# Antioxidants in fruits and vegetables

## Antioxidanter fra planter

## Contents

# Dansk forord

Nærværende rapport markerer afslutningen på et tre-årigt rammeprogram, der løb fra juni 1995 til maj 1998 under det fødevareteknologiske forskningsprogram, FØTEK2, med titlen, "Sundhedsmæssig vurdering af biologisk aktive non-nutritive indholdsstoffer i fødevarer: Antioxidanter fra planter" med acronymet, PLANTOX. Selvom rammeprogrammet ikke er stort i sammenligning med så mange andre danske forskningsprogrammer er det det hidtil største projekt indenfor dette område herhjemme. Rammeprogrammet har haft deltagelse fra både sektorforskningsinstitutter og universiteter, og har haft en programkomite bestående af repræsentanter fra såvel disse forskningssektorer som fødevareindustrien. Projektets organisation og deltagere fremgår af appendix 1 og 2.

Rapporten er udfærdiget på engelsk af hensyn til muligheden af en international evaluering af projektet og dets forløb, men med et udvidet dansk resume af hensyn til danske interessenter, der ikke er inde i det videnskabelige engelske fagsprog. Rapportens disposition følger den, der var udstukket af forskningsrådene (FELFO) i forbindelse med midtvejsevalueringen af dette projekt med undtagelse af, at dansk og engelsk forord og dansk resume er tilføjet og en samlet faglig rapportering er tilføjet som afsnit B2.b. Rapporten er således primært udarbejdet og disponeret med henblik på en efterfølgende faglig vurdering af rammeprogrammet.

Rapporten er designet til at give et samlet overblik over projektets organisation, økonomi og faglige udbytte med vægten på det målbare. Da emner som naturlige antioxidanter og sundhedsfremmende fødevarer imidlertid er meget oppe i medierne og da der også er stor interesse for området indenfor fødevareindustrien og blandt studerende på levnedsmiddeluddannelserne ved KVL og DTU har vi valgt at trykke rapporten i nogle hundrede eksemplarer samt at lægge den ud på internet under Veterinær- og Fødevaredirektoratets hjemmeside, http:/www.vfd.dk. Dermed er resultaterne gjort tilgængelige for den bredest mulige gruppe af interessenter både i Danmark og i udlandet. Teksten kan anvendes frit med kildeangivelse.

Vi håber med denne rapport at styrke interessen for forskning i fødevarers naturlige indholdsstoffer og deres tekniske og sundhedsmæssige egenskaber. Især håber vi at bidrage til den igangværende debat om vurderingen af sundhedsfremmende kostfaktorer, både i functional foods og som kvalitetsparameter for almindelige fødevarer.

Søborg 22/12 -98

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# Dansk sammendrag og konklusion

Baggrund: Sundhedsfremmende komponenter i frugt og grønt
Et af de primære mål med rammeprogrammet var at medvirke til at skabe grundlag for evaluering af fødevarer, der har sundhedsfremmende egenskaber som følge af et indhold af komponenter, der ikke i sig selv er vitaminer (non-nutritive faktorer). Sådan et grundlag er en forudsætning for at der kan udvikles fødevarer, hvor den sundhedsmæssige kvalitet er styrket, fx. gennem bedre stabilisering af deres indhold af sundhedsfremmende naturlige antioxidanter.

Figur 1.: Eksempler på forekomsten af muligt sundhedsfremmende stoffer i fødevarer

Det er kendt, at frugt og grønt i kosten modvirker folkesygdomme som kræft, hjertesygdom m.v., det fremgår fx. af Veterinær- og Fødevaredirektoratets anbefalinger om indtag af frugt og grønt fra 1998. Det er imidlertid ikke kendt med sikkerhed, hvorfor frugt og grønt beskytter. Vi ved, at en række non-nutritive faktorer i frugt, grønt og krydderier kan påvirke vores evne til at forsvare os mod naturlige og menneske-skabte giftstoffer i maden og miljøet {Dragsted, Strube, et al. 1993 ID: 290}. Eksempler på sådanne stoffer er vist i figur 1. Der findes også stoffer, der påvirker vores hormonsystem, som selektivt dræber kræftceller, som forhindrer blodets koagulation i årerne eller som på anden vis kan tænkes at forebygge kræft og hjertesygdom. Det er specielt interessant, hvordan de virker i kroppen og skaber et forbedret forsvar. Flere sådanne virkningsmekanismer er foreslået, se tabel 1 for eksempler.

Tabel 1. Eksempler på virkningsmekanismer for sundhedsfremmende stoffer fra frugt og grønt.
Virkningsmekanisme Forklaring Eksempler
Antioxidation reagerer med og fjerner frie radikaler Plantefenoler*, terpener*, karotenoider* m.fl.
Styrkelse af vort eget antioxidative forsvar øget dannelse af antioxidanter og antioxiderende enzymer Karotenoider*
Styrkelse af vort giftforsvar Induktion af enzymer, der nedbryder giftstoffer og øger deres udskillelse Indoler, isothiocyanater, flavonoider*
Neutralisering af gift eller giftvirkning Evne til at fjerne visse særlig skrappe giftstoffer eller reparere skaderne Ellaginsyre, flavonoider*, thioler
Hæmning af cellevækst Forhindring af for kraftig cellevækst ved at påvirke hormoner eller ved selektiv forgiftning af kræftceller. Fibre (i tarmen)
Simple polyphenoler*
Visse flavonoider *
Binding af giftstoffer Forhindring af, at giftstoffer optages Chlorophyllin
Modvirkning af blodpropper Udvidelse af blodkar eller forhindring af koagulation Flavonoider
'*Resultater, der til dels kommer fra dette rammeprogram.

Selvom alle er enige om, at der findes non-nutritive faktorer som med stor sandsynlighed har sundhedsfremmende egenskaber stiller det sig anderledes når der skal tages stilling til hvert enkelt af disse stoffer. Det er vanskeligt og overordentlig kostbart at påvise videnskabeligt at et bestemt indholdsstof i frugt og grønt er sundhedsfremmende. Samtidig er opdelingen i giftstoffer og "gift-

hæmmende" for naiv. De fleste af de muligt sundhedsfremmende komponenter har også sig selv visse giftvirkninger, og disse kan paradoksalt nok være en forudsætning for deres sundhedsfremmende virkninger. En svag giftvirkning kan fx. fremme vores forsvarsevne, så vi også klarer de mere farlige stoffer. Men med anvendelsen af forsigtighedsprincippet er enhver giftvirkning uacceptabel, når det gælder komponenter i fødevarer. Hvis vi derfor skal vurdere sundhedsfremmende stoffer står vi over for to vanskelige opgaver: At bevise at de gavner i de mængder, vi får fra kosten, og at godtgøre at de er sikkerhedsmæssigt acceptable. Problemstillingen kan illustreres som vist i figur 2.

Figur 2: Mindst tre videnskabelige felter indgår i bedømmelsen af naturlige antioxidanter

De vigtigste antioxidanter

I rammeprogrammet er der forskningsmæssigt focuseret på to grupper af naturlige antioxidanter fra planter, flavonoider og karotenoider. De er kendt for at bidrage til rødlige, gullige og brunlige farver hos bær, frugter og grøntsager samt i afledte produkter, fx. vin, juice, ketchup, te. Antallet af stoffer indenfor disse grupper er imidlertid så stort, at vi har måttet indskrænke os yderligere til tre grupper af vigtige flavonoider (flavoner, flavonoler og flavanoner) samt til de mest almindeligt forekomende karotenoider i kosten. Hermed udgik anthocyaninerne, der er de flavonoid-baserede farvestofferne i bl.a bær og vin, ligesom vi kun har beskæftiget os i mindre grad med catechinerne, der er vigtige polyphenoler i te og vin. Da antallet af mulige fysiologiske og sundhedsmæssige virkninger er meget stort, har vi også her målrettet forskningsindsatsen. Rammeprogrammet har derfor kun omfattet de virkninger, der kunne tænkes at have indflydelse på risikoen for kræft og her især på antioxidative virkninger.

Programmets fire projekter.

1. Opbygningen af en litteraturdatabase samt databaser over forekomst af flavonoider og karotenoider i fødevarer på baggrund af egne og andres analyser.
2. Undersøgelser in vitro, herunder undersøgelser af antioxiderende virkninger, toksiske og mutagene virkninger, samt biotransformation.
3. Undersøgelser in vivo hos forsøgsdyr og mennesker af stoffernes disposition, metabolisme og antioxiderende virkninger i organismen.
4. Langtidsforsøg med stoffernes kræfthæmmende virkninger hos forsøgsdyr.

Projekt 1: Litteraturanalyse og fødevareanalyser.

En gennemgang af den internationale videnskabelige litteratur afslørede en betydelig mangel på viden om indtagelsen med kosten, optagelsen og biotransformationen af flavonoiderne {Strube, Dragsted, et al. 1993 ID: 10880}. Her er betydeligt mere viden om indtaget og optagelsen af karotenoider. For begge grupper af antioxidanter er der modstridende oplysninger i litteraturen om deres egenskaber som antioxidanter såvel i fødevarer som i organismen. Når det kommer til virkninger hos højerestående dyr eller hos mennesker af sundhedsmæssig karakter, som kan have relation til forebyggelse af kræft er der kun forholdsvis få undersøgelser til rådighed i litteraturen for begge stofgruppers vedkommende, bortset fra epidemiologiske undersøgelser over virkningen af karotenoider, hvoraf der er omkring hundrede. Dette skal ses i kontrast til, at vi samlet fandt omkring 15.000 videnskabelige artikler om flavonoider og karotenoider som indledningsvis blev samlet i to store litteraturdatabaser til brug for det videre arbejde. En meget stor del af artiklerne handler om stoffernes kvalitative tilstedeværelse i forskellige dele af planteriget, men der er også en stor del, der drejer sig om stoffernes fysiologiske eller fysisk-kemiske egenskaber.

Tabel 2. Vigtige kilder til flavonoider og karotenoider i kosten

| Indholdsstof | Vigtige fødevarer |
|---|---|
| Flavonoider: | |
| Quercetin | løg, te, vin, æbler |
| Kaempferol | broccoli, blomkål, porre |
| Myricetin | solbær, te |
| Naringenin | appelsiner |
| Catechiner | te, alt grønt |
| Apigenin | persille, bladselleri |
| Karotenoider | |
| alpha- og beta-karoten | gulerødder |
| luteolin | spinat og andet grønt, æg, mælk |
| lycopen | tomater, vandmelon |
| beta-kryptoxanthin | citrusfrugter |
| zeaxanthin | majs |
| astaxanthin | laks, skaldyr |

Et vigtigt element i undersøgelserne var at kortlægge vores indtag med den almindelige danske gennemsnitskost. Der er derfor gennemført omfattende analyser af frugt, grøntsager, drikkevarer og krydderurter for at fastlægge kilderne til de udvalgte flavonoider. For karotenoiderne har vi kun gennemført analyser af fødevarer når der forelå modstridende analyseresultater fra andre laboratorier. Vigtige kilder til flavonoider og karotenoider kan ses af tabel 2. For begge grupper af stoffer er der ikke overraskende, hvad der kan betegnes som en nord-syd gående gradient i indtaget. Indtaget i Danmark ligger ligeså højt eller lidt højere end i andre nordiske lande, men lavere end i mellemeuropæiske lande, fx. Holland, og i Middelhavslande som Italien. F. eks. ligger gennemsnitsindtaget af de tre grupper af flavonoider i Danmark på omkring 28mg daglig, heraf ca. 18mg flavonoler og flavoner. Dette skal ses i sammneligning med et dagligt middelindtag af flavonoler og flavoner på ca. 4mg i Finland, ca. 23mg i Holland ,ca. 26mg i Wales og omkring 30mg eller mere i flere områder i landene omkring middelhavet.

Middelindtaget af karotenoider i Danmark ligger noget lavere, omkring 4,3mg, hvoraf det meste udgøres af beta-caroten, se figur 3. Middelindtaget er sammenligneligt med det i Sverige og Finland, mens lande med højere indtag af tomatprodukter, majsprodukter eller grøntsager generelt har et højere indtag og en ændret fordeling med mindre beta-caroten, mere lutein og mere lycopen (tomat) eller zeaxanthin (majs). Til sammenligning er middelindtaget herhjemme af de naturlige antioxidanter som er kendt som vitaminer omkring 75mg daglig for vitamin C og omkring 8mg daglig for vitamin E. Flavonoiderne er således kvantitativt meget vigtige antioxidanter i kosten og dermed potentielt betydningsfulde både teknologisk og sundhedsmæssigt.

Figur 3. Indtaget af de vigtigste karotenoider i Danmark fra forskellige fødevaregrupper

Projekt 2: In vitro undersøgelser

Leveren er et af de vigtigste organer for omdannelsen af fremmedstoffer i organismen. Omsætningen af flavonoiderne i leveren blev først undersøgt med leverpræparationer fra rotter og derpå

med genmodificerede cellelinier, der indeholdt specifike leverenzymer fra mennesker af typen, monooxygenaser. I disse systemer blev flavonoider som hovedregel oxideret indtil de havde en catechol-struktur (to hydroxylgrupper ved siden af hinanden) i deres B-ring. Catecholer er kendt for at være giftige gennem at katalysere dannelsen af reaktivt ilt, de er pro-oxidanter, altså det modsatte af antioxidanter. Ved forsøg med intakte dyr og med mennesker viste det sig imidlertid at omsætningen tilsyneladende stopper et trin tidligere, så fx. apigenin og kaempferol med én hydroxylgruppe i B-ringen ikke oxideres yderligere i leveren. Samtidig sker der en meget hurtig methylering af flavonoider med catecholstruktur, en omdannelse der afgifter dem, se figur 3. Risikoen for en pro-oxidant virkning i organismen er dermed relativt lav, undtagen ved ekstreme doser.

Figur 4. Omsætningsveje for flavonoider. Hurtige omsætningsveje er vist med en kraftig pil. Omsætningsveje med enkelt overstregning forløber kun in vitro, mens dobbelt overstregning betyder, at den hverken forløber in vitro eller in vivo.

Flavonoidernes kan også interagere med de jernholdige hæmoproteiner, som jo forekommer udbredt i organismen (blod, muskler) såvel som i animalske fødevarer. I omhyggelige undersøgelser af denne interaktion nåede vi frem til en sammenhængende forklaring af flavonoidernes virkemåde som både pro-oxidanter og antioxidanter og dermed til en forklaring af en del af de modstridende informationer om disse virkninger i den videnskabelige litteratur. Dette er også i tråd med andre grundlæggende fysisk-kemiske undersøgelser af antioxidanternes virkningsmekanismer under dette rammeprogram, der viser, at flavonoiderne nok er gode antioxidanter, men de er ikke i stand til at regenerere vitamin E i et isoleret system. De er dermed som udgangspunkt ringere antioxidanter end både vitamin E og vitamin C. Den konkrete antioxidant virkning er dog noget afhængig af det system, der undersøges, og der kan derfor være omstændigheder, hvorunder flavonoiderne er særligt effektive antioxidanter. Fra EU-FAIR programmet 'antioxidanter i fødevarer', som var tæt knyttet til nærværende program, ved vi, at flavonoidholdige planteekstrakter kan have en meget gavnlig effekt på holdbarheden af fødevarer, f.eks farsretter og lignende.

Projekt 3: Kortvarige undersøgelser i dyr og mennesker

For at påvise et optag af flavonoider hos mennesker blev deres udskillelse i urin undersøgt og til dette formål blev der udviklet nye og meget følsomme analysemetoder. Kun 0,5-1% af de undersøgte flavonoider i kosten udskilles intakt med urinen hos mennesker, hvilket antyder en relativt lav optagelse. For det vigtigste flavonoid i kosten, quercetin, er der tilsyneladende en direkte sammenhæng mellem indtag og urinudskillelse. Noget tilsvarende gælder catechinerne. Det betyder, at vi har gode biomarkører for flavonoidindtaget, hvilket vil tillade gennemførelsen af direkte undersøgelser af flavonoidindtaget hos personer, hvor kosten er ukendt, eller hvor vi ønsker at se effekten af fx. nye og forbedrede fødevarer.

Man har længe vidst, at flavonoiderne kan nedbrydes af tarmbakterier til simplere phenoler, der er letoptagelige og som også har antioxiderende egenskaber. Selvom flavonoiderne muligvis ikke optages særlig effektivt i organismen kan de derfor stadig have betydning som antioxidanter som følge af deres bakterielle nedbrydningsprodukter. Dette emneområde har vi kun i begrænset omfang kunnet forfølge gennem internationalt samarbejde, men det åbner selvsagt en del perspektiver af både teknologisk og sundhedsmæssig art.

Når antioxidanter forebygger en oxidationsproces går de som regel til i processen, dvs. de omdannes oxidativt til forskellige nedbrydningsprodukter. Hvis flavonoiderne virker som antioxidanter i fødevarer eller i organismen udsættes vi for nedbrydningsprodukterne. Vi har

isoleret nedbrydningsprodukter af nogle flavonoider og de havde tilsyneladende meget lav cytotoxicitet og var heller ikke toksiske overfor bakteriers arveanlæg (genotoksiske). Der er således ikke noget der tyder på, at der kan være negative toksikologiske konsekvenser af, at udnytte flavonoider som antioxidanter, hverken i fødevarer eller i organismen.

I cellekulturer kunne vi vise en betydelig cytotoxicitet af flavonoider, formentlig som følge af en pro-oxiderende virkning sammen med metaller som fx. jern eller kobber. Da jern og kobber ikke normalt forekommer frit i organismen er det ikke sandsynligt, at flavonoider kan virke pro-oxiderende gennem denne mekanisme, undtagen under specielle forhold, fx. overfor hæmoproteiner. Interessant er det dog, at flavonoiderne tilsyneladende er mere giftige for kræftceller end for normale celler. Det vil kræve yderligere undersøgelser at vise, om dette kan have praktisk betydning i forebyggelse eller behandling af kræft.

Hos forsøgsdyr virker flavonoider som antioxidanter ved doser, der er 50-100 gange højere end vores kostindtag. Det er dog ikke klart om der er tale om en direkte antioxidantvirkning eller om flavonoiderne virker mere indirekte i den konkrete dyremodel, vi opstillede. Ved de høje doser har flavonoider også en virkning på dyrenes eget antioxidative forsvar, der tilsyneladende svækkes i blodet, men er uændret i leveren og i andre organer. Om dette er udtryk for at forsvaret i blodet reguleres ned, fordi flavonoiderne tager over, eller om det må betragtes som en giftvirkning, er ikke klart. Hos mennesker sker der ved intervention med flavonoid-rige kostkomponenter i hovedsagen det modsatte, nemlig en svag, men signifikant forøgelse i det antioxidative forsvar i form af øget aktivitet af antioxidative enzymer i blodet. Da forsøgene ikke blev udført med rene flavonoider, men med flavonoidrige kostkomponenter, bl.a. juice og persille, kan det dog ikke udelukes, at det er andre stoffer i disse fødevarer, der har haft denne virkning. I disse studier sås derimod ikke nogen entydig antioxiderende virkning af flavonoidrig kost i blodprøver målt ved hjælp af biomarkører: Ved intervention med juice fandt vi f. eks. en lavere oxidativ skade i lipoproteinet LDL (der kan have relation til hjerte-kar sygdom), men samtidig en øget oxidativ skade (pro-oxidant virkning) i blodets albumin, se fig. 4. Sidstnævnte virkning skyldes højst sandsynlig juicens indhold af C-vitamin, der tilsyneladende giver denne virkning, men virkningerne af C-vitamin og flavonoider kan ikke klart adskilles i disse studier.

Hos forsøgsdyr er der en virkning af karotenoidet lycopen (fra tomater) på antioxiderende enzymer, selv ved relativt lave doser, og i biomarkørstudier hos mennesker har vi set en tilsvarende virkning som mest sandsynligt kan tilskrives karotenoidet, lutein (fra grøntsager, æg og mælk). Karotenoider kan derfor muligvis styrke vores eget antioxidative forsvar. Lutein har derimod tilsyneladende ingen virkning som selvstændig antioxidant i blodprøver fra mennesker ved normale kostindtag, målt med de biomarkører, vi har til rådighed.

Figur 4. Interventionsforsøg hos fem frivillige forsøgspersoner med æble- og solbærjuice, der har et højt indhold af flavonoider. Dosis er i ml per dag, og blodprøverne er taget umiddelbart inden intervention (dag -1) og på dag 1, 2, 4 og 7. A. Måling af fedtoxidationsproduktet MDA i plasma lipoproteiner (nmol/mg protein). B. Måling af et proteinoxidationsprodukt i plasmaproteiner (pmol/mg protein).

Projekt 4: Længerevarende undersøgelser hos forsøgsdyr
Både flavonoider og karotenoider kan endvidere hos forsøgsdyr påvirke andre enzymer (fx

monooxygenaser), der kan være af betydning for kræftudvikling. Foreløbige resultater af et langtidsforsøg med stofferne viser, at både flavonoider og karotenoider kan forebygge leverskader forårsaget af stegemutagener. Om denne forebyggelse også leder til forebyggelse af kræft på længere sigt vil vi først vide, når forsøgene er endeligt afsluttet i foråret 1999.

Projektets organisation, ressourcer og produkter
Organisation: Programmet har i det daglige været koordineret af en programleder, men har iøvrigt haft deltagere fra to institutter under Veterinær of Fødevareministeriet, fra to institutter under Levnedsmiddelcentret ved Veterinær- og Landbohøjskolen, og fra et institut ved Roskilde Universitet. En repræsentant fra hver af disse institutter har siddet i styringsgruppen, og der har desuden været en programkomite med repræsentanter fra forskningsinstitutioner og fra fødevareindustrien, der har fungeret som bestyrelse. Et organisationsdiagram er vist i appendix 1. Det daglige arbejde har været udført i fire til seks projektgrupper, der har været sammensat alt efter de aktuelle opgaver. I den afsluttende fase var sammensætningen af grupperne som vist i appendix 2. Her fremgår også, hvem der har været ansat på projektmidlerne.
Økonomi: Programmet har haft en samlet bevilling på 6 mill. kr over de tre år. Heraf er ca. 3.7 mill kr brugt ved Veterinær- og Fødevaredirektoratets institutter, 1.9 mill kr ved institutter under Levnedsmiddelcentret ved Landbohøjskolen og 0.4 mill kr. ved Roskilde Universitet. Hertil kommer en medfinanciering på ca. 3 mill kr fra Veterinær- og Fødevaredirektoratet. Der er betalt knap 12 årsværk af bevillingen. Økonomien fremgår iøvrigt af appendix 4.
Fagligt udbytte: Den samlede produktion fra projektet indtil videre er vist i appendix 3. Der er indtil nu indleveret ca. 30 manuskripter til videnskabelige tidsskrifter, bøger eller rapporter, og der har været præsenteret 24 arbejder fra dette rammeprogram ved videnskabelige konferencer og lignende. Hertil kommer et antal manuskripter under udarbejdelse. Der er tillige organiseret forskellige videnskabelige møder i programmets regi. Endelig er der gennemført en række undervisningsaktiviteter, herunder uddannelse af to ph.d studerende. Samlet set må det videnskabelige udbytte betegnes som meget tilfredsstillende. Der har været kontakt til en del virksomheder om konkret udnyttelse af resultater fra rammeprogrammet, og der har været udarbejdet tre ansøgninger om samarbejdsprojekter m.v. om udvikling af fødevarer med et dokumenteret højt naturligt indhold af antioxidanter. Det har dog ikke været muligt at opnå tilskud til projekterne i et sådant omfang, at de har kunnet gennemføres. Det fødevareteknologiske udbytte til udvikling af nye og sundere fødevarer er det derfor for tidligt at spå om endnu, men de konkrete projektforslag, der har været udarbejdet sammen med danske virksomheder viser, at branchen har en tro på, at det kan lade sig gøre at udvikle og anvende denne teknologi.

Diskussion
Omfattende forsøg, såvel i reagensglas som i dyr og mennesker peger på, at såvel flavonoider som karotenoider kan have beskyttende virkninger, der kan føre til sundhedsfremme, men at vi må se i øjnene, at de enkelte naturlige antioxidanter ikke nødvendigvis er ensidigt sundhedsfremmende. Ligesom det gælder for flere af vitaminerne har de non-nutritive komponenter både positive og negative egenskaber, der må vejes op mod hinanden i en samlet vurdering.

Blandt de vigtigste resultater af projektet skal fremhæves følgende:

- Indtaget af flavonoider og karotenoider i Danmark er på linie med indtaget i andre Nordiske lande, men lavere end i landene sydpå. Løg, æbler, vin, te og citrusfrugter er de vigtigste kilder til flavonoider mens gulerødder, tomatprodukter og andre grøntsager er de vigtigste kilder til karotenoider.

- Både flavonoider og karotenoider er effektive antioxidanter, der kan udnyttes til at stabilisere fødevarer. En model for virkningsmekanismerne for flavonoider og til dels karotenoider som antioxidanter er opstillet.

- Omsætningsvejene for de undersøgte flavonoider i organismen er kortlagt.

- Flavonoider og karotenoider kan virke som antioxidanter ved høje doser hos forsøgsdyr, men det er usikkert om flavonoiderne er antioxidanter hos mennesker ved almindelige indtag gennem kosten. Karotenoiderne kan muligvis styrke vores eget forsvar ved jævnt højt indtag med kosten.

- Med små, kontrollerede ændringer i kosten kan vi observere signifikante ændringer i biomarkører for oxidativ skade og antioxidativt forsvar. Dermed er der grundlag for at basere undersøgelser af sundhedsmæssig kvalitet til dels på kontrollerede studier af frivillige forsøgspersoner.

- Flavonoider, men ikke karotenoider, kan være pro-oxidanter (det modsatte af antioxidanter) under visse betingelser. De har større cytotoksicitet overfor kræftceller end overfor almindelige celler, muligvis som følge heraf. Flere flavonoider har også en hormonlignende virkning som kan være beskyttende mod kræft.

I en samlet vurdering af muligt sundhedsfremmende faktorer i fødevarer vil viden om virkningsmekanismer indgå med stor vægt. De grundlæggende fysisk-kemiske studier, dyreforsøgene og de biomarkør-baserede kostinterventionsstudier, der er gennemført som en del af dette rammeprogram og af EU-FAIR programmet, kan derfor komme til at danne model for udredning af virkningsmekanismer for sundhedsfremmende stoffer generelt. Rammeprogrammet har derfor nået sit mål med at bidrage til at skabe et evalueringsgrundlag for non-nutritive kost-komponenter.

Det er dog også klart, at et endeligt evalueringsgrundlag vil kræve en meget mere omfattende forskningsindsats. På nogle områder, især m.h.t. kræft og hjertekar-sygdom, mangler der stadig et optimalt forsøgsdesign. Der er initiativer igang for at opnå international koncensus om, hvilke undersøgelser, der skal til for at kunne sige, der er en sundhedsfremmende effekt af en kostkomponent. Under Nordisk Ministerråd er der udsendt en række monografier med vurderinger af kræftforebyggende virkninger, heraf den seneste om karotenoider som en del af dette rammeprogram. Den internationale kræftforskningsorganisation, IARC, under WHO har nu udsendt tre rapporter med sådanne vurderinger, hvor også erfaringerne fra nærværende rammeprogram har været inddraget. Samtidig hersker der næppe tvivl om, at et sådant grundlag kan og vil blive tilvejebragt internationalt gennem både offentlig og privat forskning i de kommende år.

De relativt store forskningsudgifter, der skal til for at udvikle produkter med øget sundhedsmæssig kvalitet er en hæmsko for investeringer i dette område. En sådan kvalitet kan fx. være et øget indhold af naturlige antioxidanter eller evnen til at forbedre vores forsvar mod frie radikaler. Det forhold, at en anprisning af sundhedsmæssig kvalitet heller ikke må forekomme på varen, gør ikke den slags investeringer mere attraktive. Hertil må føjes den usikkerhed og mangel på eksakte metoder der stadig råder med hensyn til at vise, at et produkt med sådanne dokumenterede virkninger tillige har langsigtede sundhedsmæssige effekter som f.eks. forebyggelse af kræft eller hjertekar-sygdom.

## Konklusion

Nærværende rammeprogram har udviklet og anvist metoder til at vurdere visse sundhedsmæssige virkninger af naturlige antioxidanter. De opnåede resultater kan danne basis for lignende evalueringer af andre kostkomponenter end flavonoider og karotenoider. Endvidere har dette rammeprogram anvist veje for hvordan den sundhedsmæssige værdi af fødevarer kan undersøges, hvilket både kan føre til udvikling af varer med en øget fødevarekvalitet samt en dokumentation af deres sikkerhed og sundhed.
Forskningsmæssigt og udbyttemæssigt må rammeprogrammet anses for at være en succes. Der er imidlertid både økonomiske, politiske og videnskabelige problemer, der skal overvindes før denne viden og teknologi vil kunne udnyttes fuldt ud af virksomhederne herhjemme, især af de mindre danske virksomheder.

# A. Qualitative report

# A1. Project organisation and facilities

The Programme (so called onwards) is organised with a project co-ordinator, a Steering Committee, consisting of a representative from each of the five research institutes involved, and a Programme Committee with members from trade and business-oriented research institutes, see appendix 1. The Programme Committee is acting as an executive board, responsible to FELFO at the Danish Research Councils. Statutes have been elaborated for the co-operative work in the programme as such and regulations have been written for the work of the Programme Committee. The two committees have each had meetings four times in 1995 and two times in each of the years, 1996, 1997 and 1998.

The five research institutes involved, and their affiliations are, Institute of Food Safety and Toxicology (IFT), and Institute of Food Research and Nutrition (IFEE) at the Danish Veterinary and Food Administration (VFD); Department of Dairy and Food Science (MLI) and Research Department of Human Nutrition (FHE), both part of the Food Centre for Advanced Studies (LMC) at the Royal Veterinary and Agricultural University (KVL); Department of Life Sciences and Chemistry, Roskilde University (RUC).

The Programme is subdivided into four projects:
1. Natural antioxidants (primarily flavonoids and carotenoids) in the diet.
2. Antioxidant effects, (anti)mutagenicity, and (anti)toxicity in vitro.
3. Disposition and antioxidant effects in vivo.
4. Antioxidation and prevention of cancer in experimental animals.

Each project is organised with one or two project groups, which have met two to four times a year to discuss research plans and other work details. The project co-ordinator is a member of all the groups. The six project groups are listed in appendix 2.

The programme is new in the sense that it did not exist under FØTEK I. The programme was funded by FØTEK 2 in January 1995, and officially started 1. June 1995. This final report covers the entire period from 1. June 1995 to 31. May 1998. A report for 1995 was made in February 1996, and a midterm report was filed in January 1997. Both were accepted by FELFO shortly afterwards.

Information to researchers and others close to the programme is mediated through newsletters,

which have been issued 2-4 times/year. More general information about the project in the form of a folder has been mailed to 140 companies in the Danish food sector in order to increase awareness about the programme. Those interested have further received a 6-page description of the programme, its projects, milestones and contact persons. Two scientific meetings have been organised, one internal to present the participants to each other, and one external with invitations to all other Danish research groups known to us, which were at that time involved in research on antioxidants and health. Companies which had shown interest in the programme were also invited to the latter meeting. A final workshop and press conference will be held when this report is presented.

Most of the laboratory facilities, instruments etc. have been placed at the programmes disposal by the research institutes involved. This includes ESR and electrochemical equipment at MLI, HPLC's and mass spectrometers, chemical laboratories, cell culture- and animal facilities at IFT, IFEE and RUC as well as kitchens and a nutrition unit at FHE. Two instruments have been bought directly for the work under this programme, a diode array detector for HPLC (IFEE), and a robot for automation of sample preparation (IFT).

# A2. General achievements

The objective of the present programme is to form an approach to the practical exposure, health and toxicity evaluation of non-nutritive antioxidant factors in foods as well as to evaluate their potential usefulness as antioxidants. Due to the great interest in food components with possible health-promoting effects and to the expected commercial importance of such factors, there is a need for investigation and documentation of their potentials. This is the case for certain traditional food components but is a major issue in designer foods and in a number of other functional foods. Furthermore, food components containing high concentrations of such factors, e.g. natural antioxidants, might be used to substitute for traditional food additives.

Several non-nutritive factors with potentially preventive effects on cancer, heart disease and certain age-related diseases are antioxidants. The present programme has focused on the effects of two groups of antioxidants, flavonoids and carotenoids. We have further chosen to focus on the general exposure in the Danish population, on some unresolved aspects of their toxicology, on antioxidation mechanisms and on anti-carcinogenesis.

a. Present achievements
The specific aims and the achievements of the projects under this Programme are described below in section B2a and B2b. One of the most important general achievements has been the establishment of a research team which spans from chemical and physical analyses of foods and antioxidant food components through experimental toxicology to studies involving health effects and human intervention. Some of the most promising results have only come about due to this convergence of research activities and know-how. Four of these results should be particularly emphasised:

-During chemical analyses of commercial fruit-derived products, we have noticed major differences in their contents of flavonoids, indicating that processing may seriously change the content (and possibly effects) of natural antioxidants. Early contacts with industry on this issue was established in order to evaluate the possibilities for a co-operative project, and a project proposal for FØTEK3 was sent in April 1998. Unfortunately the proposal was turned down. A new proposal focusing only

on technical aspects will be prepared by others.
-During the establishment of a biomarker for flavonoid exposure, we have noticed a general pattern of biotransformation (in rats) and possibly also major differences in their excretion in humans, both related to structure. These results will aid in the general toxicological and health evaluations of flavonoids.
-While determining the electrochemical half-potentials of certain flavonoids we have established a method to study chemically the structural changes taking place in flavonoids as they act as antioxidants. We have also unambiguously identified the oxidation products of quercetin and kaempferol. This has only been treated theoretically in the literature until now, and the results may give a clue to some unresolved toxicological problems with flavonoids and significantly increase the practical possibilities for using them as food antioxidants.
- Human intervention studies with flavonoid containing juices and with parsley have shown clearly that biomarkers for oxidative damage and markers for our defence against oxygen radicals may be influenced by these food items. So far, the influence of diet on health have focused on the final outcome: health or disease. Our results indicate that biomarker intervention studies in combination with animal studies should aid in establishing the mechanisms of action of potentially health-promoting dietary food components.

We have published or are in the process of publishing these and several other results of the programme, see appendix 3.

b. Possibilities for future expansion of the programme
Health effects of non-nutritive factors is a vast area of research with intense international competition. The strength of the present programme is our ability to treat questions related to the technological use of a given compound from a very broad angle, related to antioxidant effects, health and toxicity. Each of the projects in the present programme could off course have benefited from being supported more strongly, without overtly changing the overall design of the programme. The design could, however, be strengthened in several other ways as well, each of which could be handled by inviting existing strong Danish research groups (in square brackets) into the programme. It must be stressed that we have not contacted all the colleagues mentioned below, but some of the ideas have been used for research grant proposals (marked with asterisks):
-The present team is not very familiar with the chemical analysis of certain fatty acid oxidation products, an area which is important for antioxidant technology as well as for health. [Prof. G. Hølmer, LMC, Danish Technical University]*.
-Aspects of food technology other than antioxidation (e.g. natural colours and flavours) and aspects of health other than cancer (e.g. heart disease, effects on reproduction) could be added to the current points of focus. [Prof. P. Grandjean and Dr. J.B. Nielsen, Odense University; Dr. Alicja Mortensen, VFD]
-The scope could be broadened to encompass hygienic aspects as well. One of the potential outcomes of this programme, use of food components as surrogates for additives might lead to food products of mixed origin, where hygienic problems might arise. [Microbiological groups at KVL and VFD]
-Food plants could be selected, grown, harvested and otherwise treated to increase their contents of selected non-nutritive factors. [Dr. Kirsten Brandt and others, Danish Agricultural Research Centre, Årslev (DAR)]*
-Food plants could be genetically engineered to increase their content of selected non-nutritive factors. [DAR; VFD]*.
-Using older studies on health effects in relation to the diet and our new data on Danish dietary

levels of natural antioxidants, epidemiological studies could be performed. [Prof. Kim Overvad and others, Danish Cancer Research Fund; Danish Cancer Registry]

We need to have co-operative projects with the agro-food industry in order to evaluate which of these areas might be the most promising for future expansion. At this time, however, the various health aspects of non-nutritive factors which are also antioxidants seem to be the most promising direction for future research. A new framework programme focusing on this aspect has been launched under FØTEK3 with Prof. Leif Skibsted, KVL, as co-ordinator.

The present research connections established are necessary and important for building up know-how which includes both technology and health. The two Ph.D. students have selected courses related to more than one of these areas of research, and the project groups and journal club established forced students as well as senior researchers involved to educate themselves on several aspects of food technology, health and toxicology.

c. National research co-operation
A contact with Associate professor Claus Cornett at Department of Analytical and Pharmaceutical Chemistry at the Danish School of Pharmacy was established early on, and co-operation on NMR-spectroscopy of metabolites and oxidation products of flavonoids has resulted in several publications.

Important co-operation has taken place with DAR at Årslev, where know-how on formation and genetics related to flavonoids in plants is abundant. Several flavonoid glycosides produced at DAR are now at our disposal for further research.

Also, together with Dr. Jesper Bo Nielsen and Dr. Helle Ravn, Dept. of Environmental Medicine, Odense University, co-operation on automation of assays for antioxidant enzymes has taken place, and these assays have been set up at FHE as well.

Lars Dragsted was also a participant in the Danish Strategic Environmental Research Programme, subprogramme on human health (1994-1996, co-ordinated by Prof. Herman Autrup, Århus University), which has focused on occupational exposures and their effects on biomarkers for oxidative damage. Several of the biomarkers which were developed in this environmental subprogramme are being used in the present research.

Part of the information collected under the present programme has been included into the work on a plant data-base, supported by a FØTEK I grant to Dr. Jørn Gry, VFD.

The information collected under the programme was used to prepare the chapters on preventive effects and on toxic effects of plant constituents in a recent report on recommendations for intake of fruits and vegetables in Denmark (Ministeriet for Fødevarer, Landbrug og Fiskeri, 1998).

d. International activities
- A project under EU-FAIR, Natural Antioxidants in Foods, running from 1 december 1995 to 30. November 1998 was co-ordinated by Prof. Brittmarie Sandström with Prof. Leif Skibsted and Dr. Lars Dragsted as partners. This project was oriented towards testing of whole foods and food extracts both in vitro and in human intervention studies. Several analytical methodologies overlap

with the present Programme, and some of the activities in the projects have been expanded by using common resources.
-Dr. J. Castenmiller and Dr. Clive West at Wageningen Agricultural University in the Netherlands initiated a study on human metabolism and disposition of carotenoids from spinach, which is rich in specific carotenoids but devoid of flavonoids. About 60 volunteers were recruited for the 3-week intervention study. For economic reasons we would not be able to perform such a large study under this Programme. We therefore have joined the Dutch study and have performed several biomarker assays on the samples collected in order to fulfil our obligation to test the effect of carotenoid-rich food on markers of oxidative damage and antioxidant defence.
- Bahram Daneshwar, (post doc at IFT) working partly on the EU-FAIR programme and partly on this Programme obtained a EU research training grant to join Dr. Garry Duthie at the Rowett Research Institute in Aberdeen, Scotland, from September 1997 to June 1998. The aim was both training and exchange of technologies. This has resulted in the establishment of the comet assay for determination of oxidative DNA damage within the VFD group as recommended in the mid-term evaluation.
- Dr. Roland Wolf, Dundee Medical School, has supplied human cytochromes P-450 in order for IFT to study the human biotransformation of flavonoids.
- Michael Strube, a research assistant at IFT working on this Programme, has had a one-year training fellowship visiting Dr. H. Verhagens group at TNO, Holland. Part of the work there on the antioxidant effects of flavonoids was finished here as part of this Programme.
- The Nordic Council of Ministers permanent Committee on Food Toxicology and Risk Assessment has supported a report on carotenoids which is also part of the activities under this Programme.
- Contacts and co-operation with an EU-FAIR project has been established (Phenolic Phytoprotectants Programme, co-ordinator Prof. H. Adlercreutz, Finland), where animal feeds free from specific plant phenolics have been designed and these have been used in the present Programme.
- Dr. Ulla Justesen (IFEE) has been on reseach exchange visit to Prof. Renato Amadó at the Swiss Federal Institute of Technology, Zürich, in order to study the degradation of flavonoids by human faecal bacteria. This visit has been sponsored in part by the present program.
- Mette Nielsen (reasearch assistant, RUC) was visiting researcher at the laboratory of Prof. R.J. Ruch, Department of Pathology, Medical College of Ohio, Toledo, USA in order to study the effect of flavonoids on gap junctional intercellular communication using scrape loading/dye transfer technique.

e. Bachelor, candidate and Ph.D. education
We have had no candidates or bachelors associated with the project. We have had one Ph.D. student and one research assistant who also formally initiated a Ph.D. study. Both have initiated their studies during the summer 1995, and both have finalised their Ph.D. studies in time (Jørgensen, 1998; Nielsen, 1998). Both have attended project group meetings and also actively participated in our journal club, and both have presented their results at national or international scientific meetings (see appendix 3).

f. Information
We have initially produced a folder, which was printed and mailed with information about the project to about 140 companies, research institutes and private laboratories. Interested parties have received further information in the form of a 6-page summary of the programme, projects, milestones etc.

An open scientific meeting on antioxidants and health has been organised in 1996, where we invited all relevant research groups as well as people from companies which had shown interest.

Scientists involved in the programme have been invited as speakers at several meetings on antioxidants and/or health effects of non-nutritive factors in foods, see appendix 3. Also a few short manuscripts in Danish informing about the programme have been printed in the quarterly newsletter (formerly 'LST Nyt') from VFD.

Lars Dragsted has submitted a paper informing about the project results and the possibilities they open for food technology to another Danish journal dealing broadly with this area.

g. Importance for trade
Our results have had no impact on trade at this time.

h. Future commercial use
There are several aspects of this programme which could be brought to future commercial use. Some of these are detailed below. The large investments necessary for developing foods with improved and documented health effects is probably a limiting factor for industry to join in such projects. The utilization of results will therefore depend on the availability of funds to support the research co-operation between academia and food industry. However, some methods are simply not available or not in practice affordable within the economy of Danish research programmes or Danish food companies at this time, e.g. methods to prove that a product can actually decrease cancer risk or stroke. Moreover the European and Danish legislation does not permit advertising of health promoting effects on any food products. There is some differences in the interpretation of the European legislation in various countries leading to differences in what is accepted in different European countries in practice. This might lead to harmonisations in the near future. The development of methods for health evaluation of non-nutritive components could prove important for the evaluation process which has to take place for novel foods in each of the EU member states. Especially the combination of in vitro studies, animal studies and short-term human intervention studies with biomarkers for positive or negative effects on health could be seen as a viable approach to be used by companies interested in testing on a scientific basis the possible health effects of a novel food or ingredient. However, at this time, the companies will have to develop healthier foods because they have superior technical qualities or because of their strategic possibilities. We have listed below some possibilities for future commercial use of the results from the program:

-Increasing the stability of foods or ready-made dishes by using ingredients rich in natural antioxidants.

- Increasing the quality of products based on processed fruits and vegetables by securing that natural antioxidants are not lost during the production process. This could be achieved by chemical analysis for antioxidants and/or by identifying components which enhance oxidation processes and/or by determining the antioxidant capacity of the products at various stages of the process.

- Documenting the increased uptake or positive short-term physiological effects of natural antioxidants from improved foods by publishing results of high-quality research on the products internationally.

- Using the knowledge on which natural antioxidants may possess beneficial effects to provide a

rationale for developing food plants rich in such components by gene technology or by traditional breeding.

- Increasing the quality of meat and dairy products by optimising animal feeds with respect to natural antioxidants.

# A3. Conclusions

a. Organization

We conclude that,
a) The research organisation was established to perform the tasks promised under this Programme. The organisational framework has been adequate to secure the necessary co-operation between the research institutions involved.
b) The research has been running according to schedule seen as a whole. One activity, the long-term animal studies, is behind schedule. The experiments started in May 1998 and will be finalised in february 1999. This is financially possible due to co-sponsoring by VFD.
c) The research is relatively basic by nature, but with some clear commercial possibilities. A strategy for information and for contact with trade and industry was established initially and continued throughout.
d) The research performed is internationally competitive with abundant national and international contacts, and it has been very productive, see appendix 3.

b. Research outcome within the four projects

a. Flavonoid and carotenoid intakes in Denmark are lower than in other European countries further south. Carotenoid intakes in Denmark are comparable to intakes in Sweden and Finland, whereas flavonoid intakes are higher in Denmark than in Finland. Tea, wine, citrus fruits apples and onions are the main dietary sources of flavonoids, whereas carrots, tomato products and other vegetables are the main sources of carotenoids in the Danish diet.

b. Both, flavonoids and carotenoids, may act as efficient antioxidants in vitro and in foods, depending on the system and on the oxidative challenge. A scheme explaining the antioxidant and pro-oxidant behaviour of flavonoids has been elaborated. Flavonoids can act as antioxidants in vivo at high doses, but there may not be antioxidant effects at human dietary levels. The carotenoids, lutein and lycopene may have physiological effects related to antioxidation at human dietary levels.

c. Some flavonoids, but not carotenoids, have potent cytotoxic effects which may be related to pro-oxidant effects towards proteins at high concentrations due to interactions with transition metals. The oxidation products identified from quercetin and kaempferol are neither cytotoxic nor mutagenic.

d. The hepatic biotransformation of flavonoids has been elucidated, and biomarkers for human exposure have been developed and validated.

# A4. Economy and staff

The Programme had a total budget of 6.000,000 DKK (6M-DKK) from FELFO and about 2.5 M-DKK from VFD for the period 1995-1999. The support from FELFO has been divided with 3.71 M-DKK to VFD, 1.91 M-DKK to LMC, and 0.38 M-DKK to RUC. Detailed budgets and expenses by year and research institution can be seen in appendix 4. These budgets were generally followed, and only minor changes have taken place, see appendix 4 and section B.2.h.

Eight persons have been employed directly under the Programme for periods of 0.3-3 years. One post-doc, one Ph.D. student, four research assistants (one of which was also immatriculated as a Ph.D. student), and two laboratory technicians. A total of 12 person-years have been directly funded by the FØTEK grant to this Programme, and a further six person-years are co-financed by the research institutions involved. The placement of human resources on the project groups can be seen in appendix 2.

# B. Quantitative report

# B1. General part

a. Summary of results, deviation from original plan

A list of the products obtained so far in the Programme is given in appendix 3 by category:

There are a few deviations from the original research plan, and they have not changed the total outcome of the Programme:
- Due to the large recently terminated analytical projects in Sweden (Konde et al., 1996), Finland (Heinonen, Carotenoids and retinoids in Finnish foods and the average diet, Dissertation, University of Helsinki, 1990), the US (Mangels et al., 1993) and other places on carotenoids in various food items, the work on carotenoids has mainly consisted of compilation of data. Analysis of carotenoids in Danish food items have been performed only when large discrepancies were observed between the published contents of carotenoids in food items of some importance for the Danish dietary intake of carotenoids. This has been particularly important for lycopene in tomato products and lutein in corn products. The efforts laid down on analysis for flavonoids has been expanded about two-fold to compensate for this.
- Due to temporary leaves of two of the researchers involved, specific parts of the work has been delayed. One is the report to the Nordic Council of Ministers on certain carotenoids, the other is some of the ESR-work involved in project 2 . The latter work has also been postponed a little, because some antioxidant studies on simple terpenoids, another important group of natural and abundant antioxidants have been included in the project.
- Animal experiments (anti-tumorigenesis experiments) have been delayed, partly due to rebuilding of the animal facilities at VFD, partly due to technical problems with stability of the natural antioxidants included for testing in the animal feed. The experiments are now ongoing and will last until the end of February 1999.
- The animal model for scoring of preneoplastic lesions in the rat colon has been expanded to include tumourigenicity, induced by temporal feeding with a food mutagen/carcinogen within one year. Since this model is less complicated than the transgenic mouse models and since it is more relevant to human exposures and risk, we decided to do the tumourigenicity studies using this model in normal rats. The transgenic mouse studies will therefore be left out of the Programme. The only drawback from this may be an increased cost of test substances.

- The emphasis on studies on biotransformation and disposition in the human has been increased and the human biomarker studies have been expanded. This change of focus is partly due to the difficulties in performing the animal studies during the rebuilding of facilities 1996-1997, partly to the fact that we have been successful in developing the biomarkers without studying the disposition of labelled compounds in animals.
- The total focus has shifted more towards the flavonoids in the experimental work and less towards the carotenoids. Still, studies on carotenoids are included under each of the four projects. This change in focus has been influenced to some extent by the findings of co-carcinogenic actions of the carotenoid, beta-carotene, in two human studies.

b. Statement by Programme Committee
The Programme Committee has not had the opportunity to gather and comment on this report. An official comment will be forwarded when the report is submitted to FELFO.

# B2. Specific part

For each of the four projects in the Programme, a description of the original aims (translated from Danish) are given below in section B2.a. A status of the research work as of December 31, 1998 is given in section B2.b, and an overview can be obtained from the table under this section, based on the original milestones from the application:

a. Description of individual projects (specific aims)

1. Natural antioxidants (primarily flavonoids and carotenoids) in the diet.
The aim of this project is to determine the intakes of natural antioxidants (primarily flavonoids and carotenoids) from the diet. An evaluation of the content of selected plant phenolics and carotenoids in the Danish diet is performed based on information from the literature and analyses of relevant dietary items. Information on the presence of individual antioxidants in the diet forms part of the basis for selection of the compounds to be investigated in project 2.

2. Antioxidant effects, (anti)mutagenicity, and (anti)toxicity in vitro.
The in vitro toxicity (mutagenicity and cytotoxicity) of selected flavonoids and carotenoids as well as their antioxidant effects in vitro in various systems (physico-chemical and biochemical) will be investigated and compared with the literature. Structural determinants will be identified. Interactions between selected antioxidants will be investigated based on mechanistic considerations. The results from this project form part of the basis for selection of compounds for projects 3 and 4.

3. Disposition and antioxidant effects in vivo.
Metabolism studies in vivo in experimental animals will be performed with labelled compounds for at least two natural antioxidants (carotenoids or plant phenols), selected from projects 1 and 2. Biomarkers will be developed to determine the excretion of these compounds or their metabolites in humans from ordinary dietary levels. These exposure biomarkers will be tested in animal experiments and in a human dietary intervention study, where several effect biomarkers related to the antioxidative defence systems will also be used, including antioxidant enzymes, fatty acid oxidation products and levels of specific oxidative damage to blood proteins.

4. Antioxidation and prevention of cancer in experimental animals.
Investigations will be performed on the inhibiting effects of selected natural antioxidants

(flavonoids or carotenoids) on tumorigenesis. Specifically, a model where foci of preneoplastic change in the colon of rats are used as the endpoint and tumourigenicity studies using transgenic mice will be employed.

b. Results

1. Natural antioxidants (primarily flavonoids and carotenoids) in the diet.
Databases: Two literature databases were built with Reference Manager software, covering literature on flavonoids and carotenoids, respectively. The literature databases were compiled from Medline (1986-present), BIOSIS (1983-present), Toxline (1980-present) and CAS (1968-present). Both databases contain more than 10.000 references, but there is about 30% redundancy making the true number somewhat smaller. A number of keywords have been added systematically to the references in order to facilitate searches. The databases were updated and distributed on CD-ROM to the participants in 1996 as a solid basis for further literature work.
Flavonoid analyses: The analyses of fruit, vegetables and beverages for flavonoids show that tea, onions and oranges are the main contributors to the intake in Denmark, which can be calculated to about 28 mg/day being in reasonable good agreement with the intake in other countries (Justesen et al., 1997). Quercetin and kaempferol were the most widely distributed compounds and also hesperetin and naringenin from oranges are of importance for the intake of flavonoids (Leth and Justesen, 1997; Justesen et al., 1997), see the table below:

Table 3. Estimated median intakes of flavones, flavonols and flavanones in Denmark. The calculated total intake amounts to 27.56 [a] 28 mg/day.
Foods Quercetin
mg/day Hesperetin
mg/day Kaempferol
mg/day Naringenin
mg/day Myricetin
mg/day Apigenin
mg/day Isorhamnetin
mg/day
Apple 0.69
Kale 0.07 0.27
Leek 0.05
Onion 7.19 0.04
Orange 4.52 1.63
Orange Juice 2.22 0.20
Pear 0.04
Red Wine 0.43 0.50
Tea 2.80 3.11 0.79
Tomato 0.63 0.66
Other 0.41 0.25 0.04 0.73 0.05 0.26
Total 12.25 6.99 3.47 3.21 1.34 0.26 0.04

Carotenoid analyses: Analysis of carotenoids has been performed on 15-20 of the foods with the greatest contribution to the dietary intake of carotenoids in Denmark in order to ascertain whether the Swedish ((Konde et al., 1996)) and Finnish ((Heinonen et al., 1989)) values could be used to calculate the Danish intake of carotenoids. The analyses of carotenoids show good agreement with

Finnish and Swedish values except for especially tomatoes, where we find 3 times higher values for the lycopene content than in Sweden, and cornflakes, where we find a much lower content of lutein than in Finland. The new Danish values cover 73% of the total intake of carotenoids, which with Danish values has been calculated to 4,3 mg/day.

2. Antioxidant effects, (anti)mutagenicity, (anti)toxicity and biotransformation in vitro.
Antioxidant effects: Antioxidant effects of natural compounds is probably the mode of action which has received the most attention. The experimental work has been presented as four scientific papers in connection with a literature review in a Ph. D. thesis entitled: "Flavonoids and other naturally occurring antioxidants. Physico-chemical aspects of their antioxidant mode of action" (Jørgensen, 1998). The study was initiated in order to compare important physico-chemical properties of the flavonoids with their antioxidant mode of action in the hope that a deeper mechanistic insight might help elucidate some of the apparent inconsistent results described for their antioxidant activity. Three topics were investigated: (1) The ranking of flavonoids in antioxidant assays; (2) the fate of the antioxidant in vitro; and (3) the nature and efficiency of a possible regeneration of oxidised phenolic antioxidants by other phenolic antioxidants in vitro.
Flavonoids work as reducing agents in aqueous solution and the reduction potential of the corresponding phenoxyl radical is therefore an important parameter for the description of antioxidant activity and hierarchy. The reduction potential of the phenoxyl radicals of fifteen flavonoids were determined by cyclic voltammetry in aqueous 50 mM phosphate buffer, pH=7.4, I=0.16 (NaCl). A possible linear free energy relationship between DG‡ and DG0 for the reaction between structurally closely related flavonoids and ferrylmyoglobin was subsequently examined. The investigations of the reduction of ferrylmyoglobin, to a mixture of metmyoglobin and oxymyoglobin, by the fifteen flavonoids, showed that at least the radicals obtained by oxidation of the flavonols after their one-electron reduction of ferrylmyoglobin, are more reducing than the phenolate anion of the parent compound. This is supported by literature data, which have reported an increased acidity compared to the parent compound of flavonoid phenoxyl radicals with a B-ring catechol structure, and can be explained by the stabilisation achieved after a second electron transfer leading to the highly favourable ortho-quinone structure. This is furthermore of importance for the stoichiometry of the antioxidant reaction, where all the investigated flavonoids with a catechol structure in the B-ring were found to reduce two equivalents of ferrylmyoglobin. The more reducing phenoxyl anion radical may, however, also initiate a prooxidant mode of action (Jørgensen and Skibsted, 1998).
The water-soluble carotenoid crocin was found to reduce the hypervalent iron in ferrylmyoglobin with a surprisingly high rate-constant compared to ascorbate despite it was expected to be less reducing due to a difference of half a volt in the reduction potentials of the corresponding radicals. The initially formed cation radical of crocin was on the other hand found to be less reducing than the parent compound (Jørgensen et al., 1996).
The fate of the antioxidant is crucial for the overall observed antioxidant activity. Identification of intermediate, as well as final oxidation products of flavonoids may therefore provide deeper insight into the mechanism of their antioxidant action. It may also form the basis for new biomarkers for the antioxidant activity of flavonoids in vivo and help elucidate some of the unresolved toxicological problems observed for certain flavonoids. Quercetin and kaempferol were oxidised electrochemically by bulk electrolysis, and the oxidation products formed after a two-electron oxidation were characterised by UV-vis, MS, 1H and 13C NMR spectroscopy. The isolated oxidation products 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone and 2-(4-hydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone from quercetin and kaempferol respectively were more polar than the parent compounds and different from previously

characterised oxidation products. They had increased the molecular weight with 16 g/mol as a result of nucleophilic attack by water on the initially formed oxidation product. Other nucleophiles may however react likewise, and investigations of antioxidant activity in e.g. methanol or ethanol is thus expected to influence the fate of the antioxidant (Jørgensen et al., 1998a).
Antioxidants may interact synergistically when combined, to produce a protective effect which is greater than would be expected if the total effect was merely additive. This can e.g. be obtained if a more reactive antioxidant can be regenerated by a less reactive antioxidant. The physico-chemical nature of such interactions have been investigated with four flavonoids and a-tocopherol in a combined ESR and electrochemical study in deaerated dimethylformamide. The electrochemically generated antioxidant radicals were characterised by ESR after spin trapping by 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). Simulations of ESR spectra based on estimated coupling constants confirmed that the antioxidant radicals are oxygen-centered. a-Tocopherol was found to be most efficient in the established antioxidant hierarchy in regenerating each of the four flavonoids from their phenoxyl radicals. None of the flavonoids investigated were able to regenerate a-tocopherol from the a-tocopheroxyl radical, as has been suggested in the literature as a protection of a-tocopherol in LDL (Jørgensen et al., 1998b).

(Anti)mutagenicity: It has been known for some time that some of the most abundant flavonoids, quercetin and kaempferol, are mutagenic in bacterial in vitro assays ((Nagao et al., 1981; MacGregor and Wilson, 1988)). Several studies have been performed in order to evaluate the molecular mechanism behind this effect in order to assess whether this effect might cause risk for humans. While quercetin is a direct mutagen and is further activated by enzymes in liver cell cytosol, it has been shown that kaempferol is an indirect mutagen which needs activation by microsomal enzymes ((Nagao et al., 1981; Stich and POWRIE, 1982)). The cause of this has now been elucidated by our findings that liver microsomal enzymes can biotransform kaempferol into quercetin (Nielsen et al., 1998). However, we found that such biotransformation does not seem to take place in rats in vivo (Nielsen et al., 1998b), and we found no quercetin in urine from humans who had ingested large amounts of kaempferol (Nielsen et al., 1997). The latter result might be caused by instability of quercetin in urine after long-term storage, but we have subsequently found no evidence that quercetin is unstable in urine samples, even after storage for a year at -20°C.

Anti-cytotoxicity: The flavonoids have been found to have cytotoxic effects in several cell lines, but systematic studies have been lacking. We performed a large screening of polyphenols in order to elucidate any structure-activity relationships. The structural features observed to confer increased cytotoxicity were 1) catechol functions 2) lack of a 3-hydroxy group, and 3) lack of a 2-3 double bond (Breinholt and Dragsted, 1998). Presence of methoxy groups in the B-ring generally decreased cytotoxicity. These features are partly similar to those which are important for increased antioxidant activity ((Rice-Evans et al., 1996)). It has been reported previously that the best flavonoid antioxidants, such as quercetin and myricetin are also the most pro-oxidant ((Canada et al., 1990)). This might indicate, that oxygen toxicity is involved in their mechanism of cytotoxic action. Although potent pro-oxidant activity has been reported for a range of flavonoids under specified in vitro conditions ((Bors et al., 1995)), we have not been able to confirm these data under other conditions (Jørgensen et al., 1998b), indicating that these actions might be dependent upon the physico-chemical environment, and consequently that the actions may take place specifically within certain subcellular structures. There were some notable exceptions to the general structure-cytotoxicity relationship outlined above. We therefore investigated whether uptake into the cells or metabolic activities might influence the results, and observed increased cytotoxicity in metabolically more competent cell lines. A literature survey indicated that cytotoxicity was more

pronounced in tumour cell lines than in non-tumourigenic cell lines. We could confirm this observation in three different tumour cell lines (Breinholt and Dragsted, 1998).

Effect of flavonoids on gap junctional intercellular communication. Inhibition of gap junctional intercellular communication (GJIC) plays a key role in the late steps of carcinogenesis, named tumor promotion. Therefore, modulation of GJIC may be a valuable in vitro assay for tumor promoters as well as substances counteracting tumor promotion. The aim of our study was to examine the effect of different flavonoids on the base line GJIC and the tumor promoter inhibited GJIC. Using two different techniques both based on the direct transfer of flourescent dye from one cell to its neighbours' cells, we analysed five different flavonoids: Quercetin, kaempferol, luteolin, myricetin, and apigenin. Luteolin has not been tested in this type of assay before. Testing the effects of the flavonoids on the base line GJIC, only kaempferol (10 µM) showed an enhanced baseline GJIC at concentrations below 80% of the cytotoxic concentrations. Previously also tangeritin and apigenin have been shown to enhance base line GJIC (Chaumontet et al., 1994). Testing the effects of the flavonoids on GJIC inhibited by the tumour promoters, phorbol ester or DDT, we found that only myricetin did not counteract the GJIC inhibition. This is in accordance with a recent report (Chaumontet et al., 1997). Luteolin (6 µM) and quercetin (10 µM) counteract the phorbol ester inhibited GJIC whereas kaempferol (5-10 µM) counteract the inhibition of GJIC by phorbol ester and DDT. As phorbol ester and DDT inhibit GJIC by different mechanisms, kaempferol showed a more general protection effect, previously showed for quercetin. (Wärngård et al., 1987). In a previous asssay, kaempferol was not found to affect GJIC (Chaumontet et al., 1997). An enhanced base line GJIC and/or counteraction of the tumor promoter inhibited GJIC may indicate decreased susceptibility to tumor promoters.

In vitro biotransformation studies: Flavonoids may be metabolically changed after uptake, and the active compounds might be quite different from the flavonoids per se. It has been shown previously that the methoxylated flavanol, tangeretin, is biotransformed by microsomal preparations under the formation of formaldehyde ((Canivenc Lavier et al., 1993)), indicating that CYP demethylase activities may be involved. Microsomal biotransformation of other flavonoids was investigated for the first time by our group (Nielsen et al., 1998). We observed that formation of a catechol moiety in the flavonoid B-ring was the end product for all flavonoids investigated with rat microsomal preparations. For instance chrysin with no hydroxyl groups in the B-ring was biotransformed into apigenin with one hydroxyl group and, in turn, into the catechol luteolin with its two B-ring hydroxyl groups. Kaempferol was hydroxylated to quercetin with a B-ring catechol structure and tamarixetin was demethylated to quercetin. The only exception to the rule was isorhamnetin which was not demethylated to quercetin. The cause of this anomaly is unknown but it may be speculated, that evolution has given priority to this less toxic methylated form of quercetin. Investigations with inhibitors of various CYP isoenzymes indicated further that CYP1A1 and possibly CYP3A4 are primarily involved in the hydroxylation and demethylation activities.
Studies using human microsomes (Breinholt, paper in prep.) or preparations of human isoenzymes (Breinholt, paper in prep.) confirmed these overall results, indicating that rat monooxygenase enzyme preparations are adequate models for human biotransformation of flavonoids.

3. Disposition and antioxidant effects in vivo.
Animal studies on disposition: A number of studies on the disposition and biotransformation of flavonoids were performed in the period from 1950-1985 (reviewed in (Griffiths et al., 1982; Hackett, 1986)). It was generally found that flavonoids are degraded to a large part in the gut bacterial microflora which is able to cleave the flavonoid C-ring, leaving two simple phenolic

moieties. Also flavonoid glycosides may be cleaved by gut bacteria into the flavonoid aglycone and a free sugar moiety. The liberated aglycone may be absorbed from the large intestine and further metabolism in the form of B-ring hydroxylations have also been described in this early literature ((Griffiths and Smith, 1972)). Most of these studies used paper chromatography and were therefore not able to identify metabolites with the sensitivity of current methodologies. We have investigated the biotransformation and excretion of eight different flavonoids in Wistar rats (Nielsen et al., 1998b) and found marked differences from the observations with microsomal incubations described above. The main excretion products were the monohydroxylated forms, whereas catechols were excreted partially as their methoxylated analogs. The flavonoids were mainly excreted as sulphate and glucuronic acid conjugates in the urine, whereas faecal excretion was mainly in the form of unmetabolised compounds. The amount excreted into the urine varied from 0.4-12 % with a preference for excretion of metabolites and parent compounds with only one free hydroxy group in the B-ring. Faecal excretion varied from 0.6-27 %. The low abundance of catechols among urinary excretion products might indicate that uptake of these compounds may be avoided due to their relative toxicity, and further, that Km for the biotransforming enzymes is too high for the formation of catechols in appreciable amounts in vivo. Methylation by the catechol-O-methyl transferase ((Zhu et al., 1994)) and phase II conjugation reactions on the other hand are relatively fast processes leading to detoxification and faster excretion of free catechols in the form of their biotransformation products.

Animal studies on enzyme induction: In the above-mentioned study the ability of the flavonoids to alter phase I and II enzymes was also investigated. Gavage administration of the natural flavonoids for two consecutive weeks resulted in differential effects on phase 1 and 2 enzymes in liver, colon and heart. Tangeretin, chrysin and BNF, which was administered for 4 days only, were found to significantly induce ethoxyresorufin O-dealkylase and pentoxyresorufin O-dealkylase activities in the liver, whereas hepatic benzyloxyresorufin-dealkylase activity was only significantly increased by tangeretin and BNF. Hepatic quinone reductase (QR) activity was inhibited 43, 58 or 56% by naringenin, genistein and quercetin, respectively, whereas BNF induced QR 4-fold. The remaining flavonoids did not alter hepatic QR activity. QR in colon was slightly, but significantly induced by chrysin and BNF, whereas in heart cytosol, QR was induced by tangeretin and BNF. Glutathione transferase (GST) activity assayed by use of the substrate 1-chloro-2,4-dinitrobenzene was slightly induced by BNF in the liver, but was not affected by any of the other tested flavonoids. None of the test compounds altered GST activity in the colon. In the heart, GST was significantly induced by apigenin, genistein, tangeretin and BNF. Overall the presented data provide evidence that flavonoids differ markedly in their ability to alter enzymatic activities in major organs, and that the effect is dependent, not only, on the structural features of the compound, but also on the tissue in question. Additionally several of the flavonoid compounds seemed to be specific protective agents in the heart and blood compartment (see below), suggesting that this group of dietary nonnutrients might be good candidates as protective agents against cardiovascular disease.
Administration of lycopene to female rats at doses ranging between 0.001 and 0.1 g/kg was found to significantly alter drug metabolizing capacity of the exposed animals. Investigation of four cytochrome P450 dependent activities, revealed that benzyloxyresorufin O-dealkylase activity in the liver was significantly induced in a dose-dependent fashion at all lycopene doses investigated. Likewise was ethoxyresorufin O-dealkylase activity induced, although only at the two highest lycopene concentrations tested. Investigation of selected phase II detoxification enzymes provided evidence that lycopene was capable of inducing hepatic quinone reductase approximately 2-fold at doses between 0.001 and 0.05 g/kg b.w., whereas no effect was observed at the remaining doses tested. Glutathione transferase (GST), using the two substrates 2,4-dichloronitrobenzene and 1-

chloro-2,4-dinitrobenzene, was significantly induced at the 0.1 g/kg b.w. dose, whereas the three lower doses did not result in GST induction. No alterations of these enzymatic activities were observed in tissue preparations from heart or colon.

Animal studies on antioxidant activity in vivo: There is a limited amount of published data on antioxidant effects of flavonoids in vivo as compared to hundreds of papers on their activity in vitro. In a study on prevention of hepatic lipid oxidation caused by hypoxia and hyperthermia in rats, quercetin was found to significantly inhibit damage and also to inhibit lipoxygenase and arachidonic acid metabolism (Luk'yanchuk and Savchenkova, 1993). In a rat liver reperfusion model, the flavonol morin was found to be more protective than trolox or ascorbate. However, the direct infusion of morin in this study was probably leading to plasma concentrations far beyond those attainable by dietary administration (Wu et al., 1993). In a rat model using asbestos induced lipid- and protein oxidation in the lung, the quercetin glycoside, rutin, was observed to be highly preventive, effectively counteracting the asbestos-induced pro-oxidant state in the lungs of the rats (Soodaeva et al., 1994). Quercetin and isorhamnetin have furthermore been shown to inhibit rat liver lipid peroxidation in both normal and cholesterol-fed animals (Igarashi and Ohmuma, 1995). Catechins and tea extracts have been shown by a number of authors to counteract lipid peroxidation in vivo both in glutathione depleted rat lung (Videla et al., 1985), in chronic alcohol-induced rat heart (Edes et al., 1986) and in high-fat diet induced damage to the plasma lipoproteins (Nanjo et al., 1993) or liver (Byun et al., 1994). Also the isoflavonid, genistein, has been found to be a potent antioxidant in vivo in a study using phorbol ester induced mouse skin oxidation (Wei et al., 1993). No systematic structure-activity studies have been reported. We therefore investigated the ability of eight flavonoids to counteract the fried food mutagen-induced oxidative stress in rats as determined by the presence of MDA in plasma lipoproteins (Breinholt et al., 1998b). The heterocyclic amine, PhIP, induced a 40% increase in plasma lipoprotein MDA, and this effect was significantly counteracted by genistein > apigenin > beta-naphtoflavone(a model compound) > chrysin > quercetin. Since beta-naphtoflavone is not an antioxidant and chrysine is only weakly so, several mechanisms may be involved in this overall antioxidant activity. Based on our knowledge of uptake and biotransformation outlined above, the low activity of quercetin may be due to its limited bioavailability, whereas the monohydroxylated compounds, genistein and apigenin are better absorbed. The activity of chrysin may be due to its biotransformation to apigenin. It could be speculated, that hydroxylated metabolites of beta-naphtoquinone cause the apparent antioxidant activity, but since the compound is known to be a potent inhibitor of CYP enzymes it is more likely, that it inhibits PhIP-induced lipid oxidation by inhibiting PhIP-metabolism.
Analysis of the antioxidant status of the blood compartment in lycopene exposed animals (se above) revealed that three out of four antioxidant enzymes were affected by the lycopene treatment. The activity of superoxide dismutase was thus significantly induced at lycopene doses of 0.005 and 0.05 g/kg b.w, whereas glutathione reductase and glutathione peroxidase was only induced at the 0.005 g/kg b.w. dose. For all antioxidant enzymes investigated, the activities seemed to drop back to the control level after exerting peak induction at doses between 0.005 and 0.05 g/kg b.w. The explanation to this remains unknown. The ability of lycopene to inhibit oxidative stress induced by the heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP), was investigated by analyzing for malondialdehyde content (MDA) in plasma. Analysis of the MDA content in plasma revealed that none of the employed lycopene levels afforded significant protection against PhIP induced oxidative stress. Following PhIP exposure, however, a significant trend, toward lower MDA levels, with increasing lycopene doses, was observed. The level of PhIP-DNA adducts in liver or colon was likewise not affected by the lycopene treatment. Overall the present study provides evidence that lycopene administered in the diet, at doses relevant to human

exposure levels, exert modifying activities toward enzymes involved in the protection against oxidative stress but not cancer induced by the suspected human dietary carcinogen PhIP. Further analyses are underway to investigate this.

Human studies on disposition: In order to exert a protective effect on cancer and coronary heart disease it is reasonable to assume that the flavonoids have to be absorbed. There have only been published a few studies on absorption and disposition of flavonoids in humans, and most of these studies have been using unrealistically large doses of flavonoids compared to the average daily intake. As the flavonoid quercetin is present in a variety of fruits and vegetables, plasma concentration or urinary excretion of quercetin would have a potential use as biomarker of habitual intake of these foods. In order to evaluate this potential, information is needed on the dose-response and on the time-response relationships.
We have performed two human intervention studies with flavonoid containing foods. The first study was a pilot human cross-over intervention study with three doses of juice (750, 1000, and 1500 mL) for one week corresponding to an intake of 4.8, 6.4, and 9.6 mg quercetin per day consumed by five subjects (4 women, 1 man) in random order (Young et al., 1999).The main aim of this study was to investigate the dose-response relationship between intake and urinary excretion of quercetin when given daily at low doses. A secondary aim was to evaluate whether fruit juice intervention had an effect on markers of antioxidative status. A 1:1 mixture of apple juice and black currant juice was used as quercetin source. This study was the first one to investigate excretion of quercetin during a low-dose intake period of several days, in contrast to previous studies, that have been based on the administration of a single large dose. In the study, we found that urinary excretion of quercetin increased significantly with dose ($p<0.003$) and with time ($p<0.0001$). The fraction of intake excreted in urine was 0.29 - 0.47%, regardless of dose and without significant variation between the five subjects. The small fraction of quercetin intake that was excreted in urine is in accordance with results reported from single dose studies. Hollman et al. (1995) have found that urinary excretion was 0.31%, 0.07% and 0.12 % of the intake of 89 mg of quercetin from onions, 100 mg of rutinoside or pure quercetin aglycon, respectively, during 13 h after ingestion of the dose. In an earlier study, Gugler et al. failed to detect any urinary excretion after intake of 4 g of quercetin aglycone with a relatively insensitive analytical method (Gugler et al., 1975). We also observed, that after 3-4 days of intervention, the fraction of quercetin intake excreted into urine reached a steady state, indicating an elimination half-life of approximately 24 h. The results obtained on urinary quercetin excretion suggest, that urinary quercetin may be a useful biomarker of quercetin intake as it fulfils two key criteria for such a marker: a constant fraction excreted, independent of dose, and a non-significant interindividual variation in response. If further investigations confirm these results the quercetin content of 24 h urine samples could be used to estimate intake, despite the small fraction excreted through this route. Such a biomarker would be most valuable, for epidemiological studies in particular, as all calculations of intake are linked with a large error due to the limited information on the quercetin content of foods and to the substantial variation in the content of certain foods. In this study, we also observed, that plasma ascorbate increased during intervention due to the intake of 150, 200 and 300 mg ascorbate daily from the juice. Furthermore, total plasma malondialdehyde decreased with time during 1500 mL juice intervention ($p<0.05$), indicating a reduced lipid oxidation in plasma. Erythrocyte glutathione peroxidase activity increased with dose ($p<0.005$) whereas other antioxidant enzymes in erythrocytes (catalase, glutathione reductase, and superoxide dismutase) did not change significantly. Plasma protein 2-adipic semialdehyde (AAS) residues, increased with time ($p<0.005$) and dose ($p<0.05$), indicating a prooxidant effect of the juice, whereas erythrocyte AAS, trolox equivalent antioxidant capacity and ferric reducing ability of plasma did not change.

The decrease of total plasma malondialdehyde suggests an improvement in antioxidant status and would indicate that even low levels of flavonoids, ascorbate or other antioxidant constituents of the juice might decrease lipid oxidation within the plasma compartment. Therefore a direct antioxidant action of these constituents on plasma lipoproteins, even after only one week of intervention, could be speculated to be involved in a beneficial effect on heart disease. Due to the short period of intervention, the increase of erythrocyte glutathione peroxidase activity might be a chance finding but could also be due to the effect of flavonoids, ascorbate or other juice constituents. However, the strong prooxidant action of juice intervention on the oxidation of plasma protein lysine residues (AAS) seemed to be a clear effect of the juice intervention.
The results of the present study on the antioxidant enzymes, AAS and MDA illustrate that even within the blood there are several subcompartments which may respond differently to a dietary challenge. These results contradicts a general pro- or anti-oxidant state in the blood compartment but indicate a differential protection or damage to specific structures, depending on their interaction with the dietary components reaching them. However, the results on enzyme activities and other markers of antioxidant status need to be confirmed and extended in long term studies.

In the second human intervention study, the effect of consumption of parsley, containing high amounts of the flavone, apigenin, on the urinary excretion of apigenin and on biomarkers for oxidative stress was investigated (Nielsen et al., 1999b). Apigenin is a flavone found in vegetables, seasonings (Kühnau, 1976) and in oranges (Fernandez de Simon et al., 1992), and it possesses antioxidant activity in vitro (Fraga et al., 1987). Potent biological effects of this flavonoid have been described in vitro and in vivo. Apigenin has been ascribed anticarcinogenic (Wei et al., 1989), anti-inflammatory (Lee et al., 1993), and antimutagenic (Kuo et al., 1992) properties. Thus development of a biomarker for intake of apigenin is important in order to evaluate the potential health effects of this particular dietary component. There has only been one previously published attempt to determine the urinary excretion of apigenin in humans after ingestion of an apigenin containing camomile extract (Tschiersch and Holzl, 1993). However, due to lack of specificity and sensitivity the method Tschiersch and Holzl failed to detect any apigenin in the urine. Our intervention study was performed as a randomised cross-over trial in seven men and seven women. The subjects received a strictly controlled diet low on flavones and other naturally occurring antioxidants during the two weeks of intervention. This basic diet was supplemented with parsley providing 3.73-4.49 mg apigenin/MJ in one of the intervention weeks.
Urinary excretion of apigenin was significantly higher during intervention with parsley ($p<0.05$) than with basic diet. The potential metabolite of apigenin, acacetin (the 4'-methoxylated derivative of apigenin) was not determined in any of the urine samples. The average urinary excretion of apigenin within 24 h was 0.58% ± 0.16 (SEM) with an elimination half-life of about 12 hours, and was found to be similar in both groups of subjects. Significant inter-individual variation in the excretion of apigenin was observed and a minor fraction of the subjects could thus be classified as high excreters (0.8-4.0% apigenin excreted on average in 24 h), and the majority as low excreters (0.1-0.5%). The maximum excretion was found in a single individual with an excretion as high as 7.45% of the dose at day seven of the parsley intervention. Urinary excretion of apigenin might thus be a useful biomarker for apigenin uptake, since intake data do not necessarily reflect the systemic apigenin levels in different individuals.
The parsley intervention resulted in significant increases in the two antioxidant enzymes, glutathione reductase and superoxide dismutase. Furthermore, an overall decrease with time in the activity of all antioxidant enzymes during the total intervention regardless of diet was observed, and this could have been due to the low intake of flavonoids specificallyt or of fruits and green vegetables in general during the study.

To determine the concentration of flavonoids in the human urine samples from the two intervention studies described above, two highly sensitive HPLC methods were developed and validated (Nielsen and Dragsted, 1998a, Nielsen and Dragsted, 1998b). The sensitivity of the two methods developed were comparable with the sensitivity in urine of the method developed by (Hollman et al., 1996) using post-column derivatisation with aluminium nitrate and fluorescence detection. However, the application of UV-detection, using a diode-array detector, in these methods enables positive online identification of the flavonoids by their UV-spectra. Furthermore, these methods allow detection of other flavonoids than flavonols, which the post-column derivatisation method is limited to. The methodology developed using SPE and column switching may be used for the simultaneous detection of low levels of other flavonoids, than the ones investigated, by changing the time frames of column switching. Detection of quercetin, apigenin and acacetin were found to be accurate and reproducible, with limits of quantification of 5, 10 or 70 ng/ml urine, respectively. The methods were successfully applied to determine the levels of these flavonoids in more than 100 human urine samples from each of the human intervention studies.

4. Antioxidation and prevention of cancer in experimental animals.
A long-term animal study has been initiated in 1998 with the aim of studying the effects of natural antioxidants (lycopene, quercetin, resveratrol) on biomarkers of antioxidation and enzyme induction as well as on final cancer induction caused by co-feeding the food mutagen/carcinogen IQ. Clinical chemistry results have already revealed that both quercetin and lycopene can decrease and modulate early effects of IQ which are potentially related to pro-oxidant actions and subsequent carcinogenicity. In particular, the hepatotoxic actions of IQ were less pronounced in animals which were given both IQ and quercetin, lycopene, or both. Since the experiment has not been terminated yet, the final preventive effects on cancer cannot be described at this time.

c. Overview of milestones and results

A schematic overview of the milestones as described in the original application and the results obtained during the project period are shown below in table 4.

Table 4. Overview of milestones and results.
Project milestones Planned start Planned end Results
1.1. Evaluation of existing dietary intake data for flavonoids and carotenoids 1995 1996 Ready. Literature databases established and distributed to all partners. Two conference proceedings on Danish intakes of flavonoids and carotenoids have been published.

1.2. Report on carotenoids to the Nordic Council of Ministers 1995 1996 Ready. The activity was delayed 15 months due to a vacancy.a)
1.3. Establishment of analytical procedures for flavonoids and carotenoids. 1995 1996 Ready. One paper on flavonoid analyses has been published.
1.4. Establishment of analytical procedures for additional plant phenolics 1996 1997 Ready. Analyses for naringenin and hesperetin in foods have been established in stead of methods for catechin and epicatechin analyses.
1.5. Analysis of food items and calculation of Danish intakes. 1995 1997 Ready. The flavonoid analyses were terminated on schedule by the end of 1996 and have been published. Carotenoid analyses, where appropriate, were performed in 1997.b)

2.1. Determination of antioxidant effects of natural antioxidants in vitro 1995 1997 Ready. One paper on terpenoids, one on a carotenoid and two on flavonoids have been published.c)
2.2. Ph.D. study on antioxidant effects of carotenoids and flavonoids. 1995 1998 Ready. The Ph.D. report has been published. c)
2.3. Toxicity testing of 12 flavonoids and carotenoids in vitro 1995 1997 Ready. A paper on cytotoxicity of more than 20 polyphenols has been published.
2.4. Mutagenicity studies of 12 flavonoids and carotenoids 1996 1997 Changed. Only six intermediate and final oxidation products of flavonoids were tested. Milestone 2.6 has been substituted for this.d)
2.5. Testing of 12 flavo-noids and carotenoids for their effects on intercellular communication in vitro. 1995 1996 Finalised. The activity has been delayede), and there has been some technical difficulties. The results have been presented as a poster and as a published extended abstract.
2.6. Metabolism studies of flavonoids or carotenoids in vitro 1996 1997 Ready. Biotransformation studies with rat liver microsomes have been published. Two more publications are in preparation, one of them on human materials.f)
3.1. Metabolism and disposition of two compounds in vivo 1996 1998 Ready. A study on six flavonoids has been completed, and a publication has been submitted. A Ph.D. thesis has been published.g)
3.2. Antioxidant effects of 2 or more compounds in vivo 1996 1997 Ready. An experiment with six flavonoids has been published. g)
3.3. Biomarkers for uptake or excretion of two selected compounds 1997 1997 Ready. Analytical procedures for determination of urinary kaempferol, quercetin, and apigenin have been established and published.b)
3.4. Diet intervention study with flavonoids 1997 1998 Ready. Cross-over studies on quercetin in apple and blackcurrant juice and on apigenin in parsley have been accepted for publication.
3.5. Diet intervention study with carotenoid 1997 1998 A study has been performed in co-operation with Wageningen Agricultural University and a publication has been submitted.b)

4.1. Intervention against preneoplastic lesions in rat colon 1996 1997 A study is presently being conducted and will be terminated in 1999. It is going to include several early markers of oxidative damage and enzyme induction, preneoplastic lesions in liver and, finally colon as well as liver tumorigenesis.g)
4.2. Intervention study against tumours in transgenic animals 1996 1997 The activity will not be performed. The rat model mentioned above (4.1.) is more relevant than the available mouse models.
Summary evaluation of the use of flavonoids as antioxidants, including health and toxicity considerations 1998 1998 Several review papers have been invited from this group as a part of the procedings of symposia on antioxidants and health.
a) The collegue employed to write this report had a one year EU training grant to visit Dr. Hans Verhagens laboratory at TNO, Holland. In the mean time the literature database was completed. After his return, however, he got a fixed position before the report was finalised, causing further delay.
b) These activities are partly co-operative between the EU-FAIR programme and this Programme. In this way the activities are expanded, and the samples generated are used for more analyses.
c) The activity has been a little behind schedule due to a maternity leave for the post-doc involved, but was finalised in due time.
d) Due to abundant information on mutagenic effects of flavonoids and carotenoids we have put more emphasis on in vitro biotransformation studies.
e) Due to a delay caused by a change in staff at RUC, the research was not initiated in 1995, and the

grant for 1995 and part of the grant for 1996 will be transferred to 1997.
f) The use of microsomes for in vitro studies was not originally a milestone, so the activity has actually been expanded to compensate for the mutagenicity studies.
g) Animal experiments have been somewhat delayed due to rebuilding of the animal facilities at VFD.

d. Cooperative projects with industry

Except for VFD's participation in an information project co-ordinated by the private consultant company, Agri Contact, and some minor tests performed for MD Foods there have not been formal co-operative projects with industry as a part of this project. The present Programme was closely co-ordinated with the EU-FAIR project CT-0154 'Antioxidants in Food' where the use of antioxidants for stabilisation of foods were exploited in more detail together with major European food industries.
Two applications for grants to improve health qualities of fruit juices together with two Danish juice manufacturers have been submitted, a proposal on frozen vgetables has been submitted and a further application on frozen vegetables is underway in co-operation with a major Danish manufacturer. Prof. Leif Skibsted has had several co-operative projects on antioxidants related to the activities launched under FØTEK I, and he is now the project co-ordinator on a grant for research on antioxidation under FØTEK 3.

e. Staff and equipment in individual projects

The employed staff in person years (Py) and the limited amount of equipment bought under the programme are stated in Table 5 below:

Table 5. Staff and equipment under the Programme

| Project no. | Staffa) | Equipmentb) | Commentsc) |
|---|---|---|---|
| 1. | Diode-array detector (IFEE) | Research assistant (2 Py), paid by VFD and Nordic grant | |
| 2. | Post-doc (1 Py, KVL)<br>Research assistant (1 Py, RUC)<br>Ph.D. student (1.5 Py KVL, 1.5 Py VFD) | Robot for sample preparation (IFT) | |
| 3. | Post doc (1 Py, KVL)<br>Research assistant (3 Py, VFD)<br>Dietician (1 Py, KVL) | Research assistant (1 Py) and technician (1 Py) are paid by VFD. | |
| 4. | Technician (1.5 Py, VFD) | Research assistant (0.5 Py), paid by VFD | |

a) Person-years (Py) and institution in brackets. The Institutions are: KVL, Danish Veterinary University; VFD, National Food Agency of Denmark; RUC, Roskilde University.
b) Equipment bought under FØTEK 2.
c) Senior researchers are supporting the work under all projects at each of the five research institutes involved.

f. Ph.D. training under FØTEK 2.

Two employees (Lars Viborg Jørgensen and Salka E. Nielsen) have prepared their Ph.D. theses as a part of the present Programme. A journal club, meeting twice a month, was part of their educational programme.
At the meeting on 'Antioxidants and Health' arranged under the Programme, both students gave oral

presentations of their work, see below. Both have also presented their work at national and international scientific meetings.

g. Ongoing international co-operation, EU-projects and other projects
See section A.2.c and A.2.d above (page 3-4) for details. An overview can be seen in the figure below:

h. Economic overview
The planned distribution of the economic resources on the various projects, institutions and years are shown in appendix 4. For technical reasons the budget for VFD was split in two, 7751 being the main project and 7752 being one of the ph.d. projects. In practice we could not hold these ressources apart, and the excess on 7752 was transferred to 7751 in 1998.
There are some comments to be made regarding the deviations from the original budget of the Programme:
- Due to unforeseen problems during building reconstruction work at VFD, where animal facilities, mutagenicity laboratories and an isotope laboratory were under reconstruction from 1995-1997, we did not use the technician at VFD paid by the Programme in 1995. Also about 150.000 DKK were accumulated to pay for test substances and animals for the long-term study initiated in 1998. We have transferred most of these ressources to 1998, now that we can use the facilities again and now that the long-term study has been initiated.
- At LMC there has also been some initial delays causing transfer of ressources from 1995/6 to 1997/8. These were mainly related to delayed recruiting of staff for the project and to a maternity leave by the employed research assistant professor.
- Due to a delay caused by a change of staff at RUC, the research was not initiated in 1995, and the total grant for this year was transferred to 1996. Some of the money for 1996 were further transferred to 1997.

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# Appendix 1: Programme Committee and Steering Committee members: Organisational diagram for the Programme.

# Appendix 2: Projects, and project group members.

The four original projects (1-4 below) were not equally well suited for the practical organization of the work. Six task forces (I-VI below) were therefore organised for the practical work. Their numbering system is not completely logical since they actually cross the borders of the described projects. As the work carried on, the task forces have dynamically changed members. As an example, task force II and VI have united due to a close overlap of the involved participants, and task force I subsequently melted into this united group, because food analyses are a very important part of the human intervention studies and since the general food analysis and literature work was finalised.

1. Natural antioxidants (primarily flavonoids and carotenoids) in the diet.

Group I. Literature survey and food analysis.
Participants: Torben Leth (IFEE), Salka E. Nielsen (IFT)a), Michael Strube (IFT)a), Pia Knuthsen (IFEE), Ulla Justesen (IFEE), LOD (Lars O. Dragsted (IFT))

2. Antioxidant effects, (anti)mutagenic effects, and (anti)toxicity in vitro.

Group III. (anti)mutagenic and (anti)toxic effects.
Participants: Mona Lise Binderup (IFT), Eva Seltzer Rasmussen (IFT), Vibeke Breinholt(IFT), Gitte Winkel Svendsena), Ole Vang (RUC), Mette Nielsen (RUC)a), LOD.

Group IV. Antioxidation in vitro and in vivo.
Participants: Leif Skibsted (MLI), Gitte Winkel Svendsena), Vibeke Breinholt (IFT), Lars Viborg Jørgensen (MLI/IFT)a), Alan Mortensen (MLI), Helle Lindberg Madsen (MLI)a), Bahram Daneshvar (IFT)a), Søren Lauridsen (IFT), LOD.

3. Disposition and antioxidant effects in humans.

Group II + group VI (united): Biotransformation in vitro/in vivo and human intervention study.
Participants: Salka E. Nielsen (IFT)a), Brittmarie Sandström (FHE), Jette Young (FHE), Helle Lindberg Madsen (MLI/FHE)a), Pia Knuthsen (IFEE), Torben Leth (IFEE), LOD.

4. Antioxidation and prevention of cancer in experimental animals.

Group V. Aberrant crypts and tumorigenesis in rats.
Participants: Annemarie Mølck (IFT), Morten Poulsen (IFT), Ole Vang (RUC), Vibeke Breinholt (IFT), Otto Meyer (IFT), LOD.

a) Researchers, whose salaries are paid wholly or partly by the FØTEK 2 grant to this Programme.

# Appendix 3: Papers, presentations and information activities.

A. Peer reviewed papers, book chapters, reports, etc.

Breinholt, V. and Dragsted, L.O. (1998) Structure-cytotoxicity relationships for dietary flavonoids.

In Vitro & Molecular Toxicology, 11(2), 193-206.

Dragsted L.O. (1996) Compounds in plants inducing detoxifying and antioxidative enzymes. Royal Society of Chemistry Special Publications, 181, 365-372.

Dragsted L.O., Knuthsen P., Nielsen S.E., Strube M., and Justesen U. (1996) Polyphenols in Danish foods and their possible health effects. In: W. Pfannhauser, Ed.: Proceedings of the Symposium on polyphenols and anthocyanins as food colorants and antioxidants. FLAIR-FLOW Europe, Brussels.

Dragsted,L.O. (1997) Natural Antioxidants in Chemoprevention. Arch. Toxicol. Suppl.20, 209-226.

Dragsted,L.O. (1997) Cancer Risk Assessment and the EPA Guidelines. Hum. Ecol. Risk Assess., 3(4), 501-505.

Dragsted L.O., Nielsen, S.E., Kall, M., and Young, J. (1998) Polyphenolic antioxidants: Biotransformation and human excretion. Proceedings of the EU-FAIR (COST916) workshop, Bioactive Cell Wall Components in Nutrition and Health - Polyphenols in Food, European Commission, pp. 91-96.

Dragsted,L.O., Strube,M. and Leth,T. (1997) Dietary levels of plant phenols and other non-nutritive components: Could they prevent cancer? Eur. J. Cancer Prevent., 6 522-528.

Dragsted, L.O. (1999) Intakes and modes of action of dietary anticarcinogenic compounds. Royal Society of Chemistry Special Publications, accepted.

Justesen, U., Knuthsen, P., Leth, T. (1997) Determination of plant polyphenols in Danish foodstuffs by HPLC-UV and LC-MS detection. Cancer Lett. 114, 165-167.

Justesen, U. (1998) HPLC-MS analysis of Flavonoids in Foods. Adv. Mass Spectr. 14, Submitted.

Justesen, U., Knuthsen, P., Leth, T. (1998) Estimation of the mean daily intake of flavones, flavanols and flavanones in Denmark. Submitted

Justesen,U.,Knuthsen,P. and Leth,T. (1998) Quantitative analysis of flavonols, flavones, and flavanones in fruits, vegetables, and beverages by HPLC with photodiodearray- and mass spectrometric detection. J. Chromatogr. A, 799, 101-110.

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Jørgensen L.V., Andersen H.J., and Skibsted, L.H. (1996) Kinetics of reduction of hypervalent iron in myoglobin by crocin in aqueous solution. Free Rad. Res. 27, 73-87.

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339-350.

Jørgensen, L.V., Madsen, H.L., Thomsen, M.K., Dragsted, L.O. and Skibsted, L.H. (1998b) Regeneration of phenolic antioxidants from phenoxyl radicals. An ESR and electrochemical study of antioxidant hierarchy. Free Radical Research, In press..

Leth,T., Justesen,U. (1997) Analysis of flavonoids in fruits, vegetable, and beverages by HPLC-UV and LC-MS and estimation of the total daily flavonoid intake in Denmark. Proceedings of the 1st workshop on Polyphenols in Food: Bioactive plant cell wall components in nutrition and Health (COST 916). European Commission.

Madsen, H.L., Bertelsen, G., Skibsted, L.H. (1996). Antioxidative activity of spices and spice extracts. Proc. A.C.S. National Spring Meeting (24-28/3 1996).

Møller, J.K.S., Madsen, H.L., and Skibsted, L.H. (1998) Dittany (Origanum dictamnus L.) as a source of water extractable antioxidants. Z. Lebensmittel Untersuch. Forsch., accepted

Nielsen,S.E., Kall,M., Justesen,U., Schou,A., Dragsted,L.O. (1997) Human absorption and excretion of flavonoids after broccoli consumption. Cancer Letters 114, 173-174.

Nielsen, S.E. (1998) Metabolism and biomarker studies of dietary flavonoids. Ph.D. Thesis.

Nielsen, S.E., Breinholt, V., Justesen, U., Cornett, C., and Dragsted, L.O. (1998) In vitro biotransformation of flavonoids by rat liver microsomes. Xenobiotica, 28(4), 389-401.

Nielsen,S.E. and Dragsted,L.O. (1998a) A column switching high-performance liquid chromatographic assay for determination of quercetin in human urine with ultraviolet absorbance detection. J. Chromatograhpy B, 707, 81-89.

Nielsen,S.E. and Dragsted,L.O. (1998b) A column switching high-performance liquid chromatographic assay for determination of apigenin and acacetin in human urine with ultraviolet absorbance detection. J. Chromatograhpy B, 713, 379-386.

Nielsen, S.E., Breinholt, V., Cornett, C., and Dragsted, L.O. (1999a) Biotransformation of dietary flavonoids in female rats and identification of metabolites with intact flavane nucleus. Submitted

Nielsen, S.E., Young, J.F., Daneshvar, B., Lauridsen, S.T., Knuthsen, P., Sandström, B., and Dragsted, L.O. (1999b) Effect of parsley intake on urinary apigenin excretion, blood antioxidant enzymes and on biomarkers for oxidative stress in humans. Br. J. Nutr., accepted.

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Strube,M. and Dragsted,L.O. (1998) Naturally Occurring Antitumourigens IV. Carotenoids, except ß-carotene. TemaNord Food series, Nordic Council of Ministers, Copenhagen, Denmark, In press.

Young, J.F., Nielsen, S.E., Háraldsdottir, J., Daneshvar, B., Lauridsen, S.T., Knuthsen, P., Crozier, A., Sandström, B., and Dragsted, L.O. (1999) Effect of Fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status. Amer. J. Nutr., accepted.

B.Informative outputs to Food Industry etc.

'Antioxidanter fra Planter' a folder (in Danish), describing the present programme, opportunities for co-operative projects, contact points, etc.

'Sundhedsmæssig vurdering af biologisk aktive non-nutritive indholdsstoffer i nye levnedsmidler: Antioxidanter fra planter', a 6-page description (in Danish) of the present Programme, including the individual projects.

Dragsted, L.O.: Ny forskning i antioxidanter, LST-nyt 1. March, 1995.

Dragsted, L.O.: Toksikologiske aspekter af functional foods, Diætisten 29(5), 13-14, 1997.

C. Scientific meetings
Ressources from the present Programme have been used in part to arrange the following meetings:

Natural antioxidants and health. Open meeting held 24/4-1996 at National Food Agency of Denmark, Søborg: 60 participants, 10 oral presentations, 6 posters.

International Symposium: Micronutrients and human cancer risk, Århus 21-24/5 1997.

Mini-symposium, "Flavonoid analysis, absorption, and biotransformation", Levnedsmiddelstyrelsen, 25/6-1997.

D. Oral and poster presentations including published abstracts

Dragsted L.O. (1995) Compounds in plants inducing detoxifying and antioxidative enzymes. Invited lecture at the QSFNE conference (Quality and Safety Aspects of Food & Nutrition in Europe), Helsinki, Finland.

Ulla Justesen, Pia Knuthsen, Torben Leth (1996) Determination of plant polyphenols in danish foodstuffs by HPLC-UV and LC-MS. Poster at: Food and Cancer Prevention II, Ede, Holland

Nielsen, S.E., Breinholt, V., Justesen, U., Dragsted, L.O., and Cornett, C. (1996) 'Metabolisme af flavonoider i rottelevermicrosomer'. Oral presentation (in Danish), Forskningens Dag, Royal School of Pharmacy.

Nielsen, S.E., Breinholt, V., Justesen, U., Dragsted, L.O., and Cornett, C. (1996) 'Metabolisme af flavonoider i rottelevermicrosomer'. Poster presentation at the fourth Danish Symposium in Analytical Chemistry, Copenhagen.

Nielsen, S.E., og Dragsted, L.O. (1996). Human absorption and excretion of kaempferol after broccoli consumption. Poster at : Food and Cancer Prevention II, Ede, Holland.

Dragsted, L.O. (1996). Polyphenols in Danish foods and their possible health effects. Invited lecture at the Proceedings of the Symposium on polyphenols and anthocyanins as food colorants and antioxidants. University of Vienna, 15/11-1996.

Dragsted, L.O. (1996) Flavonoider, förekomst, toxikologi och hälsommässiga aspekter. Oral presentation (in Swedish) to the Swedish Society of Nutrition, Lunds hospital, 24/4-1996.

Dragsted, L.O. Flavonoids, health and disease. Invited presentation at the COST916 conference, "Biologically active cell wall components", Aberdeen, Scotland 18. april 1997.

Dragsted, L.O. Dietary Levels of Plant Phenols and other Non-Nutrive Components: Could they Prevent Cancer? Invited lecture at "Micronutrients and Human Cancer Risk" in Århus, Denmark, 22/5-97. Abstract publ. in Eur. J. Cancer Prev., 6, 490.

Breinholt V., Lauridsen S., Nielsen A., Dragsted L.O. (1997) Differential effects of dietary flavonoids on drug metabolizing and antioxidant enzymes. Oral presentation at "Micronutrients and Cancer Risk" in Århus, Denmark, 22/5-97. Abstract publ. in Eur. J. Cancer Prev., 6, 499.

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# Appendix 4: Economic overview (budget and expenditures).

LST 7751 LST
7752 RUC LMC I alt
Budget
1995 1,332,600 11,040 98,640 238,406 1,785,686
1996 786,600 203,400 285,178 852,305 2,127,483
1997 704,568 221,400 - 735,464 1,661,432
1998 92,002 234,768 - 98,630 425,400
Budget total 2,915,770 775,608 383,818 1,924,805 6,000,001

Expenditures
1995 1,177,193 99,471 - 200,438 1,477,102
1996 786,567 164,362 353,178 559,546 1,863,653
1997 655,735 151,979 30,640 839,905 1,678,259

1998 519,335 160,762 - 325,916 1,006,013
Total 3,138,830 576,574 383,818 1,925,805 6,025,027

Advance payments

1995 1,349,489 99,471 98,640 238,400 1,786,000
1996 826,155 164,362 285,178 852,305 2,128,000
1997 773,557 151,979 735,464 1,661,000
1998 164,602 160,762 99,636 425,000
Total 3,113,803 576,574 383,818 1,925,805 6,000,000

Difference: Budget - expenses:

1995 155,407 16,569 98,640 37,968 308,584
1996 33 39,038 (68,000) 292,759 263,830
1997 48,833 69,421 (30,640) (104,441) (16,827)
1998 (427,333) 74,006 - (227,286) (580,613)
Total (223,060) 199,034 - (1,000) (25,026)

Difference: Payments - expenses:

1995 172,296 - 98,640 37,962 308,898
1996 39,588 - (68,000) 292,759 264,347
1997 117,822 - (30,640) (104,441) (17,259)
1998 (354,733) - - (226,280) (581,013)
(25,027) - - - (25,027)