respectively (average value for plough layer soil samples taken in March in both growth years) measured in soil samples using standard procedures *(31)*.

The carrots were grown in three different agricultural systems: one conventional (C) and two organic (OA and OB) growth systems with three replicates of each in the field resulting in 9 plots per year and 18 plots in total (each of 120 m^2). The replicates from each system were located in three separate blocks geographically close to each other. Each block contained all three agricultural systems, which were all stock-less cash crop production systems with an identical sequence of main crops (8-year rotation). In the conventional system (C), pesticides and inorganic fertilizer were used (120-20-60 kg N-P-K/hectare). The OA system relied on fertilization with animal pig manure (55-5-20 kg N-P-K/hectare), while the nutrient supply was based on the use of cover crops as green manures (mainly legumes) in the OB system. Cover crops were grown in the autumn after the main crops and incorporated into the soil in the spring before carrots were grown. The organic systems were managed in full compliance with the Danish guidelines for organic farming administered by the Danish Plant Directorate (http://pdir.fvm.dk/).

The carrots were harvested at maturity at the same day from all growth systems. A 15 kg sample was collected from each plot with 50−250 g fresh weight of individual roots (marketable quality) as inclusion criteria in both growth years. Sampling representativeness was tested by double sampling of random plots and yielded an average relative standard deviation (RSD) of 8.9 % for FaDOH, FaDOAc and FaOH (n = 6 for each compound).

Only the eatable parts of the carrots were analysed and the samples were prepared for analysis as described above. However, samples from the field trial experiment were freeze-dried at 0.08 kPa for 1−2 days at a commercial freeze drying company (Danish Freeze Dry A/S, Kirke Hyllinge, Denmark).

Extraction methods

EtOAc was used as extraction solvent throughout the method comparison and in the final optimized method.

Extraction method with stirring

0.5 g freeze-dried material was extracted with 30 mL EtOAc overnight under continuous stirring at room temperature. Before stirring, the sample was exposed to ultrasound for 10 min (120 W, Brasonic 5200, Soest, The Netherlands). The extract was filtered and the residue was extracted further with 30 mL EtOAc for 3 hours. The extracts were combined and evaporated to dryness *in vacuo* at 30 ^{o}C on a rotary evaporator. The extraction procedure was based on the principles suggested by Christensen and Kreutzmann *(26)*.

Extraction with Ultrasonic Liquid Processor (ULP)

0.5 g freeze-dried material (1 g in optimized ULP method) was transferred to a cylindric plastic tube and 30 mL of EtOAc was added. The microprobe was immersed into the tube and the extraction was performed at room temperature for 60 s using an ULP operated at 10 W (Microson XL 2000, Misonix, Newtown, CT, US). The sample was centrifuged (5 min, 3000 *g*, 20 ^{o}C) and 20 mL of the supernatant was evaporated to dryness *in vacuo* at 30 ^{o}C on a rotary evaporator.

Residues from both extraction methods were redissolved in MeOH (5 mL) and filtered through a 0.45 and a 0.20 µm filter before chemical analysis.

Quantification of polyacetylenes by HPLC-UV

A Waters 2695 Alliance Separations Module in combination with a Waters 2996 Photodiode Array Detector (PDA) and a Waters 2487 Dual λ Absorbance Detector (Waters, Milford, MA, US) was used for the chromatographic analysis of polyacetylenes. Empower 2 was used for instrument control and data acquisition. The chromatographic separation was carried out at a flow rate of 1.0 mL/min at 40 ^{o}C with an injection volume of 15 µL (temperature of autosampler: 5 ^{o}C). The column was a Phenomenex Prodigy RP-C18 column (4.6 × 250 mm, 5 µm). The A- and B-eluents were Milli-Q water and ACN, respectively. The gradient program was as follows: 70 % B for 5 min, a linear gradient to 86 % B for 13 min, a linear gradient to 95 % B for 2 min, isocratic elution for 8 min, followed by a 2-min ramp back to 70 % B, and re-equilibration for 3 min giving a total run-time of 33 min. The PDA collected data from 190-400 nm and the Dual λ Absorbance Detector was used for quantification of the polyacetylenes (205 nm), which were quantified relative to FaDOH.

Identification of polyacetylenes by HPLC-UV and MS

Structure elucidation of polyacetylenes in carrots was based on accurate mass measurements, isotopic pattern fit of measurement compared to theoretical (i-FIT values, Elemental Composition 4.0 software, Waters, USA) and fragmentation patterns. An Ultra Performance Liquid Chromatograph (UPLC) interfaced to a time of flight tandem mass spectrometer (TOF-MS) was used for exact mass determinations. Afterwards, the identities were confirmed with available standards by comparison of retention times, UV, and MS data.

UPLC-TOF. The same mobile phases as those used for the HPLC-UV analyses were applied for the UPLC (Acquity UPLC, Waters) analyses, except addition of 0.1 % formic acid to the A-eluent in order to enhance ionisation. This change in solvent did not have any significant effects on the retention times. The separation of analytes was performed at 40 °C on a Waters Acquity HSS C18 column (2.1 × 100 mm, 1.8 µm). The HPLC gradient was scaled according to the column dimensions and gradient flow rates were adjusted to UPLC mode. The gradient program was as follows (0.25 mL/min): 70 % B for 1.7 min, a linear gradient to 86 % B for 4.3 min, a linear gradient to 95 % B for 0.7 min, isocratic elution for 2.9 min, followed by a 1.2-min ramp back to 70 % B and re-equilibration for 3.6 min giving a total run-time of 14.4 min. A sample volume of 3 µL was injected using the partial loop with needle overfill mode. The TOF-MS (LCT Premier/XE mass spectrometer, Waters) was operated in electrospray ionisation positive ion mode (ESI^+). The operating conditions were: ion source temperature: 120 ^oC , desolvation gas (N_2): 350 ^oC, 500 L/h, cone gas: 25 L/h, capillary voltage: 2 kV and scan time: 1.0 scan/s. MassLynx software version 4.1 was used for instrument control and data acquisition and leucine enkephaline (556.2771 g/mol) was used as lock-spray mass (reference standard). External mass calibration (m/z 100-1000) was carried out before the analyses using a sodium formate solution containing 5 mM sodium hydroxide and 0.5 % formic acid in 2-propanol:water (90:10, v/v).

Method optimization and validation

Optimization of extraction with ULP. The following ULP parameters were optimized: sample weight (0.5 and 1.0 g freeze-dried material), extraction time (15 s, 60 s and 120 s) and number of extractions (1, 2, and 3 times). They were optimized with regard to extraction efficiency (the quantity of extractable polyacetylenes per unit weight), precision (determined as the RSD from a

number of replicate analysis measured under repeatable conditions) and sensitivity (the capability of the method to discriminate small concentration differences of the polyacetylenes).

Optimization of chromatographic method. The HPLC method used by Christensen and Kreutzmann *(198)* was optimized by gradient adjustments in order to reduce the total run time. A pilot study was also conducted to test the applicability of a faster chromatographic technique, UPLC (Waters), with a PDA detector for quantification at 205 nm. The columns tested were Waters Acquity UPLC BEH C18 (2.1×50 mm, 1.8 μm) and Waters Acquity UPLC HSS C18 (2.1×50, 100 and 150 mm, 1.8 μm), which were retained at 40 °C. The same mobile phases as those used for the HPLC-UV analyses were used, and the gradient was scaled and optimized according to the column dimensions and flow rates applied (0.2─0.8 mL/min). The best achievable separation with regard to resolution and speed was obtained on a Waters HSS C18 (2.1×100 mm, 1.8 μm) column at 0.2 mL/min with the following gradient: 70 % B for 2 min, a linear gradient to 86 % B for 8 min, a linear gradient to 95 % B for 2.5 min, isocratic elution for 1.5 min, followed by a 0.5-min ramp back to 70 % B, and re-equilibration for 2.5 min giving a total run-time of 17 min.

Method validation of ULP method. The selectivity was studied by extraction of blank samples (*i.e.* extractions without addition of carrot material) because carrot samples without polyacetylenes were not available. The precision was determined by triplicate analyses of the same sample in three series (*i.e.* repeatability of measurements of the same sample using the same method on various days). The sensitivity and linear range of measurement were determined for the standard FaDOH as well as FaDOH, FaDOAc, and FaOH in a sample by varying the injection, which provided comparable results to serial dilution of standards. The limit of detection (LOD) and quantification (LOQ) was determined as the intercept of the standard curve plus three and ten times, respectively, the standard deviation of the intercept. The trueness of the method was determined by recovery experiments because no certified reference samples or samples without or with a low concentration of the polyacetylenes were available. The recovery was determined by extraction of triplicates in three series with and without standard addition of FaDOH, which was the only commercially available standard. We did not use an internal standard because it was not possible to find a suitable reference standard, which had similar chemical and physical properties, did not co-elute with the compounds of interest, and was not present in the matrix.

Quality assurance was performed by including an in-house carrot sample as reference material (average RSD: 2.8 %, n = 3) and duplicate measurements (incl. weighing and extraction) of

randomly selected samples in each series of analyses yielding an average RSD of 4.6 % for FaDOH, FaDOAc, and FaOH (n = 9 for each compounds).

Elemental nitrogen analysis

Nitrogen was measured using isotope ratio mass spectrometry (IR-MS, Europa Scientific, Crewe, UK). Approximately 4 mg of pulverised material was weighed in tin capsules and introduced to the MS via a combustion interface. Quality assurance was performed using certified reference material, frequent quality control samples as well as duplicate measurements of all samples.

Statistical analysis

The responses y_{ysb} were modelled as: $y_{ysb} = \mu + \alpha_b + \beta_y + \delta_s + \varepsilon_{ys} + \varepsilon_{yb} + \varepsilon_{ysb}$, where μ is the generalized intercept, α_b, $b = 1, 2, 3$ is the effect of the blocks, β_y, $y = year\ 1, year\ 2\ is$ the effect of year, δ_s, $s = C, OA, OB$ is the effect of growth system. Errors (ε) are considered independently and normally distributed and represent corresponding variance components of interaction. The pair wise comparisons and their confidence intervals between the systems were adjusted to obtain a family wise error rate of 5%. The model was fitted using the proc mixed procedure in the SAS/STAT software packages (Version 9.2, SAS Institute Inc., Cary, NC, USA).

Results and discussion

Identification of polyacetylenes in carrots

An HPLC-chromatogram and the corresponding UV spectra confirmed the presence of three aliphatic C_{17}-polyacetylenes (FaDOH, FaDOAc, and FaOH) in carrots (Figure 2) in agreement with previous results *(18)*. Three absorption maxima were observed in the UV spectra of the identified polyacetylenes at 230−243 nm, 243−246 nm, and 257−260 nm (Table 1) as a consequence of their conjugated triple bonds *(18)* and in agreement with previous obtained spectra of the same compounds *(22, 26)*.

The MS analysis showed that the identified polyacetylenes had low abundance of the protonated molecular ions $[M+H]^+$ (Table 1). The $[FaOH+H]^+$ ion was not observed in ESI^+ mode, which is the most appropriate mode compared to ESI^-, where polyacetylenes are non-detectable *(20)*. Generally,

fragments due to loss of one or two water molecules from the protonated molecular ion ([M-H_2O+H]$^+$ or [M-(H_2O)$_2$+H]$^+$) had high abundance together with adducts of ACN ([M+CH_3CN+H]$^+$ or [M-H_2O+CH_3CN+H]$^+$) similarly to a previous study of polyacetylenes using positive atmospheric pressure chemical ionization (APCI) mode and a MeOH-eluent *(32)*. The latter adduct of ACN ([M-H_2O+CH_3CN+H]$^+$) has also previously been observed as the most abundant ion of FaOH in ESI$^+$ and the formation of ACN or MeOH adducts seem to be typical for aliphatic C_{17}-polyacetylenes in positive APCI or ESI ion mode *(20)*. The exact masses of the polyacetylenes were determined based on the fragment [M-H_2O+H]$^+$ due to the low abundance of the protonated molecular ions and were within an acceptable range (< 5 ppm) from the theoretical values.

Comparison of extraction methods

The extraction efficiencies with ULP were 8─15 % higher than extraction with stirring (Figure 3), while the sensitivities of these two methods were comparable, but can probably be improved by increasing the sample weight. The precisions were similar and in an acceptable range (RSDs: 1─5 %) for this type of analyses. The slightly higher extraction efficiencies with ULP could be because it is a very fast method (60 s extraction time) in comparison with the traditional methodology with stirring and long extraction times (overnight extraction and extra 3 hours). Thereby, the risk of analyte degradation is reduced due to the sensitivity of polyacetylenes towards oxidation *(28)* as well as heat and light *(23)*.

Ultrasonication has previously been used for extraction of polyacetylenes *(14)*. However, it was not tested in this study because it resembles the same basic principle as used in ULP, but ULP is a much faster extraction method and also expected to cause stronger tissue disruption than ultrasonication. Pressurized liquid extraction has also recently been used *(20)* and could be advantageous for high-through-put applications, where larger sample sets are handled. However, considering the size of the sample set in this study, we considered ULP to be a better choice for extraction of polyacetylenes because of its advantages with regards to efficiency and speed.

Method optimization and validation

Extraction times of 15 s, 60 s, and 120 s were tested using the ULP, but no improvements for FaDOH, FaDOAc, and FaOH were observed at increasing extraction times and the precisions were

similar (RSDs: 0.7─4.0 %) (Figure 4). An increase in sample weight from 0.5 to 1.0 g increased the method sensitivity without affecting the extraction efficiency and precision (data not shown). It was also found that in one extraction cycle more than 95 % of the extractable polyacetylenes were detected with acceptable precision (RSDs: 1.7─6.0 %), while less than 4 % and 1 % of the polyacetylenes were detected in the second and third extract, respectively (data not shown). In contrast, two and three extraction cycles were considered necessary in previous studies using extraction with stirring *(14)* and ultrasonication *(26)*. So, the optimum extraction conditions were 1.0 g sample and one extraction cycle of 60 s, whereby more than 95 % of the extractable polyacetylenes were obtained with acceptable precision and sensitivity.

A good chromatographic separation of the three polyacetylenes was achieved within 33 min (Figure 2) yielding a satisfactory resolution between the compounds of interest and interfering peaks. The marked decrease in chromatographic run time represents a significant analytical improvement in comparison with earlier studies, where run times from 45 min to 95 min have been applied *(14, 20, 26)*.

A pilot-study of UPLC resulted in a further reduction in run-time by a factor of 2 from 33 min to 15 min (data not shown). However, the selectivity of the HPLC and UPLC columns were different and interfering peaks disturbed the separation in the UPLC chromatogram produced, yielding poor resolution of FaDOH and FaDOAc. Thus, it was decided to continue with the HPLC separation, because a compatible UPLC column with a similar stationary phase as the one used in the HPLC analysis was not commercially available.

The linear range of measurement for FaDOH was 0.3─58 µg/mL (equal to 4.5─870 µg/g dry weight; $R^2 > 0.999$) (Table 2). It was in the same range for FaDOAc and FaOH when tested by varying the injection volume of a sample, but exact concentration levels could not be obtained due to lack of available standards. The sensitivity for FaDOH was 8.6×10^4 AU/(µg/mL) and the selectivity was satisfactory as interfering compounds (co-eluting compounds or adduct ions) were neither immediately detected in blank samples nor in the alternative UPLC-MS analysis. The sensitivity and selectivity was fully acceptable for the current study and a more sensitive and selective detection principle, *e.g.* MS, was not necessary as reported in previous studies *(20, 30)*.

LODs and LOQs ranged from 3.6 to 7.2 µg FaDOH-equivalents/g dry weight (µg FaDOH-eq/g dw) and 7.8 to 49 µg FaDOH-eq/g dw, respectively (Table 2). Comparable LODs and LOQs were obtained when adjusting for a possible matrix effect by diluting the precision samples to a

concentration close to the expected LOQ and determination of LOD and LOQ as three and ten times the standard deviation. The LOQs were within the linear range of measurement and the precisions were acceptable for our purpose (RSD: 2.3─3.6 %, Table 2).

Recovery experiments with FaDOH were used to estimate the trueness of the method because no certified reference samples or samples without or with a low concentration of the polyacetylenes were available and FaDOH was the only commercially available standard. The recovery of FaDOH was 93 % (Table 2), which we consider satisfactory. Furthermore, as previously stated, the ULP method yielded satisfactory extraction efficiencies compared to the stirring based method (Figure 3) and more than 95 % of the extracted polyacetylenes were detected within one extraction cycle.

Polyacetylenes in organically and conventionally grown carrots

The developed analytical method was used to compare the content of polyacetylenes in organically and conventionally grown carrot roots. The average concentrations of polyacetylenes across growth systems (± standard deviation, n = 9) in year one were 222±33, 30±3.7, and 94±19 µg FaDOH-eq/g dw of FaDOH, FaDOAc, and FaOH, respectively (Figure 5), which corresponds to 26±4.0, 3.6±0.4 and 11±2.3 µg FaDOH-eq/g fresh weight. The concentration levels were 15, 12, and 3 % lower in year two, respectively, and generally in agreement with previously reported values *(5, 14, 20, 21, 27)*. However, the concentration of FaDOH in peeled carrots of the cultivar *bolero* has previously been determined to 10.1 µg/g fresh weight *(21)*. These concentration differences are likely to be caused by variations in soil type, climate, nitrogen fertilization etc.

In the present study, FaDOH was the most abundant polyacetylene in peeled carrot roots in agreement with previous studies *(5, 22, 27)*, but in contrast to a recent study of peeled carrots, where FaOH was found to be the most abundant analyte *(26)*. Previous studies have shown that FaDOH and FaDOAc are primarily found in the carrot peel, but it should be noted that only peeled carrot roots were investigated in the present study.

No significant differences ($p > 0.05$) in the content of polyacetylenes between the three growth systems (C, OA, and OB) were observed. The concentration levels of FaDOAc were significantly lower in year two compared to year one ($p = 0.003$), while there was no significant difference between the concentrations of FaOH and FaDOH in year one and two ($p = 0.42$ and 0.07, respectively), though the statistical analysis indicated a lower concentration of FaDOH in year two ($p < 0.10$).

The comparable content of polyacetylenes in the conventional and organic growth systems might be explained by a high general fertility of the soil, which reduces the impacts of differences in fertilizer application rates between growth systems. This observation was substantiated by the values obtained for harvest yields and dry matter contents, which showed no significant effect of growth year or system (Table 3). The only significant difference between the conventional and organic growth systems was found for the nitrogen content of carrot roots, which was significantly higher in conventionally grown carrots as a consequence of a higher nutrient supply and availability due to the application of inorganic NPK fertilizer. Thus, the nitrogen content of the organic systems was suboptimal compared to norm values found in the literature (0.85-0.95 % nitrogen of dry matter *(33)*) and was consistently lower in all systems compared to previous studies *(24, 34)*. Kaack *et al*. *(24)* found a positive correlation between nitrogen application by green manures and the harvest yield, content of nitrogen, amino-acids, β-carotene and nitrate as well as a reduced dry matter content at increasing nitrogen supply. However, the significant changes observed were only present at larger differences in nitrogen content than those observed in the present study. The content of polyacetylenes might have differed significantly if larger differences in fertilizer application rates between systems had been applied, but the used fertilizer levels reflects common farmer practise in Danish carrot production at similar soil types.

Root size has previously been shown to affect the concentration of polyacetylenes in carrots *(5)*, but the same tendencies were not observed in the present study though the carrots from the OB system were significantly larger than the other growth systems (Table 3). However, the differences in root size were considerable lower in the present study (average root sizes in year one and two: 90.2, 88.7, and 102 g fw/carrot in the C, OA, and OB system, respectively) compared with the root sizes investigated by Kidmose *et al*. (50−100 and >250 g fw/carrot) *(5)*.

In conclusion, three polyacetylenes (FaDOH, FADOAc, and FaOH) in carrots were identified and analysed with an optimized and validated ULP extraction method yielding very fast extractions with improved extraction efficiency in comparison with a traditional stirring based method. The ULP method was combined with the optimized HPLC method leading to a considerable reduction in run time. The method is selective, sensitive, and precise for analysis of polyacetylenes in carrot roots. The new method was applied for analysis of carrot roots grown either organically or conventionally, but no statistical differences were found in the content of the possible health-promoting polyacetylenes. However, a year-to-year variation was observed for FaDOAc and a year-to-year

difference in the content of FaDOH was indicated, which illustrates the importance of weather on the content of polyacetylenes in carrots.

Abbreviations Used

ACN: acetonitrile

APCI: atmospheric pressure chemical ionization

C: crops grown conventionally with the use of inorganic fertilizers and pesticides

DMSO: dimethyl sulfoxide

dw: dry weight

ESI: electrospray ionization

EtOAc: ethyl acetate

FaDOAc: falcarindiol-3-acetate

FaDOH: falcarindiol

FaOH: falcarinol

HPLC: high-performance liquid chromatography

i-FIT: isotopic pattern fit of measurement compared to theoretical

LOD: limit of detection

LOQ: limit of quantification

MeOH: methanol

MS: mass spectrometry

OA: crops grown organically with application of animal manure

OB: crops grown organically with the use of cover crops as green manure

PDA: photodiode array detector

RSD: relative standard deviation

TOF-MS: time of flight tandem mass spectrometer

ULP: Ultrasonic Liquid Processor

UPLC: Ultra Performance Liquid Chromatograph

UV: ultraviolet (radiation)

Acknowledgement

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Figure captions

Figure 1. Chemical structures of the main polyacetylenes in carrots. The systematic names are listed in the foot notes.

Figure 2. Chromatogram of a carrot extract (HPLC-UV, 205 nm).

Figure 3. Extraction efficiency (expressed in FaDOH-equivalents/g dry weight) of the methods tested: ■ : extraction with stirring and ■ : extraction with ultrasonic liquid processor (ULP). The error bars describe the standard deviation (n = 4).

Figure 4. Extraction efficiency (expressed in FaDOH-equivalents/g dry weight) with ultrasonic liquid processor (ULP) in relation to extraction time. ■ : 15 s, ■ : 60 s, and ■ : 120 s. The error bars describe the standard deviation (n = 4).

Figure 5. The average concentrations (ug FaDOH-equivalents/g dry weight) of FaDOH (■), FaDOAc (■), and FaOH (■) in three different growth systems (C: conventional, OA: organic using animal manure and OB: organic using cover crops) and in two growth years (one and two). The error bars describe the standard deviation of replicates from the field (n = 3).

Table captions

Table 1. Polyacetylenes present in the carrots.

Table 2. The linear range of measurement, limit of detection (LOD) and quantification (LOQ), precision (expressed as the relative standard deviation, RSD, n = 9) and the recovery (%, n = 9) of the analyses of polyacetylenes in carrots. Concentrations are expressed in µg falcarindiol-equivalents/g dry weight (µg FaDOH-eq/g dw).

Table 3. Harvest yield expressed as ton fresh weight/hectare (ton fw/ha), carrot root size (gram fw/peeled carrot), nitrogen content (% in dry matter) and dry matter content (%) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (one and two). The averages ± standard deviations are shown (n = 3). The two lower rows represent the results from the statistics across growth systems (year effect) or years (system effect). Growth systems followed by different letters are significantly different ($p < 0.05$).

Table 1

	Formula	t_R [a] (min)	UV_{max} (nm)	Mass of [M-H_2O+H]$^+$ (amu) Calcu-lated	Mass of [M-H_2O+H]$^+$ (amu) Measu-red	Δ m [b] (ppm)	i-FIT [c]	Identified fragments (relative abundance, %)
FaDOH [d]	$C_{17}H_{24}O_2$	8.8	233 245 259	243.1739	243.1749	-4.1	4.5	m/z 227 [M-(H_2O)$_2$+H]$^+$ (55) m/z 243 [M-H_2O+H]$^+$ (75) m/z 261 [M+H]$^+$ (2) m/z 284 [M-H_2O+CH_3CN+H]$^+$ (100) m/z 302 [M+CH_3CN+H]$^+$ (11)
FaDOAc	$C_{19}H_{26}O_3$	14.5	234 246 260	285.1866	285.1855	3.9	7.5	m/z 225 [M-$C_2H_3O_2$-H_2O+H]$^+$ (8) m/z 243 [M-$C_2H_3O_2$+H]$^+$ (69) m/z 285 [M-H_2O+H]$^+$ (100) m/z 303 [M+H]$^+$ (9)
FaOH	$C_{17}H_{24}O$	17.8	230 243 257	227.1788	227.1800	-5.3	16	m/z 227 [M-H_2O+H]$^+$ (21) m/z 268 [M-H_2O+CH_3CN+H]$^+$ (100) m/z 286 [M+CH_3CN+H]$^+$ (17)

[a] Retention time

[b] Calculated from [M-H_2O+H]$^+$ due to low abundance of [M+H]$^+$

[c] Isotopic pattern fit of measurement compared to theoretical

[d] Identity confirmed with standard

Table 2

	Linear range of measurement (µg FaDOH-eq/ g dw)	**LOD (µg FaDOH-eq/g dw)**	**LOQ (µg FaDOH-eq/g dw)**	**Precision (RSD, %)** [a]	**Recovery (%)**
FaDOH	4.5-870	7.2	49	2.3	93
FaDOaC	4.5-870	3.6	7.8	2.5	- [b]
FaOH	4.5-870	6.5	22	3.6	- [b]

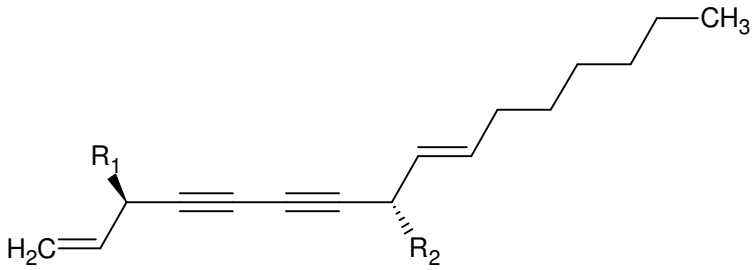
[a] Concentration level in samples used for determination of the precision: 230, 32 and 97 ug FaDOH-eq/g dw for FaDOH, FaDOAc, and FaOH, respectively.

[b] Recovery not determined due to unavailability of standards.

Table 3

	Harvest yield (ton fw/ha)		**Carrot size (gram fw/carrot)**		**N (% in dry matter)**		**Dry matter (%)**	
	Year 1	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**
C	94.7±3.2	106±4.4	98.1±4.4	82.2±5.0	0.93±0.08	0.96±0.12	11.8±0.2	11.3±0.2
OA	84.8±5.6	99.1±4.8	91.9±5.6	85.5±15	0.71±0.05	0.71±0.10	11.5±0.1	11.5±0.2
OB	89.5±4.9	92.4±4.1	110±12.3	94.6±13	0.80±0.04	0.80±0.03	11.8±0.1	11.5±0.2
Year effect	p = 0.13		p = 0.28		p = 0.84		p = 0.31	
System effect	p = 0.26		**p = 0.015** **C** b**, OA** b**, OB** a		**p = 0.0005** **C** a**, OA** b**, OB** b		p = 0.76	

Figure 1



	Abbreviation	R_1	R_2
Falcarinol [a]	FaOH	OH	H
Falcarindiol [b]	FaDOH	OH	OH
Falcarindiol-3-acetate [c]	FaDOAc	$OCOCH_3$	OH

[a] 1,9-heptadecadiene-4,6-diyn-3-ol

[b] 1,9-heptadecadiene-4,6-diyne-3,8-diol

[c] 1,9-heptadecadiene-4,6-diyne-3,8-diol, 3-acetate

Figure 2

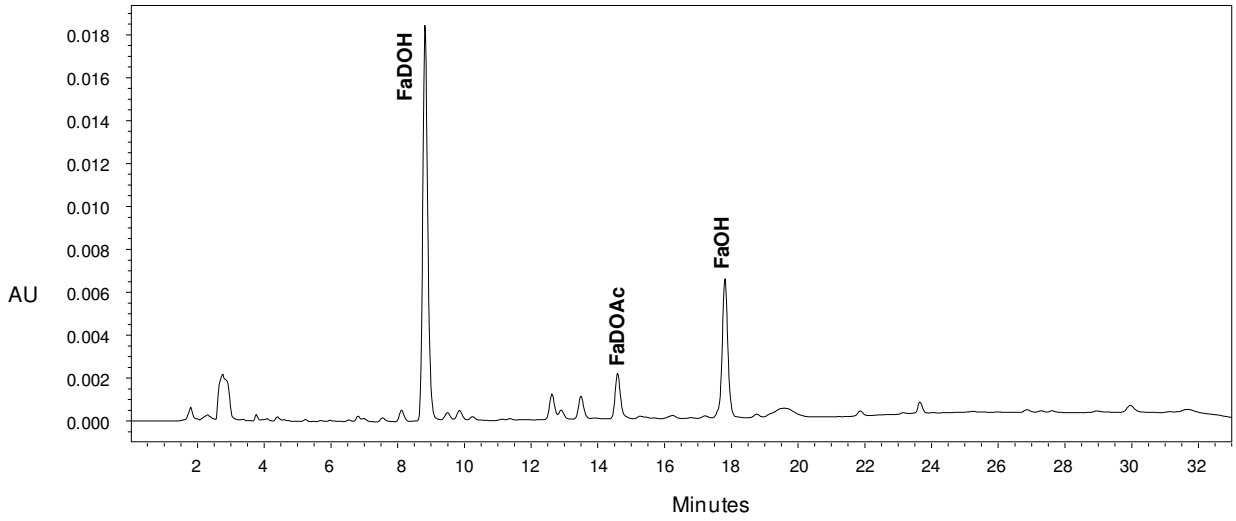


Figure 3

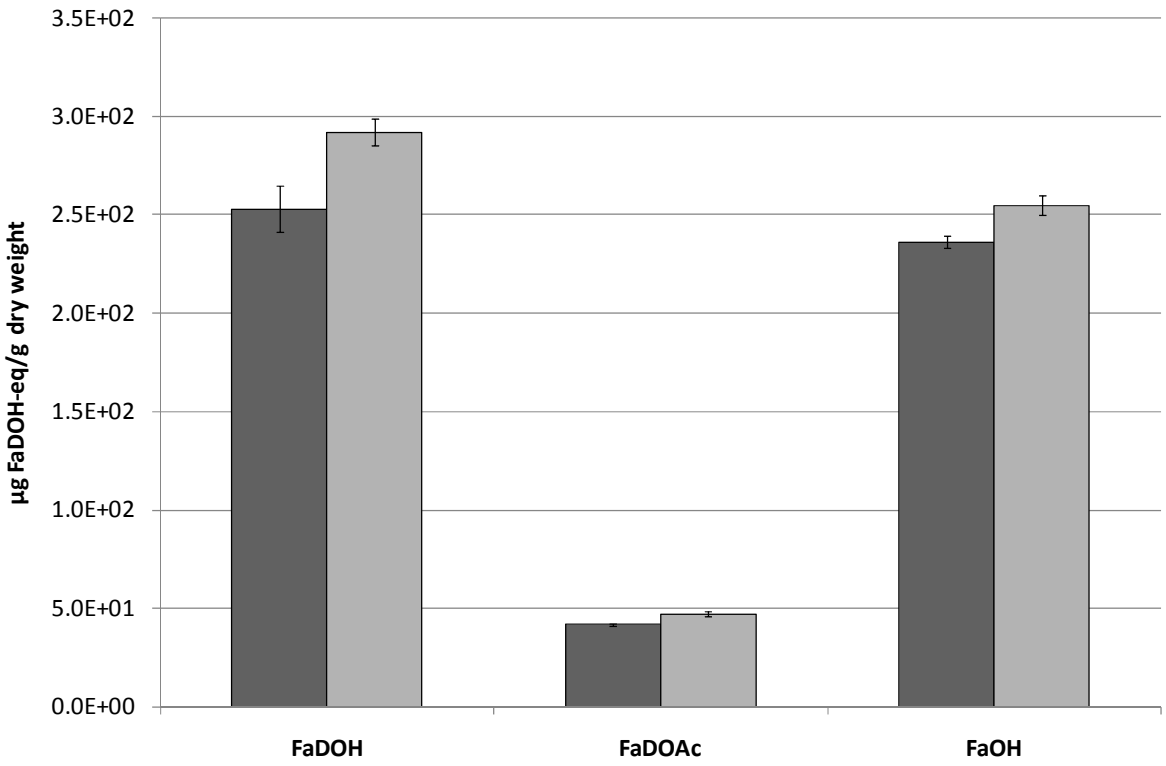


Figure 4

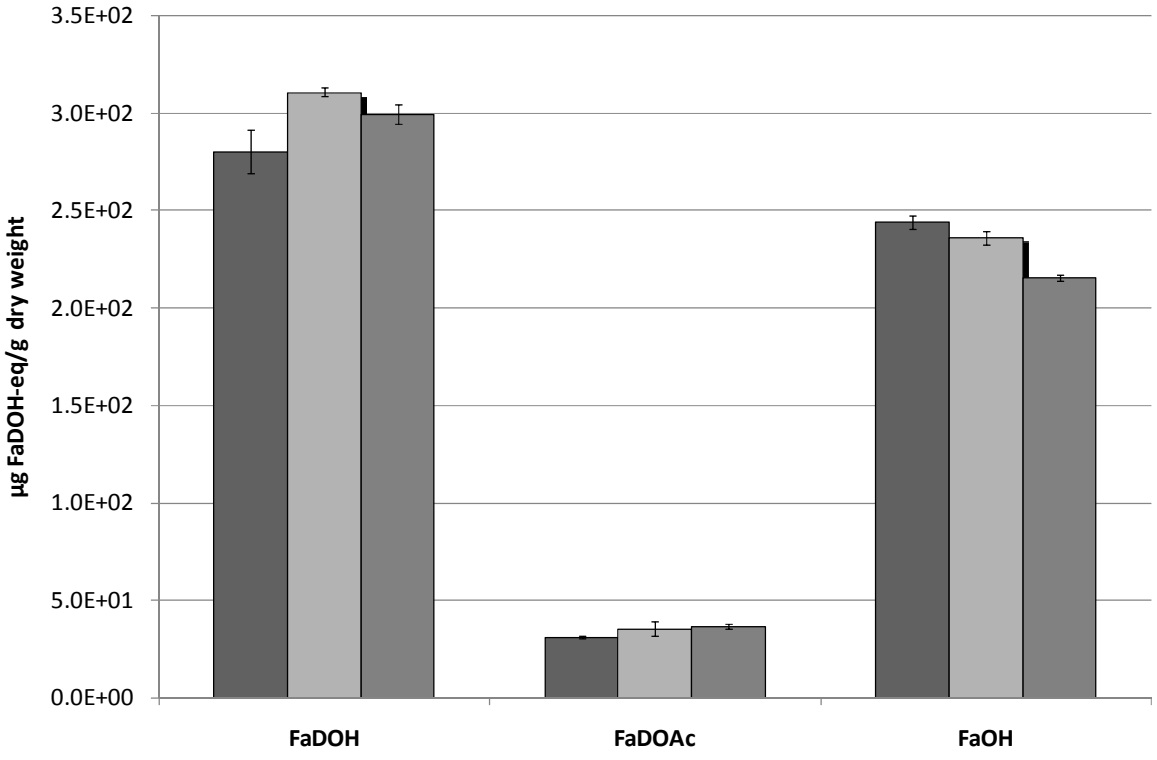
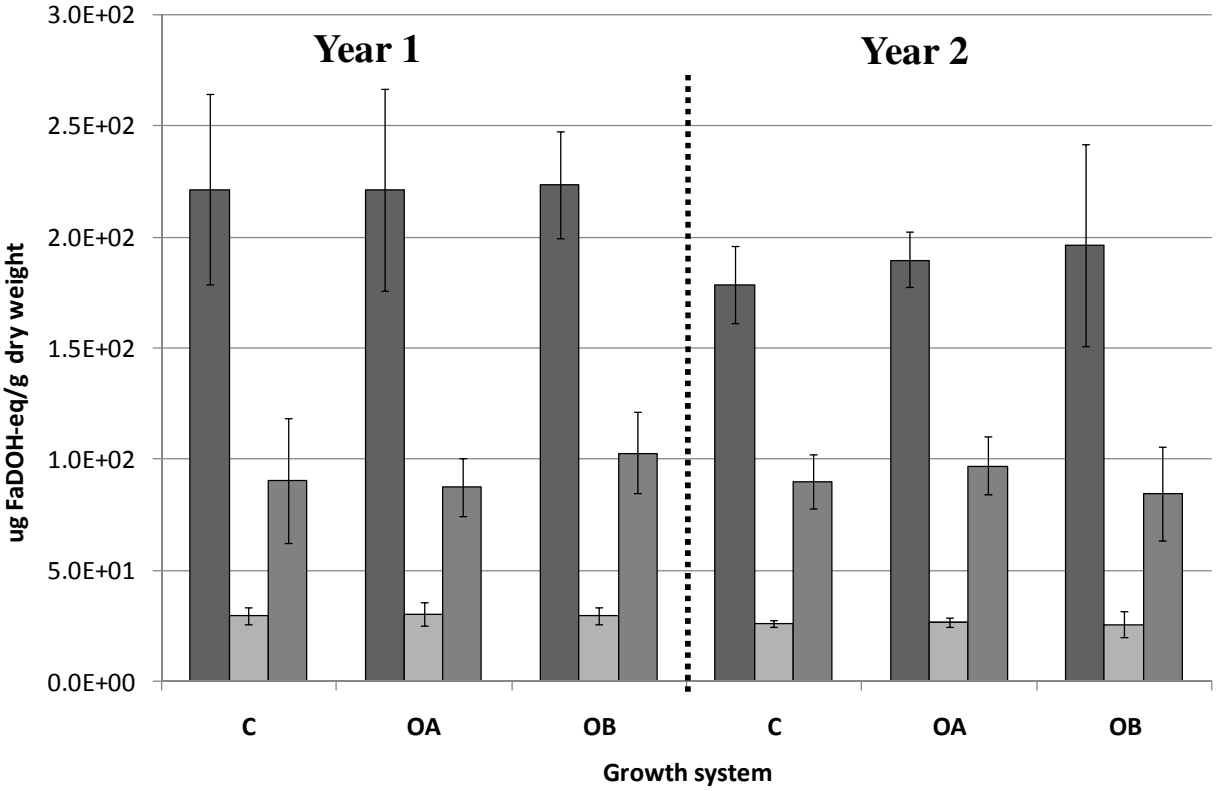


Figure 5


Year 1
Year 2
3.0E+02
2.5E+02
2.0E+02
1.5E+02
1.0E+02
5.0E+01
0.0E+00
ug FaDOH-eq/g dry weight
C
OA
OB
C
OA
OB
Growth system

Manuscript II

Effects of organic and conventional growth systems on the content of flavonoids and phenolic acids in vegetables

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Abstract

The demand for organic food products is steadily increasing partly due to the expected health benefits of organic food consumption. Polyphenols, such as flavonoids and phenolic acids, are a group of secondary plant metabolites with presumably beneficial health effects, and contents in plants are affected by *e.g.* plant nutrient availability, climate, pathogen infection, and pest attack. In the current study, onions, carrots, and potatoes were cultivated in two-year field trials in three different geographical locations, comprising one conventional and two organic agricultural systems. The content of flavonoids and phenolic acids in plants were analyzed by pressurized liquid extraction and HPLC-UV quantification. In onions and carrots, no statistically significant differences between growth systems were found for any of the analyzed polyphenols. However, the content of 5-*O*-caffeoylquinic acid was significantly higher in potatoes in one of the organic growth systems compared with the conventional system, thereby overruling geographical and yearly variation.

Keywords

Carrots (*Daucus carota* L.), flavonoids, onions (*Allium cepa* L.), organic agriculture, potatoes (*Solanum tuberosum* L.), phenolic acids

Introduction

Plant production in organic agricultural systems relies on organic manures and biological pest control, excluding the use of inorganic fertilizers and synthetic pesticides. Organic agriculture has developed rapidly in most parts of Europe since the 1990s, especially in the Scandinavian and Mediterranean countries *(1)*, and the market shares were around 5 % in Denmark at the end of 2007 *(2)*. Improved animal welfare, environmental protection, human health as well as taste and freshness are the most important reasons for the increasing demand of organic food products *(3)*. However, the possible health benefits of organic food consumption are still controversial and not considered scientifically well-documented *(4)*.

The fundamental differences in organic and conventional agricultural systems, particularly regarding fertilization strategy and soil fertility management, affect the nutrient composition in plants *(5)*. However, previous studies on the nutrient content of organically and conventionally grown plants have generated contradicting results *(6, 7)*. In order to make valid comparisons of organic and conventional plants, important variation caused by several factors such as geographical location and growth season must be included to ensure that the possible differences found are systematic and reliable, but this has not been the case in most previous studies.

Little consideration has previously been given to bioactive secondary plant metabolites *(8)*, which play an important role in the growth, development, and defence system of plants *(9)*. Despite being non-essential to humans, various secondary plant metabolites have been proposed to prevent numerous diseases *(10)*. Polyphenols have one or more phenol units in their chemical structure *(11)* and are a group of secondary plant metabolites, frequently speculated to have positive health effects including a decreased risk of heart diseases, dementia *(12)*, and cancer *(13, 14)*.

Flavonoids are a group of polyphenols, which occur widely across the plant kingdom *(15)*. Onions are one of the main sources of polyphenols in the daily food intake *(16, 17)* and a rich source of flavonoids *(18)*. Quercetin mono- and diglucosides account for up to 80 % of the total content of flavonols in onions, but kaempferol *(19)*, isorhamnetin derivates *(20)*, and myricetin *(9)* have also been found (Figure 1).

Another group of widespread polyphenols are phenolic acids e.g. caffeic, p-coumaric, ferulic, and chlorogenic acids (Figure 1), which are found in various beverages, fruits, and vegetables *(21)*. 5-*O*-caffeoylquinic acid (Figure 1) is the predominant phenolic acid in potatoes *(22)* and carrots *(23*), where it constitutes 80–90 % of the total content of phenolics.

The content of polyphenols in plants is affected by factors such as cultivar *(24)*, pathogen infection and pest attack *(25)*, time of harvest as well as storage and processing procedures *(26)*. The content of nutrients and secondary plant metabolites in food products is also affected by e.g. growth conditions, use of fertilizers, climate *(27)*, and plant nutrient availability *(28)*.

The objective of our study was to compare the content of selected flavonoids and phenolic acids in organically and conventionally grown onions, potatoes, and carrots, and to evaluate if the ability of the crops to synthesize selected secondary metabolites is systematically affected by growth system across different growth years as well as geographic locations (soil types).

Materials and methods

Reagents and chemicals

Methanol (HPLC-grade, Rathburn Chemicals Ltd., Scotland) and Milli-Q water (18 MΩ, Millipore, USA) were used for standards and eluents. Dimethyl sulfoxide (DMSO, >99 %) and formic acid (98−100 %) were purchased from Merck (Germany). The following standards were purchased: quercetin (Q) from Extrasynthése (France), quercetin-3,4'-diglucoside (Q-3,4'-diglu) from Polyphenols (France), quercetin-7,4'-diglucoside (Q-7,4'-diglu) from Apin (United Kingdom), quercetin-4'-glucoside (Q-4'-glu) from Plantech (United Kingdom); and quercetin-3-glucoside (Q-3-glu, purity > 90%), 5-*O*-caffeoylquinic acid (5-CQA, purity > 95%) and caffeic acid (CA, purity >99 %) from Sigma-Aldrich (Germany).

Individual standard stock solutions were prepared by dissolving the solid compounds in DMSO (100 µg/mL). Working solutions were prepared by ten-times dilutions of the stock solutions with methanol. Stock solutions were stored at −80 ^{o}C (<1 year), while the working solutions were prepared shortly before analyses and kept at −20 ^{o}C until analyses.

Samples

Potato, carrot, and onion samples were obtained from field trial studies undertaken in 2007 and 2008 (year 1 and 2). The potatoes (*Solanum tuberosum* cv. *sava*) were grown in the long-term CropSys crop rotation experiment *(29)* at three different geographical locations (Flakkebjerg, Foulum, and Jyndevad) (http://www.cropsys.elr.dk/uk/). The carrots (*Daucus carota* cv. *bolero*) and onions (*Allium cepa* cv. *hytech*) were grown at one location (Aarslev) in the VegQure rotation

experiment (http://www.vegqure.elr.dk/uk/). The crops were grown in three different agricultural systems at all locations: one conventional system (C) and two organic systems (OA and OB). The systems were all based on stock-less cash crop production systems with an identical sequence of main crops (8-year rotation in Aarslev and 4-year rotation at the other locations). In the conventional system, pesticides and inorganic fertilizer were used. The OA system relied on import of animal manure. In the OB system, nutrient supply was based on the use of cover crops (mainly legumes), but animal manure was also applied to onions in order to satisfy the high nitrogen demand of this crop. Cover crops were generally grown in the autumn after the main crops and incorporated into the soil in the spring before the main crops were grown. The organic systems were managed in full compliance with the Danish guidelines for organic farming administered by the Danish Plant Directorate (http://pdir.fvm.dk). The potatoes were grown with two replicates of each growth system resulting in 6 plots per year per location (36 plots in total), while carrots and onions were grown with three replicates of each growth system resulting in 9 plots per year (18 plots in total). Table 1 in the Supporting Information presents details on field trial characteristics such as geographical locations, soil types, fertilizer applications etc.

The crops were harvested at the same day for all systems. A 15 kg sample was collected from each plot using the following inclusion criteria both years (marketable quality): potatoes: 35−60 mm diameter, carrots: 50−250 g fresh weight (fw), and onions: 40−80 mm diameter. Representative sampling was ensured by stepwise mass reduction as described in Petersen *et al. (30)*. Only the eatable parts of the crops were analysed, i.e. the bulb of the onions were used for analysis by removing the shoots and outer leaves and the shoot of the carrots was discarded. The samples were washed in Milli-Q water, peeled (only carrots and potatoes), cut into 0.5 cm thick slices, and freeze-dried at 0.08 kPa for 1-2 days at a commercial freeze drying company (Danish Freeze Dry A/S, Kirke Hyllinge, Denmark). Afterwards, the samples were crushed, homogenised, and stored at −20 ^{o}C in an inert nitrogen atmosphere until analysis. In general, the samples were protected from light and oxygen during the entire sample preparation by wrapping in aluminium foil and storing in a nitrogen atmosphere.

Chemical analysis of flavonoids and phenolic acids

The principles of the chemical analysis are summarized below and further details can be seen in Søltoft *et al. (31)*. Extraction of flavonoids and phenolic acids was performed by pressurized liquid

extraction using an ASE-200 (Dionex, Sunnyvale, CA, US). The sample material (0.5 g) was added to 5 ml extraction cells together with 0.5 g C18-material (Sepra E-C18, 50 µm; Phenomenex, Allerød, Denmark) and extracted with 65 % aqueous methanol. The volume of the extracts was adjusted to 20 mL and filtered (0.20 µm, Sartorius Minisart, Aubagne, France) before quantification by HPLC-UV on a Phenomenex Prodigy RP-C18 column (4.6×250 mm, 5 µm, 30 ^{o}C) with 0.1 % formic acid in Milli-Q water (v/v) and methanol (90:10, v/v, A-eluent) and 100 % methanol (B-eluent). The flavonoids and phenolic acids were quantified relative to quercetin according to common practise for analyses of bioactive compounds *(32)*. The chemical structure of the flavonoids and phenolic acids was elucidated by mass spectrometry (MS) and MS/MS analysis, and by comparison of retention times, UV, and MS data with available standards (Q, Q-3,4'-diglu, Q-7,4'-diglu, Q-4'-diglu, Q-3-glu, 5-CQA, and CA) according to the principles detailed in Søltoft et al. (31), which also presents the validation parameters used.

Quality assurance was performed by including an in-house sample as reference material (a mix of freeze-dried potato, onion, and carrot) in each series of analyses and yielded an average RSD of 6.2 % for all quantified polyphenols (n = 4). Furthermore, duplicate measurements (incl. weighing and extraction) of randomly selected samples in each series of analyses were included, yielding an average RSD of 6.8 %, 11 %, and 2.7 % for potatoes, carrots, and onions for all quantified polyphenols in each crop (n = 6 for each compound in potatoes, n = 2 for carrots and onions).

Elemental nitrogen analysis

Nitrogen was measured using isotope ratio mass spectrometry (IR-MS; Europa Scientific, Crewe, UK). Approximately 4 mg of pulverised material was weighed in tin capsules and introduced to the MS via a combustion interface. Quality assurance was performed using certified reference material, frequent quality control samples as well as duplicate measurements of all samples.

Statistical analysis

The responses y_{ysb} from the Aarslev location were modelled as: $y_{ysb} = \mu + \alpha_b + \beta_y + \delta_s + \varepsilon_{ys} + \varepsilon_{yb} + \varepsilon_{ysb}$, where μ is the generalized intercept, α_b, *b = 1, 2, 3* is the effect of the blocks, β_y, *y = year 1, year 2* is the effect of year, and δ_s, *s = C, OA, OB* is the effect of growth system. The responses y_{yslb} from the other locations were modelled as: $y_{yslb} = \mu + \beta_y + \delta_s + \gamma_l + \alpha_{lb} + \varepsilon_{ys} + \varepsilon_{yl} + \varepsilon_{sl} + \varepsilon_{ylb} + \varepsilon_{yslb}$,

where μ is the generalized intercept, $\beta_{y,}$ *y = year 1, year 2* is the effect of year, $\delta_{s,}$ *s = C, OA, OB* is the effect of growth system, γ_l is the effect of location, *l = Foulum, Jyndevad, Flakkebjerg* is the location, and $\alpha_{lb,}$ *b =1, 2* is the effect of the blocks within each location.

Errors (ε) are considered independently and normally distributed and represent corresponding variance components of interaction. The pair wise comparisons and their confidence intervals between the systems were adjusted to obtain a family wise error rate of 5%. The model was fitted using the proc mixed procedure in the SAS/STAT software packages (Version 9.2, SAS Institute Inc., Cary, NC, USA).

Results and discussion

Flavonoids in onions

The flavonoid profile of onions was similar to profiles previously identified in southern Italian red onions *(20)* and white onions *(33)* with a high abundance of Q-3,4'-diglu and Q-4'-glu, but traces of quercetin-3,7,4'-triglycoside (Q-3,7,4'-trigly), Q-3-glu, isorhamnetin-3,4'-diglycoside (I-3,4'-digly), and isorhamnetin-4'-glycoside (I-4'-gly) were also detected (Figure 2). Q-7,4'-diglu was only present in concentrations below the limit of quantification (6.6 µg quercetin-equivalents/g fw (µg q-eq/g fw)). The average concentrations (± standard deviation (sd), n = 9) of Q-3,4'-diglu and Q-4'-glu in year 1 were 262 ± 38 and 208 ± 41 µg q-eq/g fresh weight, respectively, and the concentrations were 9 % and 8 % lower in year 2, respectively. The concentrations correspond to 530 ± 77 µg Q-3,4'-diglu/g fw and 330 ± 65 µg Q-4'-diglu/g fw, and the concentration levels of flavonoids were thus in accordance with levels previously found in onions *(18, 34)*.

No significant differences ($p > 0.05$) in the content of any of the flavonoids in onions between the three growth systems (C, OA, and OB) were observed. However, a large variation (average RSD for all quantified flavonoids: 16 %, n = 3) within growth systems was seen in most cases, despite the replicates being located geographically close to one another in order to minimize the effects of differences in soil fertility and microclimate.

A year-to-year variation was observed for Q-3,7,4'-trigly with a significantly higher content in year 2 ($p = 0.046$). The variation in flavonoid content of onions between growth years has previously been shown by Mogren *et al. (34, 35)*. A significant difference in harvest yield ($p = 0.02$) and onion size ($p = 0.03$) between the two growth years was also found (Table 2 in Supporting Information).

The observed year-to-year variation could, for instance, be related to the weather conditions since year 1 was a growth year with higher temperatures and more precipitation in comparison with year 2.

The use of inorganic nitrogen in conventional agriculture often results in a higher plant nitrogen availability *(5)*, harvest yield *(36)*, and nitrogen content of the crop *(5)*. Higher nitrogen availability has previously caused lower accumulation of flavonoids in tomatoes *(5)*. The harvest yield was significantly affected (Table 2 in Supporting Information) by the different amounts of fertilizer and fertilizer types used for onions in the growth systems in the current study (Table 1 in Supporting Information). Thus, the application of high amounts of inorganic fertilizer in the growth system C resulted in the highest harvest yield in both years. However, no significant differences between the conventionally and organically grown onions in the content of nitrogen and flavonoids, size, and dry matter content were found ($p > 0.05$). The nitrogen levels at harvest were within norm values (1.2−1.4 % in dry matter *(37)*), but the differences in harvest yield indicated that fertilization of the organic crops during the growth season was sub-optimal and below the yield plateau as is often the case in organic farming *(36)*.

The results were in accordance with previous onion cultivation studies, where neither the source (organic or inorganic) nor the levels of nitrogen fertilization affected the content of flavonoids *(34, 38)*. In contrast, a higher content of myricetin and quercetin-3-rhamnoside has previously been found in organically in comparison with conventionally grown onions, but the significance of the growth system was difficult to determine as the crops were grown at two different farms *(39)*.

Phenolic acids in carrots

The only polyphenol found in carrots was 5-CQA, which is in accordance with previous observations *(23, 40)*, where it accounted for up to 80% of the total phenolic acid content (23). The average concentration (± sd, n = 9) of 5-CQA in year 1 was 15 ± 4.8 µg q-eq/g fw, which corresponds to 55 ± 18 µg 5-CQA/g fw (refer to Figure 3), and the concentration levels were in agreement with previous levels reported for carrots *(23, 40)*. The concentrations were on average 28 % lower in year 2, but no significant year-to-year variation was found across the growth systems ($p > 0.05$).

The very different amounts of fertilizer and fertilizer types applied to the three growth systems resulted in a significantly ($p = 0.0005$) higher content of nitrogen in conventional carrots (Table 2

Supporting Information). However, no significant differences in the harvest yield, dry matter content, or the concentrations of 5-CQA were found ($p > 0.05$). Similar results have been observed in a previous study, where no difference in the content of 5-CQA was found for potatoes cultivated at different fertilization levels (0–300 kg N/ha) *(41)*. Furthermore, a large average variation within growth systems was observed for 5-CQA in carrots (RSD: 12 %) (Figure 3) as was also seen for flavonoids in onions.

Phenolic acids in potatoes

5-CQA was the most abundant phenolic acid identified in potatoes in agreement with previous studies (22, 41), but also small quantities of 4-O-caffeoylquinic acid (4-CQA) were found (Figure 4). The average concentrations (± sd, n = 6) of 5-CQA in year 1 were 21.7 ± 1.8, 19.6 ± 2.5, and 28.2 ± 1.5 µg q-eq/g fw at the three different locations Foulum, Jyndevad, and Flakkebjerg, respectively, which corresponds to 80.2 ± 6.6, 72.2 ± 9.4, and 104 ± 5.4 µg 5-CQA/g fw. The average concentrations in year 2 were 22 % lower at the Foulum location, and 17 % higher at both the Jyndevad and Flakkebjerg locations. The concentration levels were in accordance to *(42)* or slightly higher *(41)* than previous concentration levels found in potatoes, and the average variation within growth systems (RSD: 7.9 %) was lower than previously seen for the carrots (RSD: 12 %) and onions (RSD: 16 %). The concentration of 4-CQA constituted 20─31 % of the concentration of 5-CQA, which was slightly higher than previously found in potatoes (15─20 %) (43). Traces of CA were also detected in the potatoes, but the concentrations were below the limit of quantification (4 µg q-eq/g fw; data not shown).

A significantly higher content of 5-CQA was found in the organic growth system with cover crops (OB) compared to the conventional growth system (C) across locations and growth years ($p = 0.03$), despite that no significant differences in nitrogen content was found at harvest (Table 3 in Supporting Information). This is in agreement with a previous study, where no correlation between nitrogen application rate and production of phenolic acids in potatoes was observed *(41)*. Instead, the significantly higher content of 5-CQA in the OB system could be related to a lower potassium fertilization level in comparison with the conventional growth system (Table 1 in Supporting Information), which has previously been observed for polyphenols in potatoes *(44, 45)*. Hajslova *et al*. *(46)* also found a higher level of 5-CQA in organically compared with conventionally grown potatoes. However, the crops were grown at two different farms, which could have induced a

variation unrelated to growth system, even though the farms were located relatively close to each other.

The potato sizes and dry matter contents did not differ, but a positive correlation between potato harvest yield and nitrogen application rate was found. Hence, the yield was significantly higher in the conventional compared with the two organic growth system ($p < 0.0001$, C>OA>OB, Table 3 in Supporting Information) and the nitrogen fertilization was most likely below optimum in the organic growth systems. Furthermore, a significant effect of the geographical location on the content of phenolic acids in potatoes has previously been observed *(46)*, but no significant location differences were found in the present study for any of the analysed parameters. Furthermore, no significant year-to-year variation was seen in the content of phenolic acids in potatoes in contrast to a previous farm study by Hajslova *et al*. *(46)*.

In conclusion, seven flavonoids were detected in onions as well as one and three phenolic acids in carrots and potatoes, respectively. A significant year-to-year variation was observed for Q-3,7,4'-trigly in onions, but no significant differences in the content of flavonoids and phenolic acids between the conventional and the two organic growth systems were found. In the organically grown potatoes fertilized with cover crops, a significantly higher content of 5-CQA was found compared with the conventional system, which overruled variation caused by geographical location and growth year. Based on the present study carried out under well-controlled conditions, it cannot be concluded that organically grown vegetables generally have higher contents of health-promoting secondary metabolites in comparison with the conventionally cultivated ones. The ability of crops to synthesize selected secondary metabolites was not systematically affected by the growth system across different growth years and geographical locations.

Abbreviations used

C: conventional growth system using pesticides and inorganic fertilizer

CA: caffeic acid

4-CQA: 4-O-caffeoylquinic acid

5-CQA: 5-O-caffeoylquinic acid

fw: fresh weight

I-3,4'-digly: isorhamnetin- 3,4'-diglycoside

I-4'-gly: isorhamnetin-4'-glycoside

MS: mass spectrometry

MS/MS: tandem mass spectrometry

OA: organic growth system using animal manure

OB: organic growth system using cover crops

Q: quercetin

q-eq: quercetin-equivalents

Q-3,7,4'-trigly: quercetin 3,7,4'-triglycoside

Q-3,4'-diglu: quercetin-3,4'-diglucoside

Q-7,4'-diglu: quercetin-7,4'-diglucoside

Q-4'-glu: quercetin-4'-glucoside

Q-3-glu: quercetin-3-glucoside

RSD: relative standard deviation

sd: standard deviation

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Figure captions

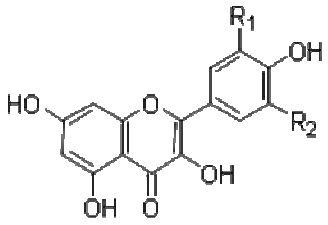
Figure 1. Chemical structures of selected flavonoids and phenolic acids. The systematic names are listed in the foot notes.

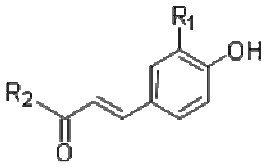
Figure 2. Average concentrations of flavonoids in onions (µg quercetin-equivalents/g fresh weight) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (A: year 1 and B: year 2). Error bars describe the standard deviation of replicates from the field (n = 3). ■: isorhametin-3,4'-diglycoside, ■: isorhamnetin-4'-glycoside, □: quercetin-3,7,4'-triglycoside, ■: quercetin-3-glucoside, □: quercetin-3,4'-diglucoside, and ■: quercetin-4'-glucoside.

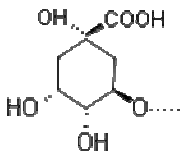
Figure 3. Average concentrations of 5-*O*-caffeoylquinic acid in carrots (µg quercetin-equivalents/g fresh weight) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crop) and in two growth years (□: year 1 and ■: year 2). Error bars describe the standard deviation of replicates from the field (n = 3).

Figure 4. Average concentrations of phenolic acids in potatoes (µg quercetin-equivalents/g fresh weight) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (A: year 1 and B: year 2) at three different locations (Foulum, Jyndevad, and Flakkebjerg). Error bars describe the standard deviation of replicates from the field (n = 2). □: 5-*O*-caffeoylquinic acid and ■: 4-*O*-caffeoylquinic acid.

Figure 1





	R_1	R_2		R_1	R_2
Quercetin [a]	OH	H	Caffeic acid [e]	OH	OH
Kaempferol [b]	H	H	*p*-coumaric acid [f]	H	OH
Isorhamnetin [c]	OCH_3	H	Ferulic acid [g]	OCH_3	OH
Myricetin [d]	OH	OH	5-*O*-caffeoyl quinic acid [h]	OH	

[a] 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one

[b] 3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one

[c] 3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)chromen-4-one

[d] 3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)chromen-4-one

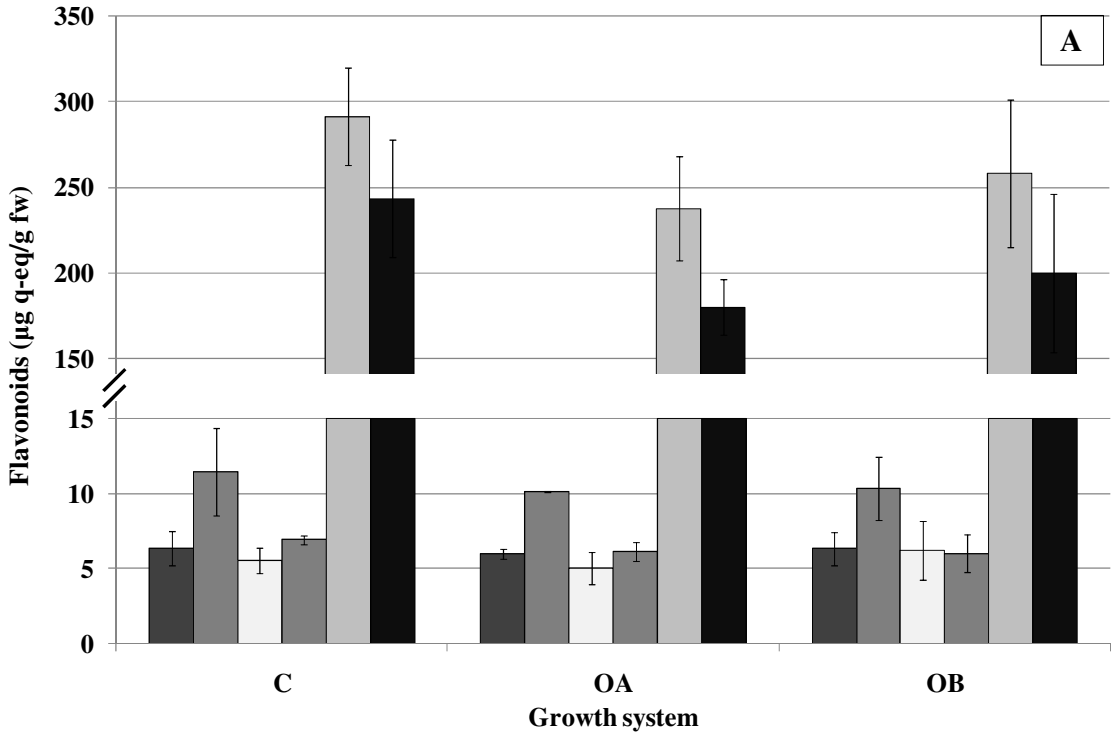
[e] 2-propenoic acid, 3-(3,4-dihydroxyphenyl)-

[f] 2-propenoic acid, 3-(4-hydroxyphenyl)-, (2E)-

[g] 2-propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-

[h] cyclohexanecarboxylic acid, 3-[[3-(3,4-dihydroxyphenyl)-1-oxo-2-propen-1-yl]oxy]-1,4,5-trihydroxy-, (1S,3R,4R,5R)-

Figure 2


A
350
300
250
200
150
15
10
5
0
Flavonoids (µg q-eq/g fw)
C
OA
OB
Growth system

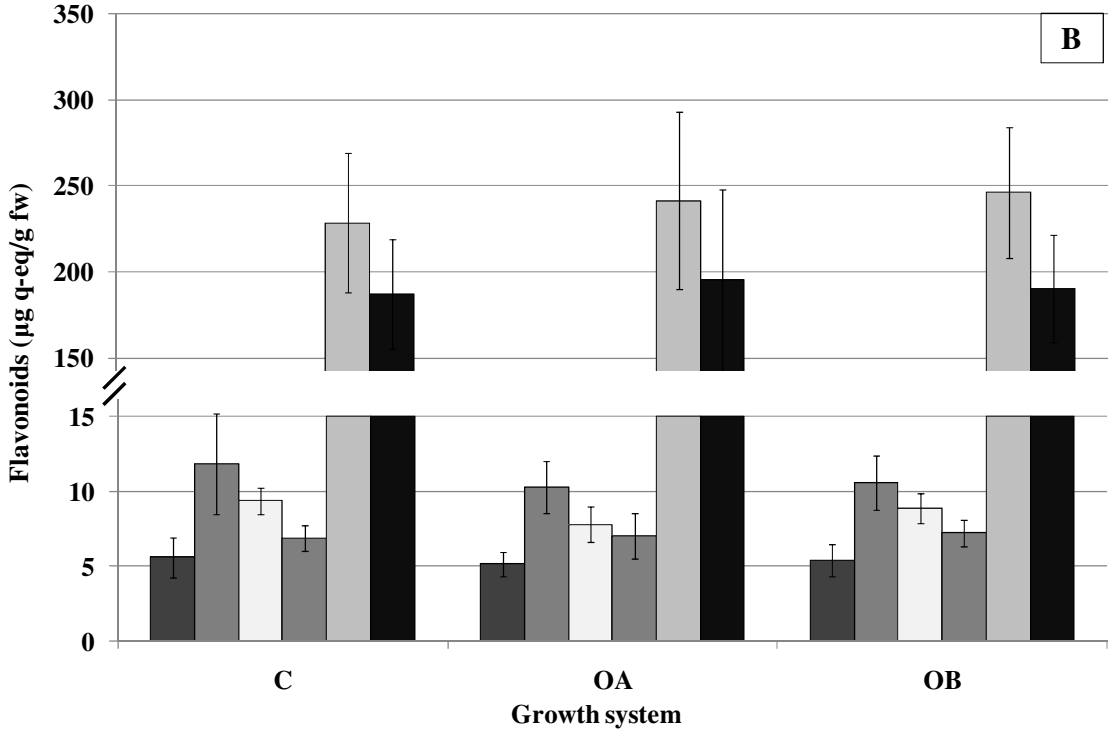

B
350
300
250
200
150
15
10
5
0
Flavonoids (µg q-eq/g fw)
C
OA
OB
Growth system

Figure 3

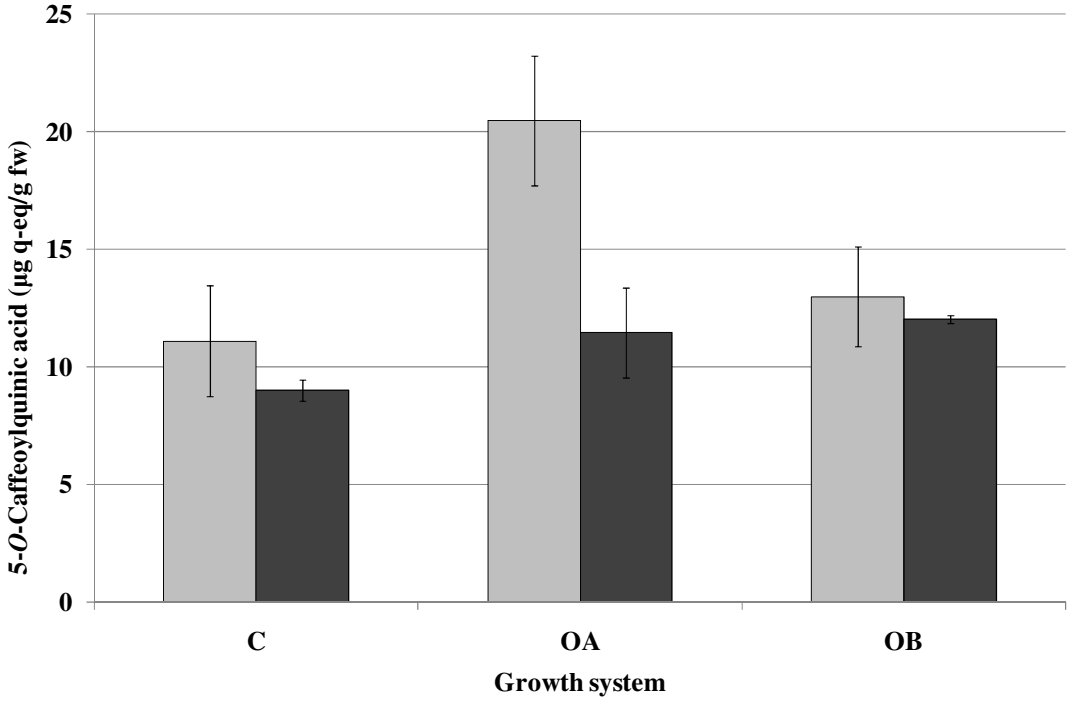

25
20
15
10
5
0
5-O-Caffeoylquinic acid (µg q-eq/g fw)
C
OA
OB
Growth system

Figure 4

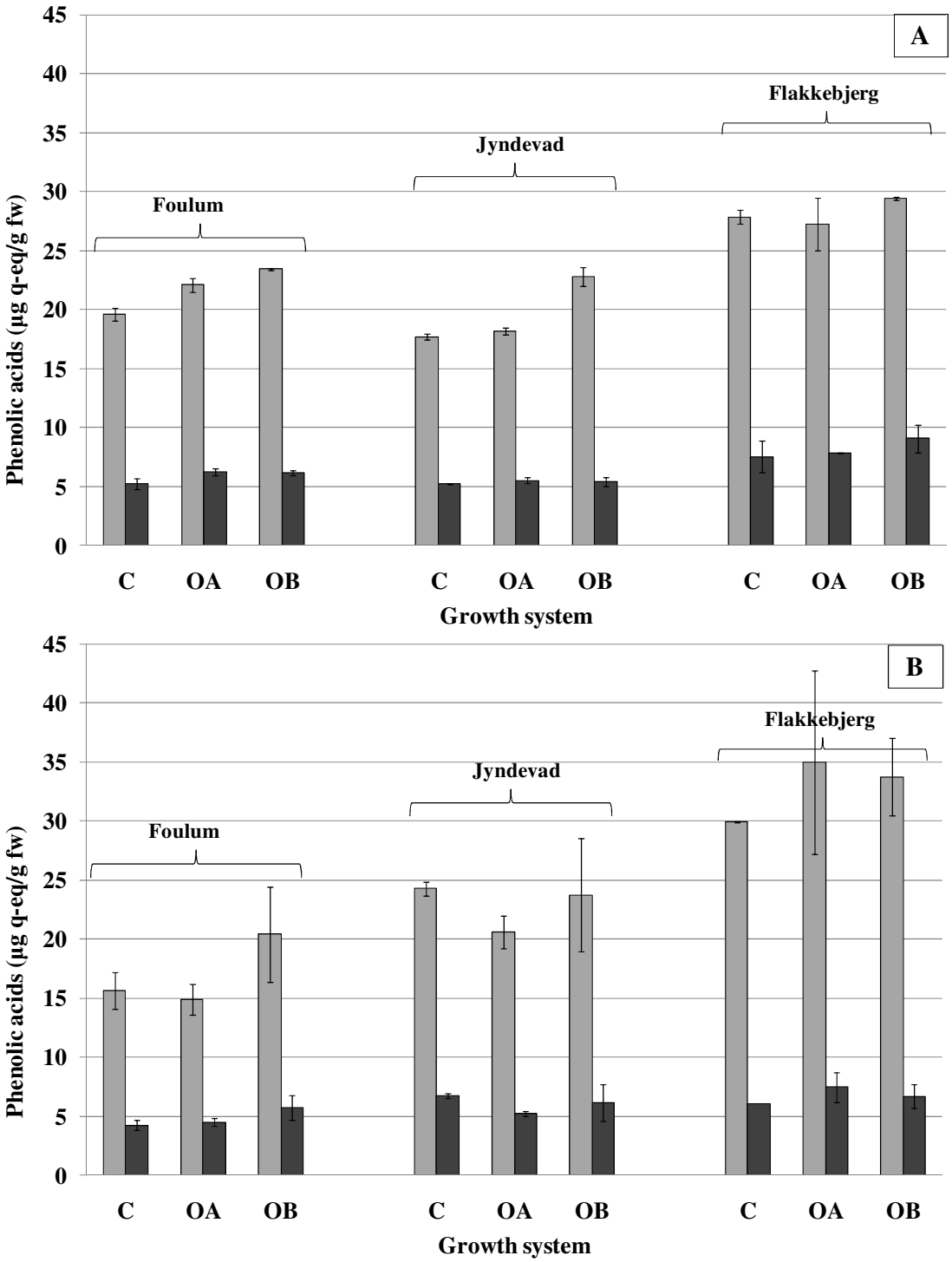

A
Flakkebjerg
Jyndevad
Foulum
Phenolic acids (µg q-eq/g fw)
45
40
35
30
25
20
15
10
5
0
C
OA
OB
C
OA
OB
C
OA
OB
Growth system
B
Flakkebjerg
Jyndevad
Foulum
Phenolic acids (µg q-eq/g fw)
45
40
35
30
25
20
15
10
5
0
C
OA
OB
C
OA
OB
C
OA
OB
Growth system

Table 1 in Supporting Information. Field trial characteristics.

Location	**Flakkebjerg**	**Foulum**	**Jyndevad**	**Aarslev**	
Region	Zealand	Central Jutland	South Jutland	Funen	
Coordinates	11°23'E, 55°19'N	09°34'E, 56°30'N	09°08'E, 54°54'N	10°27'E, 55°18'N	
Crop	Potato	Potato	Potato	Onion and carrot	
Plot size (m^2)	169	216	378	120	
Soil type	Sandy loam	Loamy sand	Sand	Sandy loam	
Clay (%)	16	9	5	15	
Silt (%)	12	13	2	27	
Sand (%)	70	74	91	55	
pH (in 0.01 M $CaCl_2$) [a]	6.5	5.6	5.7	6.2	
P (mg/100 g soil) [a]	2.3	4.4	4.2	2.6	
K (mg/100 g soil) [a]	9.5	12	5.4	12	
Mg (mg/100 g soil) [a]	4.4	4.2	5.3	4.3	
	Fertilizer application (average of year 1 and 2)				
Crop	Potato (Flakkebjerg)	Potato (Foulum)	Potato (Jyndevad)	Onion (Aarslev)	Carrot (Aarslev)
N/P/K [b] **system C (kg/ha)**	140/50/250	140/30/175	140/35/200	170/80/65	120/20/60
N/P/K [c] **system OA (kg/ha)**	110/15/120	115/25/145	125/20/155	110/10/40	55/5/20
N/P/K [d] **system OB (kg/ha)**	2/0/55	1/0/90	1/0/90	70/5/25	- / - / -

[a] : average for plough layer soil samples taken in March across all plots in both growth years and analysed as described by Husted *et al. (47).*

[b] : applied as inorganic fertilizer.

[c] : applied as animal manure. K was supplemented as vinasse for potatoes.

[d] : K was applied as vinasse for potatoes. The vinasse also contained trace amounts of N. Animal manure was used for onions.

Table 2 in Supporting Information. Onion and carrot harvest yields expressed as ton fresh weight/hectare (ton fw/ha), crop unit size (gram fw/onion or carrot), nitrogen content (% in dry matter), and dry matter content (%) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years. The averages ± standard deviations are shown (n = 3). The two lower rows for each crop represent the results from the statistics across growth systems (year effect) or years (system effect). Growth years or systems followed by different letters are significantly different ($p < 0.05$).

Onion	**Harvest yield (ton fw/ha)**		**Onion size (gram fw/onion)**		**N (% in dry matter)**		**Dry matter (%)**	
	Year 1	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**
C	74.4 ± 1.1	96.1 ± 2.8	147 ± 13	194 ± 12	1.3 ± 0.1	1.7 ± 0.1	10.7 ± 0.3	11.4 ± 0.3
OA	46.7 ± 7.3	64.3 ± 0.9	103 ± 31	191 ± 17	1.2 ± 0.2	1.4 ± 0.2	9.9 ± 0.7	11.2 ± 0.2
OB	46.8 ± 6.7	65.1 ± 8.8	131 ± 3.4	191 ± 11	1.3 ± 0.1	1.4 ± 0	9.8 ± 0.5	11.3 ± 0.4
Year effect	**p = 0.02** **Year 1** b, **Year 2** a		**p = 0.03** **Year 1** b, **Year 2** a		p = 0.15		p = 0.10	
System effect	**p < 0.001** **C** a**, OA** b**, OB** b		p = 0.42		p = 0.33		p = 0.38	
Carrot	**Harvest yield (ton fw/ha)**		**Carrot size (gram fw/carrot)**		**N (% in dry matter)**		**Dry matter (%)**	
	Year 1	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**
C	94.7 ± 3.2	106 ± 4.4	98.1 ± 4.4	82.2 ± 5.0	0.93 ± 0.08	0.96 ± 0.12	11.8 ± 0.2	11.3 ± 0.2
OA	84.8 ± 5.6	99.1 ± 4.8	91.9 ± 5.6	85.5 ± 15	0.71 ± 0.05	0.71 ± 0.10	11.5 ± 0.1	11.5 ± 0.2
OB	89.5 ± 4.9	92.4 ± 4.1	110 ± 12	94.6 ± 13	0.80 ± 0.04	0.80 ± 0.03	11.8 ± 0.1	11.5 ± 0.2
Year effect	p = 0.13		p = 0.28		p = 0.84		p = 0.31	
System effect	p = 0.26		**p = 0.015** **C** b**, OA** b**, OB** a		**p = 0.0005** **C** a**, OA** b**, OB** b		p = 0.76	

Table 3 in Supporting Information. Potato harvest yields expressed as ton fresh weight/hectare (ton fw/ha), crop unit size (gram fw/potato), nitrogen content (% in dry matter), and dry matter content (%) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years. The averages ± standard deviations are shown (n = 2). The two lower rows represent the results from the statistics across years and growth systems (location effect), locations and growth systems (year effect) or locations and years (system effect). Growth systems followed by different letters are significantly different ($p < 0.05$). Fl = Flakkebjerg, Fo = Foulum, and Jy = Jyndevad.

Potato	**Harvest yield (ton fw/ha)**		**Potato size (gram fw/potato)**		**N (% in dry matter)**		**Dry matter (%)**	
	Year 1	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**
C (Fl)	45.6 ± 1.0	38.4 ± 11.9	89.5 ± 8.8	81.0 ± 0.1	1.4 ± 0.1	1.4 ± 0	21.0 ± 0.2	21.5 ± 0.1
OA (Fl)	28.7 ± 0.2	32.3 ± 1.0	76.0 ± 3.4	90.7 ± 4.6	1.5 ± 0	1.5 ± 0	19.3 ± 0.2	22.0 ± 0.5
OB (Fl)	20.1 ± 0	21.4 ± 4.4	67.4 ± 4.6	85.0 ± 2.8	1.4 ± 0.1	1.5 ± 0.1	20.3 ± 0	22.1 ± 0.1
C (Fo)	43.5 ± 3.8	61.5 ± 0.6	74.0 ± 13	94.1 ± 11.2	1.8 ± 0	1.5 ± 0	17.7 ± 0.4	19.6 ± 0.1
OA (Fo)	29.3 ± 1.4	46.4 ± 3.7	64.3 ± 1.2	104 ± 3.4	2.0 ± 0.1	1.7 ± 0.2	17.2 ± 0.8	18.5 ± 0.3
OB (Fo)	27.2 ± 2.2	38.9 ± 4.0	65.7 ± 3.9	102 ± 11	1.7 ± 0.1	1.4 ± 0.1	18.5 ± 0.5	20.1 ± 0.1
C (Jy)	43.2 ± 1.3	44.0 ± 3.0	74.1 ± 6.4	73.8 ± 3.1	1.5 ± 0	1.1 ± 0	20.3 ± 0.3	23.1 ± 0.2
OA (Jy)	28.3 ± 5.1	29.0 ± 4.1	77.4 ± 13	79.7 ± 1.5	1.8 ± 0.2	1.5 ± 0.1	18.0 ± 0.8	20.0 ± 0
OB (Jy)	25.3 ± 1.0	23.0 ± 0.5	60.6 ± 2.8	63.5 ± 4.2	1.4 ± 0	1.4 ± 0.2	20.3 ± 0.1	19.7 ± 0.1
Location effect	p = 0.42		p = 0.60		p = 0.24		p = 0.06	
Year effect	p = 0.46		p = 0.28		p = 0.26		p = 0.09	
System effect	**p < 0.0001** **C** a, **OA** b, **OB** c		p = 0.52		p = 0.10		p = 0.20	

Manuscript III

Effects of organic and conventional growth systems on the content of carotenoids in carrot roots, and on intake and plasma status of carotenoids in humans

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Running title: Carotenoids in organic and conventional carrots, and plasma status in humans

ABSTRACT

BACKGROUND: The demand for organically grown food products has increased steadily during the last two decades due to their probable health effects among others. A higher content of secondary metabolites such as carotenoids in organic food products has been claimed, but not documented, to contribute to the increased health effects of organic foods. The aim was to study the impact of organic and conventional agricultural systems on the content of carotenoids in carrot roots and human diets as well as the plasma status of carotenoids in humans consuming diets containing carrots from the organic and conventional agricultural systems.

RESULTS: The main carotenoid found in carrot roots, diets, and plasma was β-carotene together with smaller quantities of α-carotene and lutein. The content of carotenoids in carrot roots and human diets was not significantly affected by the agricultural production system or year, despite differences in fertilization strategy and levels. The plasma status of carotenoids increased significantly after the consumption of the diets based on organic and conventional food products, but no systematic differences between the agricultural production systems were observed.

CONCLUSION: The expected higher content of presumed health-promoting carotenoids in organic food products was not documented in this study.

KEYWORDS

Carotenoids, carrots (*Daucus carota* L.), human intervention study, organic agriculture, plasma status

INTRODUCTION

Organic agriculture promotes biodiversity and natural biological cycles, and the use of external inputs such as inorganic fertilizers and synthetic pesticides is minimized.[1] The abundance of organic farming has increased in most parts of Europe since the 1990s.[2] The reasons for the increasing demand of organic food products are mainly improved environmental protection, animal welfare, human health as well as better taste and freshness of the products.[3] Several consumers perceive that organic food products are more nutritious and healthier than conventional, but the possible health effects of organically grown products have not been scientifically documented with certainty [4,5] and more studies are needed to verify or refute this perception. Human intervention studies are especially needed as a limited number of studies have been carried out until now.[6]

Bioactive secondary metabolites play an important role in the growth, development, and defense mechanisms of plants,[7] but the composition of secondary metabolites in plants may in the same way as nutrients be affected by the differences between organic and conventional agricultural systems, especially the soil fertility management.[8] They are non-essential to humans, but various secondary metabolites have been proposed to play an important role in the prevention of diseases,[9] for instance, carotenoids are believed to contribute to the beneficial effects of increased fruit and vegetable consumption.[10] Epidemiological studies indicate that consumption of foods rich in carotenoids is associated with reduced risk of several diseases, including cancer, cardiovascular diseases, and cataracts.[11] However, human intervention studies with carotenoids are still lacking to support these protective effects [12] and negative health effects of carotenoids have even been shown in heavy smokers.[13]

Carotenoids are naturally occurring fat-soluble pigments [12] and have characteristic colors (mainly yellow to red with orange tones) due to their multiple conjugated double bonds.[11] They act as accessory pigments for light harvesting, attractants for pollinators, and prevent photo-oxidative damage in plants.[14] Carotenoids are a primary source of vitamin A in fruits and vegetables,[15] although only some of the compounds possess provitamin A activity (*e.g.* α- and β-carotene).[12] Humans obtain carotenoids exclusively from the diet,[11] where they are especially abundant in yellow-orange fruits and vegetables as well as in dark green leafy vegetables. The composition and content of carotenoids in plants are affected by *e.g.* the cultivar, geographic location, climate, physiological age, and growing conditions such as fertilizer application and plant nutrient availability.[12]

The average intake of carotenoids in Denmark has been estimated to 4.8 mg day^{-1},[16] whereas the average intake in five other European countries (UK, Spain, France, Ireland, and the Netherlands) was 14 mg day^{-1}.[17] The main contributors to the intake in Denmark are β-carotene, lycopene, α-carotene, and lutein (Figure 1), and the main sources are carrots, tomato, tomato products, and other vegetables.[16] The processing of food might change the structural integrity of the matrix, which can have negative or positive effects on the content of carotenoids in the diet.[14] The content of carotenoids might be reduced due to their instability towards oxidation as well as heat and light,[18] while their bioavailability can be enhanced due to *e.g.* homogenization and heat treatment.[14] The bioavailability of carotenoids in humans is affected by factors such as storage and postharvest procedures, composition of the diets as well as the nutritional and physiological status of the consumer.[12]

The objective of our study was to compare the content of carotenoids in organically and conventionally grown carrot roots and human diets based on organically and conventionally grown food products across different growth years. Furthermore, the effect of growth system and growth year on plasma status of carotenoids in humans consuming diets based on organically and conventionally grown crops was studied. To the best of our knowledge, this is the first human intervention study based on a fully controlled organic or conventional diet, where the effect of agricultural system on plasma status of carotenoids is investigated.

EXPERIMENTAL

Crop study

Study design. The crops were from a two-year field trial study undertaken in year 1 (2007) and year 2 (2008). The carrots (*Daucus carota* cv. *bolero*), onions (*Allium cepa* cv. *hytech*), oat (*Avena sativa* cv. *freddy*), and white cabbage (*Brassica oleracea* cv. *impala*) were grown in the VegQure crop rotation experiment (http://www.vegqure.elr.dk/uk/) located at the Aarslev field trial station, Denmark (10^{o}27'E, 55^{o}18'N). The barley (a mix of the varieties: *Hordeum vulgare* cv. *simba*, *smilla* and *power*), faba beans (*Vicia faba* cv. *columbo*), potatoes (*Solanum tuberosum* cv. *sava*), rape seeds (*Brassica napus* cv. *elan*), and wheat (*Triticum* cv. *tommi*) were grown in the long-term CropSys crop rotation experiment [19] at the Foulum field station, Denmark (09^{o}34'E, 56^{o}30'N) (http://www.cropsys.elr.dk/uk/). Soil analyses (pH, phosphorous, potassium, and magnesium; data not shown) were performed and can be seen together with the soil profiles in Soltoft *et al.* [20]

The crops were grown in three different agricultural systems: one conventional (C) and two organic growth systems (OA and OB). In the conventional system, pesticides and inorganic fertilizers were used (carrots: 120-20-60 kg N-P-K ha^{-1}, total: 1310-280-900 kg N-P-K ha^{-1}), while the OA system relied on import of animal manure and potassium was supplemented as vinasse (carrots: 55−5−20 kg N−P−K ha^{-1}, total: 806−115−675 kg N−P−K ha^{-1}). In the OB system, nutrient supply was based on the use of cover crops (mainly legumes), but animal manure was also applied to the onions and white cabbage, and vinasse, which also contained trace amounts of nitrogen, was applied to barley, faba beans, potatoes, and wheat in order to satisfy the high nutrient demands of these crops (total: 209−15−440 kg N−P−K ha^{-1}) (Table 1 in Supplementary Material). Cover crops were grown in the autumn after the main crops and incorporated into the soil in the spring before the main crops were grown. The field setup is described in Soltoft *et al.* [20] and each individual crop was harvested at the same day for all growth systems. The organic growth systems were managed in compliance with the Danish guidelines for organic farming administered by the Danish Plant Directorate (http://pdir.fvm.dk).

Samples for chemical analysis of crops. A representative sample was collected from each plot and crop, but prior to that the potatoes, carrots, and onions were sorted with the following inclusion criteria: potatoes: 35−60 mm, carrots: 50−250 g, and onions: 40−80 mm. Representative sampling was ensured by stepwise mass reduction as described in Petersen *et al.* [21] and double sampling of random plots was performed to test the sampling representativeness, yielding an average relative standard deviation (RSD) of 4.3 % for α-carotene, β-carotene, and lutein in carrots (n = 6). Only the eatable parts of the crops were analysed, *i.e.* the shoot and root tip of the carrots were discarded and they were peeled. The sample preparation also involved washing and freeze-drying followed by homogenization and storage at −20 ^{o}C in an inert nitrogen atmosphere.[20] In general, the samples were protected from light and oxygen during the entire sample preparation by wrapping in aluminium foil and storing in a nitrogen atmosphere.

Human intervention study

Study design. A double-blinded, cross-over, human intervention trial was performed at The Department of Human Nutrition, University of Copenhagen (Denmark) for two conseecutive years (year 1 and 2) from January to April with diets prepared from the crop study described above. The interventions were performed as 3×12 days dietary periods with a wash-out period of minimum 2

weeks. The subjects (n = 18) were assigned by drawing lots to the pre-defined order in which the diets were distributed in a balanced way. Energy requirement for each individual subject was calculated from the reported physical activity, weight, and age prior to the beginning of the study.[22]

The subjects were weighed on day 1 and 4 of the first period and energy intake was adjusted if necessary to maintain constant weight. The subjects were asked to consume fully controlled diets during each intervention period, and no foods or drinks other than those provided were allowed. The subjects consumed lunch at the department under supervision, while the other meals were distributed daily for consumption at home, except weekend meals, which were given out Friday afternoons. The subjects were instructed to consume all foods served as well as report and also bring back any left overs to the department. The study protocol was approved by the Danish National Committee on Biomedical Research Ethics of the Capital Region of Denmark (J. no. H-C-2007-0078).

Subjects. Adult men were recruited from the area of Copenhagen (Denmark) each year in the autumn before the start of the human intervention study. All subjects were healthy, omnivorous, non-smokers, did not exercise more than 10 h per week or consume more than 21 units of alcohol per week. Neither of the subjects took any medication or vitamin, mineral or other supplements, and did not donate blood for at least 2 months prior to the study or during the study. The weight of the subjects was measured in underwear with an electronic digital scale (Lindertronic 8000, Sweden) to the nearest 100 g on day -1, 1, 4, and 12 during the first dietary period and on day 1 and 12 during the last two periods. Height was measured to the nearest centimetre with a wall-mounted stadiometer at the beginning of the study.

Intervention diets. One conventional (C) and two organic (OA and OB) intervention diets were tested in the human trial with two replicates of each diet resulting in six diets in total. The crops and growth systems were the same as described above in the crop study, but only two of the three replicates of the crops from the Aarslev location were used in order to match the two replicates of the crops at the Foulum location. The diets included two different menus consumed during each intervention period on day 1, 3, 5, 8, 10, and 12 (menu 1) and on day 2, 4, 6, 7, 9, and 11 (menu 2) (Table 1). The menus and the food quantities used in all six diets were identical. The content of carbohydrates, protein, and fat in the diets was 52 %, 15 %, and 33 % of total energy intake, respectively, as calculated using the Dankost 2000 dietary assessment software (Danish Catering

Center, Herlev, Denmark). The individual portions of the meals were weighed according to the estimated energy requirement prior to the study.

The average daily content of the investigated crops in the diets is presented in Table 2. All other ingredients of the diets were kept at a minimum, but the following food products (the stated amounts are for a daily 10 MJ energy intake) were purchased from local distributors besides the food products already mentioned in Table 1 (skimmed milk, butter, and meat balls): low fat (0.5 %) organic milk for cooking (107 mL; ARLA, Denmark), minced pork meat (40 g; Danish Crown, Randers, Denmark), organic eggs (23 g; Danæg, Christiansfeld, Denmark), sugar (41 g; Dansukker, Copenhagen, Denmark), lemon juice (8 g; Valsølille, Hvidovre, Denmark), baking yeast (5 g; Malteserops, De Danske Gærfabrikker, Grenå, Denmark), salt (4 g; fine salt added iodine, Brøste, Lyngby, Denmark), baking powder (1 g; Tørsleff, Hvidovre, Denmark), gelatine powder (0.4 g; Santa Maria, Brøndby, Denmark), and pepper (0.12 g; Easycuisine a/s, Denmark). In year 2, the harvested amount of wheat was lower than required and it was necessary to purchase supplementary portions of wheat from an organic producer (33.5 g $10MJ^{-1}$ d^{-1}; Aurion, Hjørring, Denmark). Subjects had free access to mineral water (Aqua D'Or; Aqua d'or mineral water, Brande, Denmark), table salt (Oscar, Rønne, Denmark), and instant coffee (Nestle Denmark, Copenhagen, Denmark), which was made by addition of mineral water. All meals were prepared shortly after harvest in the metabolic kitchen and stored at −20 °C until the day of consumption, except coleslaw, barley, and bean salads as well as raw carrots, which were stored at 2 °C until serving.

A 10 MJ portion of each of the six diets was collected, homogenized, freeze-dried (Christ Freeze Dryers, Beta 1-8, Montreal Biotech Inc., Dorval, Canada), and stored in an inert nitrogen atmosphere at −80 ˚C until chemical analysis.

Blood sampling. Baseline blood samples were collected in the morning (07.30─09.00 hours) on day 1 and end-time blood samples were taken in the morning after the last meal (day 13) of each dietary period. Fasting blood samples were drawn from a forearm vein puncture into EDTA tubes, centrifuged at 2200 *g* and 4 ˚C for 15 min, and stored at −80 ˚C until analyses. The subjects were instructed to be fasting (>12 hours), not to drink alcohol or heavily exercise for 48 hours before the blood sampling.

Chemical analysis of carotenoids

Reagents and chemicals. Methanol (MeOH, HPLC-grade; Rathburn Chemicals Ltd., Walkerburn, Scotland), methyl *tertiary*-butyl ether (MTBE, HPLC-grade; Merck, Darmstadt, Germany), and Milli-Q water (18 MΩ; Millipore, Billerica, MA, US) were used for eluents. Magnesium carbonate ($MgCO_3$, 99 %) and butylated hydroxytoluene (BHT, 2,6-bis(1,1-dimethylethyl)-4-methylphenol, >95 %) were purchased from Sigma-Aldrich (Steinheim, Germany), ethanol (EtOH, 96 %) from Kemethyl A/S (Køge, Denmark), and petroleum ether (PE, p.a. grade), chloroform (p.a. grade), sodium sulphate (Na_2SO_4), and tetrahydrofuran (THF, p.a. grade) from Merck (Darmstadt, Germany).

The α-carotene standard was purchased from CaroteNature (Lupsingen, Schwitzerland). A standard stock solution was prepared by dissolving the solid compound in chloroform with 0.1 % BHT (50 μg mL^{-1}). Working solutions were prepared by evaporating aliquots of the stock solution to dryness under nitrogen. The residue was dissolved in THF with 0.1 % BHT (v/v) and added mobile phase A (MeOH with 4 % water (v/v):MTBE, 80:20, v/v) containing 0.1 % BHT (v/v) (1:4).

Extraction of food samples. An aliquot of the freeze-dried material (crop samples and intervention diets) was suspended in water with 2 % BHT (v/v). The suspension was extracted with a mixture of THF and EtOH (1:1, v/v) on an ultrasonication bath at room temperature for 15 min (120 watt; 5200 Ultrasonic Cleaner, Branson, Soest, the Netherlands). Afterwards, Na_2SO_4 and $MgCO_3$ were added, and the sample was centrifuged at 3000 *g* and 20 ^{o}C for 5 min. The supernatant was collected and the residue was reextracted twice. The three supernatants were combined and filled to a fixed volume with extraction solvent. An aliquot of the sample was evaporated *in vacuo* at 30 ^{o}C on a rotary evaporator. The residue was dissolved in THF with 0.1 % BHT (v/v) and added mobile phase A containing 0.1 % BHT (v/v) (1:4). The samples were analysed on HPLC-UV at the day of extraction.

Extraction of plasma samples. The plasma samples were extracted following the principle used by Toft *et al*.,[23] but additional details are given here. 400 μL of plasma was mixed with an equal volume of EtOH containing 0.1 % BHT (v/v). The mixture was extracted with 800 μL PE and shaken for 10 min. The sample was centrifuged at 2000 *g* and 4 ^{o}C for 10 min and the PE phase collected. Afterwards, the extraction with PE was repeated twice. The combined PE phases were evaporated to dryness under a steam of nitrogen at room temperature. The residue was dissolved in

THF with 0.1 % BHT (v/v) and mobile phase A containing 0.1 % BHT (v/v) (1:4). The samples were analysed on HPLC-UV at the day of extraction.

Quantification of carotenoids by HPLC-UV. The HPLC system (Waters, Milford, MA, US) was equipped with a gradient pump system (Waters 600) and an autosampler (4 ^{o}C; Waters 717). Empower 2 (Waters) was used for instrument control and data acquisition. The chromatographic separation of carotenoids was achieved using a C_{30} carotenoid column (5 µm, 4.6×250 mm; YMC, Wilmington, NC, US) maintained at 25 ^{o}C. Mobile phase A and B was MeOH with 4 % water (v/v) and MTBE mixed in the ratios 80:20 and 30:70 (v/v), respectively. The gradient elution was as follows (1 mL min^{-1}): a 25 min linear gradient from 0 % B to 40 % B, a 10 min linear gradient from 40 % B to 100 % B, a 10 min hold at 100 % B, and a 2 min gradient back to 0 % B. The total run time was 55 min. A photo diode array detector (Waters 996) was applied for identification of the carotenoids (200─600 nm) and an UV detector (Waters 2487) was used for quantification of α-carotene, β-carotene, β-cryptoxanthin, lutein, and zeaxanthin at 450 nm, and lycopene at 470 nm. The concentrations of the individual carotenoids were calculated using an external standard curve of α-carotene and the specific molar extinction coefficients.[24]

Quality assurance was performed by including in-house samples as reference samples. The average RSD was 1.8 % (n = 6) and 6.2 % (n = 9) for α-carotene, β-carotene, and lutein, in food samples and plasma, respectively, which is acceptable compared with the RSD of α-carotene and β-carotene normally seen in food samples (11 %). Duplicate measurements (incl. weighing and extraction) of randomly selected samples in each series of analyses were performed and yielded an average RSD of 9.5 % (n = 12) and 3.3 % (n = 14) for α-carotene, β-carotene, and lutein in food samples and plasma, respectively.

Statistical analysis

The carotenoid contents of the carrots (responses, y_{ysb}) were modelled as: $y_{ysb} = \mu + \alpha_b + \beta_y + \delta_s + \varepsilon_{ys} + \varepsilon_{yb} + \varepsilon_{ysb}$, where μ is the generalized intercept, α_b, $b = 1, 2, 3$ is the effect of the blocks, β_y, $y =$ *year 1, year 2* is the effect of year, and δ_s, $s = C, OA, OB$ is the effect of growth system. Errors (ε) are considered independently and normally distributed and represent corresponding variance components of interaction. The pair wise comparisons and their confidence intervals between the systems were adjusted to obtain a family wise error rate of 5%. The model was fitted using the proc

mixed procedure in the SAS/STAT software packages (version 9.2, SAS Institute Inc., Cary, NC, USA).

The individual intervention diets were tested for a significant difference and the replicates of each growth system combined if no differences were identified. The effect of growth system in each year was analyzed by one-way analysis of variance (ANOVA).

A paired t-test was applied to test for a significant difference between plasma status at baseline and the end of the dietary periods. A linear mixed model was used to test the effect of the intervention diets on plasma status with the following variables: diet, order of diets, period, group, subject, and baseline concentration. Stepwise backwards model reduction was applied to remove non-significant variables except the diet, which was kept in the model regardless of its significance. Restricted Estimation of Maximum Likelihood and its related F tests were used to test for significance of the intervention diets and post-hoc t-tests were applied to determine, which diets differed. Variance homogeneity and normal distribution of the plasma carotenoid concentrations were checked by construction of residual and normal probability plots. The statistical analyses of intervention diets and plasma samples were performed in the Base SAS software (version 9.1.3, SAS Institute, Cary, US) with a 5 % significance level.

RESULTS AND DISCUSSION

Carotenoids in carrot roots

The main carotenoids found in carrot roots were β-carotene and α-carotene, respectively. Lutein was also detected in small quantities (Figure 2), but neither zeaxanthin, β-cryptoxanthin, nor lycopene were quantified in the carrot roots. The relatively percentages of β-carotene, α-carotene, and lutein were 75 %, 23 %, and 1.9 %, and this distribution is in agreement with previous studies of carrots.[14,25] The average concentrations (± standard deviation (sd), n = 9) of β-carotene, α-carotene, and lutein in the carrot roots in the three growth systems were 90 ± 5.5, 27 ± 3.2, and 2.6 ± 0.42 mg kg^{-1} fresh weight in year 1 and the corresponding average concentrations were 3.3 %, 7.4 %, and 19 % higher in year 2 (Figure 2). The concentrations are comparable to previous levels of carotenoids reported.[12,26,27]

The concentrations of carotenoids in the carrots were not significantly different between the three growth systems ($p > 0.05$), despite differences in fertilization strategy and level, but the results were

similar to previous fertilization studies of carrots [28] and tomatoes.[29,30] In contrast, a positive correlation between the level of nitrogen fertilization and the concentration of carotenoids in carrots has been observed in other studies of carrots.[31,32] Our results were in agreement with the harvest yield and dry matter content in the carrot roots because no significant differences were observed,[33] even though the plant available nitrogen in the carrots at harvest was higher in the conventional than in the organic growth systems.[33]

In contrast to our study, Leclerc *et al.* [34] found a higher content of carotenoids in organically compared to conventionally grown carrots, but the crops were grown at two different farms, which could have induced a variation not related to growth system, even though the farms had relatively close geographical locations. However, another cultivation study of organic and conventional carrots by Warman *et al.* [35] supported our findings, although a significant difference was observed in one out of the three growth years. No significant year-to-year variation ($p > 0.05$) was observed in the content of carotenoids in the organically and conventionally grown carrots in the present study in contrast to previous studies.[28.36]

Carotenoids in intervention diets

The dry matter contents of the intervention diets were 38 ± 0.3 % and 40 ± 0.6 % in year 1 and 2, respectively. No significant difference between the replicates or the three growth systems was observed ($p > 0.05$), but a significant year-to-year variation was observed ($p = 0.001$), even though no significant differences in dry matter content between the carrot roots was observed.[33]

The main carotenoid found in the intervention diets was β-carotene, but also smaller quantities of α-carotene and lutein were detected (Figure 3). Neither zeaxanthin, β-cryptoxanthin, nor lycopene were quantified in the intervention diets similarly to the carrots. The average concentrations (± sd, n = 6) of β-carotene, α-carotene, and lutein in menu 1 and menu 2 in all six diets were 15 ± 1.8, 4.5 ± 0.71, and 1.8 ± 0.26 mg $10MJ^{-1}$, respectively, in year 1, while the average levels in year 2 were 2.4 % and 4.0 % higher for α-carotene and β-carotene, respectively, and 9.8 % lower for lutein (Figure 3). The concentrations of carotenoids were not significantly different between the replicates or the three growth systems ($p > 0.05$), and no significant differences between the two growth years were observed ($p > 0.05$) in agreement with the insignificant effects of growth system on the content of carotenoids in carrot roots.

The concentration of β-carotene determined by chemical analysis was comparable to the concentration obtained by calculations in the Dankost 2000 dietary assessment software (17 mg 10MJ $^{-1}$) and considerably higher than the estimated average daily intake of β-carotene for Danish men (3.7 mg 10MJ $^{-1}$).[37] The total intake of carotenoids (9.5 mg day^{-1} in year 1 and 5 % higher in year 2; sum of α-carotene, β-carotene, and lutein) was also much higher than the estimated total intake of carotenoids in Denmark (4.8 mg day^{-1}),[16] so the intervention diets were rich in carotenoids compared to a habitual Danish diet.

Carrots were considered the main dietary source of carotenoids. Butter was also a dietary source of β-carotene (5.0 mg kg^{-1}), but the concentration and consumption was of minor importance in comparison with the carrots. The average concentration levels of α-carotene and β-carotene were slightly lower (5 %) than the expected concentration calculated from the intake and content in the carrots (Figure 2). Hence, the storage and processing procedures of the intervention diets only had a very small impact on the content of α-carotene and β-carotene, despite both increased and decreased concentrations of carotenoids after food processing such as steaming, boiling, and frying have been observed.[12,14,38]

The concentration of lutein found in the intervention diets was on average 260 % higher than the expected concentration assuming that carrots were the main source of carotenoids. The relative percentage of lutein (8.1 %) in the intervention diets was also higher in comparison with the carrots. Chemical analysis of faba beans and rape seeds showed that these crops contained small quantities of lutein. Thus, the sources of lutein in the intervention diets were carrots as well as faba beans and rape seeds, but the other crops did not contribute considerably to the content of carotenoids in the intervention diets. Hence, the carrots could be considered the main dietary source of carotenoids, apart from lutein.

Plasma status of carotenoids

The baseline characteristics of the subjects are shown in Table 3, and no significant changes in the body weight were observed during the study. The main carotenoid found in plasma at baseline was β-carotene (0.31 ± 0.19 and 0.28 ± 0.15 μg mL^{-1} in year 1 and 2, respectively), but also smaller quantities of α-carotene (0.14 ± 0.08 and 0.11 ± 0.05 μg mL^{-1} in year 1 and 2, respectively) and lutein (0.13 ± 0.06 and 0.11 ± 0.05 μg mL^{-1} in year 1 and 2, respectively) were detected. Furthermore, zeaxanthin, β-cryptoxanthine, and lycopene were detected in the plasma samples at

baseline, but the focus here will be on lutein, α-carotene, and β-carotene due to their abundance in the carrot roots and intervention diets. The carotenoids found in plasma at baseline were among the predominant carotenoids normally found in human plasma.[39] The baseline levels of lutein were in agreement with levels found in a similar group of subjects.[40] In contrast, the baseline concentrations of α-carotene were higher than in comparable studies, while both higher and lower baseline levels of β-carotene have previously been found.[40,41]

The distribution of the three carotenoids in plasma at baseline and after the intervention was similar with β-carotene as the most abundant compound (Table 4). The plasma status increased significantly after the intervention ($p < 0.001$) probably due to increased intake of food products rich in carotenoids. This is in agreement with previous intervention studies with supplementation of food products rich in carotenoids.[41,42,43] The plasma levels after the intervention were higher than levels found in a similar 2-week study, where the subjects were supplemented with 200 g carrots day^{-1},[41] but higher baseline levels were also detected in the current study. The large differences in plasma status observed between the subjects (Table 4) could be due to various factors affecting the bioavailability of carotenoids, for instance, physiological factors and nutritional status of the subjects [12] as well as the ability of subjects to absorb carotenoids.[44]

In year 2, the plasma status of α-carotene and β-carotene at end-time was significantly higher in period 3 compared to period 1 and 2 ($p = 0.002$ and 0.003, respectively), but otherwise no significant differences in the plasma status at end-time between the periods were observed after correction of concentration differences at baseline ($p > 0.05$). The period effect was probably related to the differences in time of the intervention periods and corrections for the period effect were included in the statistical model. Carry-over effects are possible in cross-over studies in contrast to parallel studies since the effect of the diet given in the previous period might persist into the following period and alter the effect of the second diet. However, no significant carry-over effects were observed in the present study ($p > 0.05$).

No significant differences in plasma status were observed between the replicates of the intervention diets from each growth system after correction for differences in baseline levels and possible period effects ($p > 0.05$), except a difference in the plasma concentration of β-carotene after consumption of the conventional diet, despite the replicates being located geographically close to each other ($p < 0.03$, Table 4). The plasma status of lutein varied significantly between the growth systems in both growth years ($p < 0.002$). The plasma status of β-carotene from the conventional growth system was

significantly different from the organic growth systems in year 1 ($p < 0.007$), but the same trend was not seen in year 2 (Table 4). Thus, systematic differences in the plasma status of carotenoids after the intervention with diets based on one conventional and two organic growth systems were generally not observed, except for lutein, in agreement with similar intervention studies with supplementation of carrots [41] and tomato puree.[45]

A comparable group of subjects were included in the intervention study recently published by Stracke *et al*.,[41] which was designed as a parallel study *i.e.* the subjects were randomized to a diet with carrots from an organic or a conventional growth system. The variation between subjects was eliminated in the present cross-over study, where the subjects served as their own controls, and the variation within subjects was expected to be lower than the variation between subjects. In our study, the subjects were given full intervention diets for 2 weeks in comparison with carrot supplementation to the habitual diet [41] and two organic growth systems with high and low fertilization inputs were compared. Nevertheless, no systematic differences in plasma status after the intervention were observed in our study in compliance with the content of carotenoids in the carrots and intervention diets.

The current study indicated that the plasma status of carotenoids is not solely influenced by the dietary intake levels as no significant correlation between dietary intake and plasma status of carotenoids was observed in contrast to previous studies.[46,47] An increased plasma status after intervention with diets rich in carotenoids has previously been seen, but no major impact on human health parameters, *e.g.* immune parameters and markers of oxidative stress, were found in those studies.[41,42] Similar health parameters were not investigated in the present study, but the few observed significant differences in plasma status after the intervention are not expected to be of considerable significance with regard to human health due to the small concentration differences observed, which are unlikely to have health implications for the consumer.[6]

CONCLUSION

The most abundant carotenoid in carrots, intervention diets, and human plasma was β-carotene, but smaller quantities of α-carotene and lutein were also found. The growth system (conventional versus organic) did not have a significant impact on the content of carotenoids in carrots and intervention diets, and no significant year-to-year differences were observed ($p > 0.05$). Carrots were the main dietary source of carotenoids in the intervention, which caused a significant increase

in the plasma status of carotenoids ($p < 0.05$). However, few significant differences in plasma status between the conventionally and organically based diets were observed, but they were not systematic and doubtfully of significance for human health. Thus, the two organic agricultural practices did not have a major impact on the content of the potentially health-promoting carotenoids in carrots and intervention diets, as well as no systematic effect on the absorption of carotenoids in humans.

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Table 1. Composition of the intervention diets (g day^{-1}) for 10MJ energy intake.

Meal	Component	Menu 1	Menu 2
Breakfast	Skimmed milk	385	385
	Carrot roll	160	160
	Carrot jam	52	52
	Butter	8	8
Lunch	Coleslaw salad	- [a]	145
	Barley and bean salad	100	- [a]
	Full grain bread	120	120
	Raw carrot	34	- [a]
	Meat balls	70	- [a]
	Hummus	- [a]	118
Snack	Carrot cake	93	- [a]
	Potato cake	- [a]	77
	Oat cookies	60	60
Dinner	Baked potatoes with carrot	286	- [a]
	Minced meat and vegetables	194	- [a]
	Fricassee with faba beans	- [a]	451
	Mashed potatoes	- [a]	275

[a]: not included in the menu

Table 2. Content of crops in the intervention diets (g day^{-1}) for 10MJ energy intake.

Crop	Menu 1	Menu 2	Average
Barley	71	26	49
Carrot	162	184	173
Faba bean	30	102	66
Oat	45	45	45
Onion	80	42	61
Potato	195	264	230
Rapeseed oil	44	65	54
Wheat	92	83	88
White cabbage	28	100	64

Table 3. Characteristics of subjects at baseline (average ± standard deviation) in the two-year trial. The ranges are shown in brackets.

	Year 1	Year 2
Number of subjects	18	18
Age (years)	25.1 ± 6.7 (18─40)	26.0 ± 7.1 (19─37)
Energy level (MJ day^{-1})	13.5 ± 1.0 (12─15)	13.8 ± 1.1 (12─15)
Weight (kg)	73.7 ± 8.9 (57.0─92.3)	80.8 ± 8.6 (67.6─103)
Height (m)	1.84 ± 0.07 (1.70─1.94)	1.83 ± 0.07 (1.76─1.93)
Body Mass Index (kg m^{-2})	21.8 ± 1.6 (19.8 ─25.0)	24.0 ± 2.3 (19.9─30.1)

Table 4. Plasma status of carotenoids (average ± standard deviation, n = 18; μg mL^{-1}) at end-time after consumption of diets produced from cultivated crops from three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (1 and 2). Plasma statuses with different letters within a row are significantly different ($p < 0.05$) after correction of possible variation in baseline concentrations and between periods.

		Growth system		
Year	**Carotenoid**	**C**	**OA**	**OB**
1	Lutein	0.23 ± 0.09 ^{A}	0.19 ± 0.09 ^{B}	0.19 ± 0.07 ^{B}
1	α-carotene	0.26 ± 0.09	0.23 ± 0.09	0.26 ± 0.08
1	β-carotene ^{a}	0.69 ± 0.29 ^{A} 0.40 ± 0.09 ^{C}	0.49 ± 0.22 ^{B}	0.55 ± 0.20 ^{B}
2	Lutein	0.18 ± 0.05 ^{A}	0.16 ± 0.05 ^{C}	0.17 ± 0.05 ^{B}
2	α-carotene	0.26 ± 0.09	0.26 ± 0.07	0.26 ± 0.09
2	β-carotene	0.62 ± 0.38	0.65 ± 0.38	0.63 ± 0.40

^{a} : the plasma status of β-carotene in growth system C was significantly different between the two replicates ($p = 0.01$). Thus, the average (± standard deviation, n = 9) of each replicate is shown.

Table 1 in Supplementary Material. Fertilization levels (N, P, and K; kg ha^{-1}) applied to the individual crops in the three growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops). The amounts are an average of the two growth years (1 and 2).

		Growth system		
	Crop	**C** [a]	**OA** [b]	**OB** [c]
N	Barley	130	55	1
	Carrot	120	55	0
	Faba bean	0	1	1
	Oat	90	45	0
	Onion	170	110	70
	Potato	140	115	1
	Rape seed	185	95	0
	Wheat	165	105	1
	White cabbage	310	225	135
	Total	**1310**	**806**	**209**
P	Barley	20	10	0
	Carrot	20	5	0
	Faba bean	25	0	0
	Oat	15	5	0
	Onion	80	10	5
	Potato	30	25	0
	Rape seed	25	25	0
	Wheat	20	20	0
	White cabbage	45	15	10
	Total	**280**	**115**	**15**
K	Barley	95	65	45
	Carrot	60	20	0
	Faba bean	125	70	70
	Oat	45	15	0
	Onion	65	40	25
	Potato	175	145	90
	Rape seed	85	115	90
	Wheat	105	120	70
	White cabbage	145	85	50
	Total	**900**	**675**	**440**

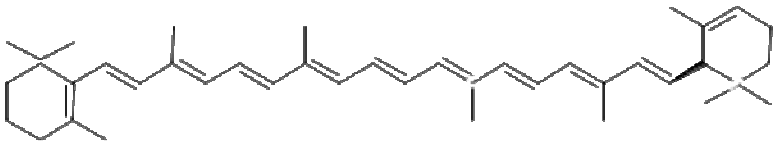
[a] : applied as inorganic fertilizer.

[b]: applied as animal manure. K was supplemented as vinasse.

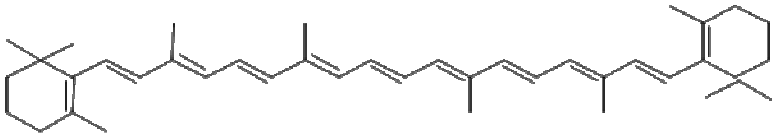
[c] : applied as vinasse, which also contained trace amounts of N. Animal manure was used for onions and cabbage.

Figure 1. Chemical structures of major dietary carotenoids.

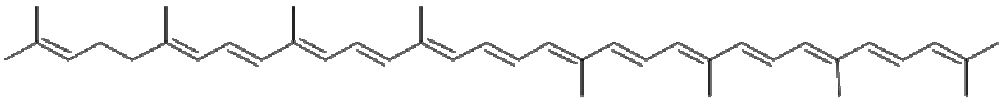
α-carotene:



β-carotene:



Lycopene:



Lutein:

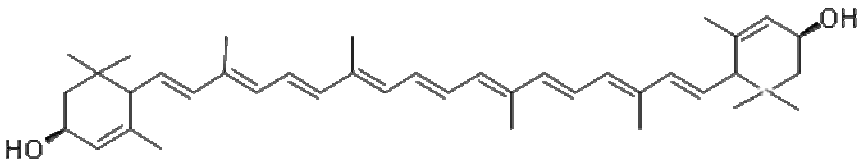


Figure 2. The concentrations of carotenoids in carrot roots (mg kg^{-1} fresh weight) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (1 and 2). The error bars describe the standard deviation of replicates from the field (n = 3). ■: lutein, ▩: α-carotene, and □: β-carotene.

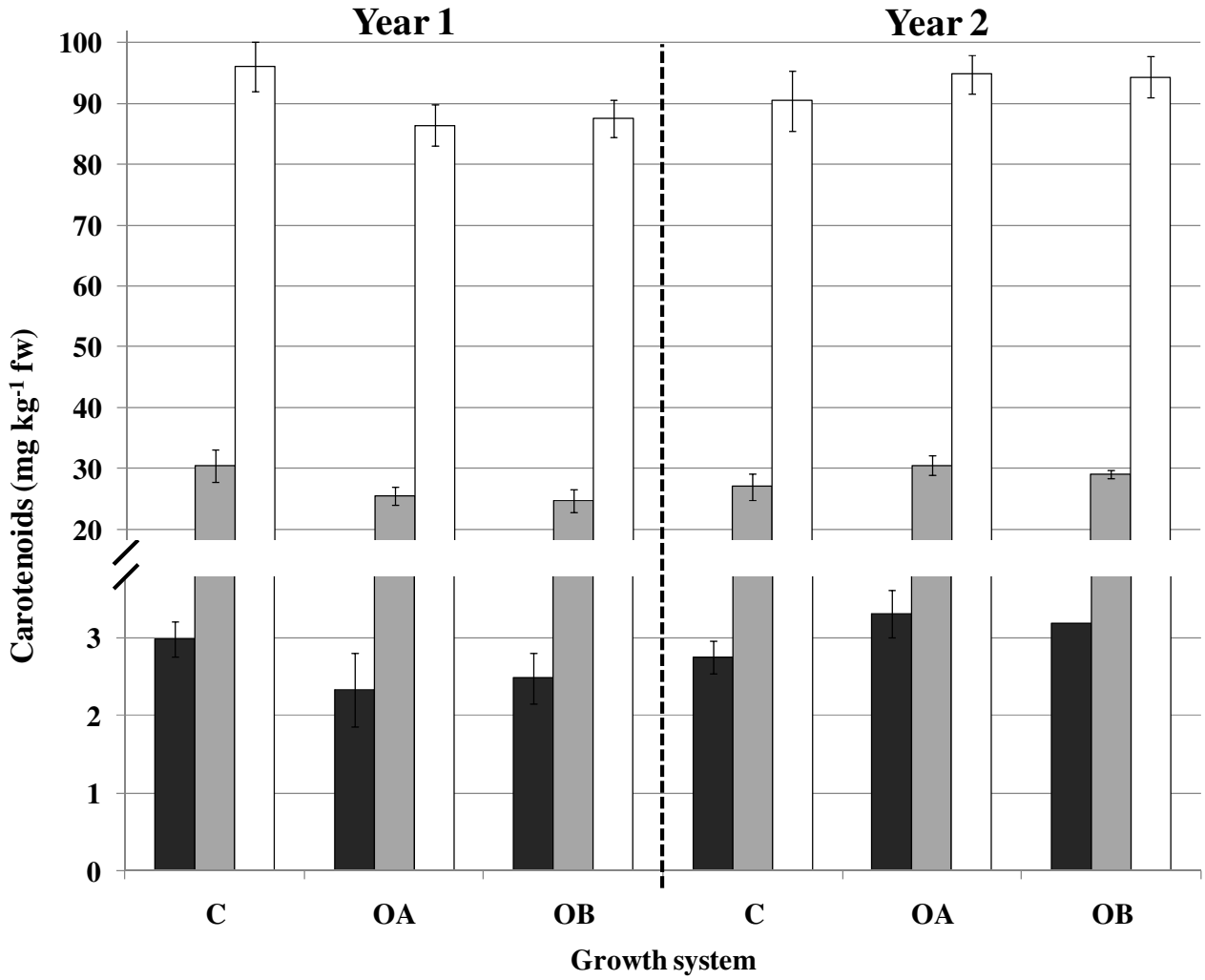


Figure 3. The concentrations of carotenoids in intervention diets (mg $10MJ^{-1}$, average of menu 1 and 2) in three different growth systems (C: conventional, OA: organic using animal manure, and OB: organic using cover crops) and in two growth years (1 and 2). The error bars describe the standard deviation of replicates (n = 2). ■: lutein, ■: α-carotene, and □: β-carotene.