Evaluation of Plant Product's Functionalities

Dr. Rong Cao
Food Research Program
Agriculturure & Agri-Food Canada
Guelph, Ontario, CANADA

September 12-13, 2005
Suranaree University of Technology
Nakhon Ratchasima, THAILAND

School of Food Technology, Suranaree University of Technology

Hosting a seminar series on

Evaluation of Plant Product Functionalities

12-13 September 2005

Meeting Room, Surasummanakarn, Suranaree University of Technology

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12 September 2005 (09:00-16:00 h)

Registration: 08:30 - 09:00 h.

1. natural sources of nutraceuticals and functional foods

2. nutraceutical chemistry

coffee break

3. nutraceutical biochemistry

4. discussion

lunch break

5. development of nutraceuticals and functional foods

coffee break

6. natural health products

7. discussion

13 September 2005 (08:30-11:30 h.)

Registration: 08:15 - 08:30 h.

1. natural sources of biopreservatives

2. chemistry and biochemistry of biopreservatives

coffee break

3. potential applications of bio-preservatives in the food industry

4 discussion

Invited Expert : Dr . Rong Cao

Food Research Program, Agriculture & Agri-Food Canada

Guelph, Ontario

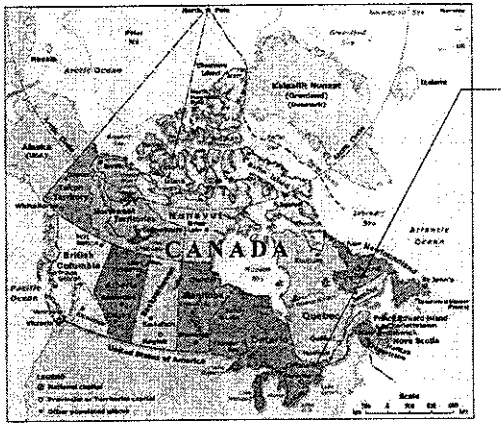
Canada

Nutraceuticals, Functional Foods and Natural Health Products

Rong Cao (R. Tsao)

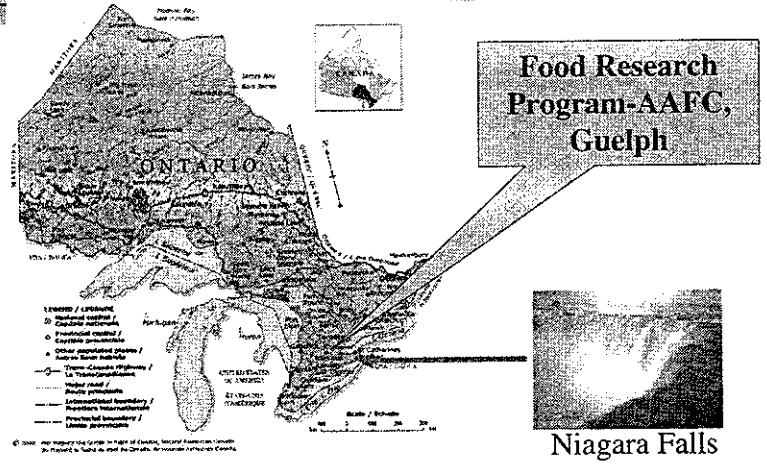
Food Research Program,
Agriculture & Agri-Good Canada,
Guelph, Ontario, Canada

Food Research Centre-AAFC



Guelph, Ontario

Food Research Program-AAFC, Guelph

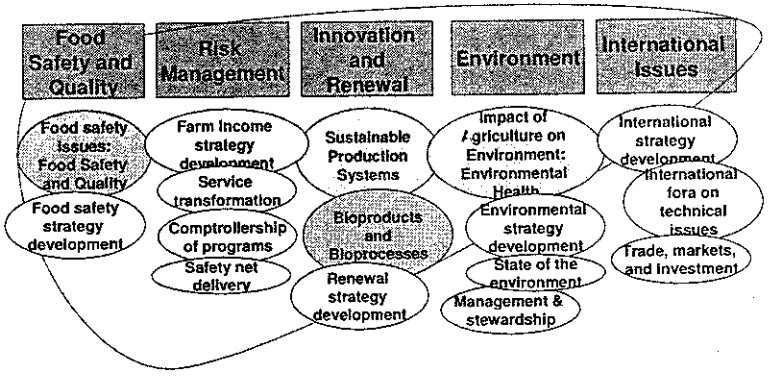


Food Research Program-AAFC, Guelph

Niagara Falls

New National Focus on Science

Linking science and policy across AAFC



4 National Programs

Food safety and quality	Sustainable production systems	Bioproducts and bioprocesses	Environmental health
Themes ✓Safety and Processing ✓Food Quality and Nutrition	*Themes* ✓ Cultivars ✓ Grains & Oilseeds ✓ Field Crops ✓High Value Crops ✓ Livestock ✓Animal behavior	*Themes* ✓Specialty Biobased products and processes ✓Genomics, bioinformatics ...	*Themes* ✓Soil ✓Water ✓Air ✓Biodiversity ✓Nutrients and organic residues ✓Integrated pest *management*

NFF-NHP and Nutrition

- Nutraceuticals, Functional Foods and Natural Health Products
- Falls into Two National Priorities
  - BioProducts & BioProcesses
  - Food Safety & Food Quality

Food Research at Guelph

- Four areas of research
  - Food safety
  - Food preservation
  - Food Functionality
  - Nutraceuticals and Functional Foods

Outlines

- Definitions
- Background
- NFF Chemistry
- NFF Biochemistry
- Examples

The Hype!

Major Driving Forces

1. **Resent studies have shown that certain diseases are preventable through diet.**
2. **More consumers have better understanding of the link between diet and disease.**
3. **Population is aging and becoming more concerned about health**
4. **Costs of health care are rising**
5. **More consumers are interested in preventing illness than in treating or curing**
6. **Advances in food science & technology continue.**

Top Causes of Death

In US & Canada

- **Heart disease**
- **Cancer**
- **Stroke**
- **Diabetes**
- **Arteriosclerosis**

Death Links with Diet/Lifestyle

- Studies have associated dietary factors or secondary lifestyle with:
  - 20-35% of cardiovascular deaths,
  - 20-60% of fatal cancers,
  - 50-80% of diabetes mellitus cases,
  - 30% diabetes deaths

Costs of Major Illnesses*

Cardiovascular diseases	$ 7.4 billion
Digestive diseases	$ 3.4 billion
Cancer	$ 3.3 billion
Musculoskeletal diseases	$ 2.5 billion
Diabetes	$ 0.6 billion
Birth defects	$ 0.3 billion
Total	**$ 17.5 billion**

*Annual, in Canada. *Source.* Health Canada, Economic burden of illness in Canada 1997

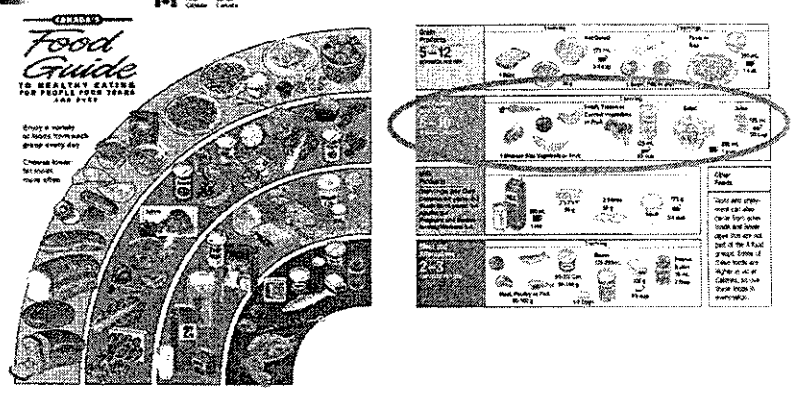
Health Claims Allowed by US-FDA

- Calcium and osteoporosis
- Sodium and high blood pressure
- Potassium and high blood pressure
- Plant stanol and sterol esters and coronary heart disease
- Dietary fat and cancer
- Dietary saturated fat and cholesterol and coronary heart disease
- Soy protein and coronary heart disease
- Fiber-containing grain products, fruits, and vegetables and cancer
- **Fruits, vegetables** and grain products that contain fiber, particularly soluble fiber, and **coronary heart disease**
- **Fruits and vegetables** and **cancer**
- Folate and neural tube birth defects
- Dietary sugar alcohols (sorbitol) and dental cavities
- Dietary soluble fiber, such as that found in whole oats and psyllium seed husk, and coronary heart disease

Health Claims-Canada

- sodium and hypertension
- calcium and osteoporosis
- saturated and *trans*- fat and cholesterol and coronary heart disease
- **Fruits and vegetable and Cancer**
- sugar alcohols and dental caries was apparent.

Canada's Food Guide for Healthy Eating



http://www.hc-sc.gc.ca/hpfb-dgpsa/onpp-bppn/food_guide_rainbow_e.html

Traditional Concept of Food

- Daily dietary intake must provide adequate quantities of certain **essential nutrients** needed to maintain optimum health.

Nutraceutical/Natural Health Product

- Is a product isolated or purified from foods/plants that is generally sold **in medicinal forms (e.g. pills, capsules)** not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease

Functional Foods

- **Similar in appearance** to, or may be, a conventional food, is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce risk of chronic disease beyond basic nutritional functions.

Neither Drugs Nor Regular Foods

- Regulatory agencies are caught between the lack of explicit guidelines and consumers demand for availability of such compounds with full approval for safety of such products.

Claims

- Approval of a claim depends on the type of claims being sought. There are three major types of claims based on
  - nutrient content
  - structure function
  - health benefit

The Nutrient Content Claim

- The nutrient content claim, which makes claim on the content of a particular nutrient of a value, is the least controversial. e.g. This spinach juice contains high concentration of lutein.

The Structure-Function Claim

- The structure -function claim promotes the role of a nutrient for a specific biological and physiological process. e.g. daily consumption of 6.5 g of soy protein for maintaining and reducing cholesterol level. Several countries have legislation in place to grant such claims.

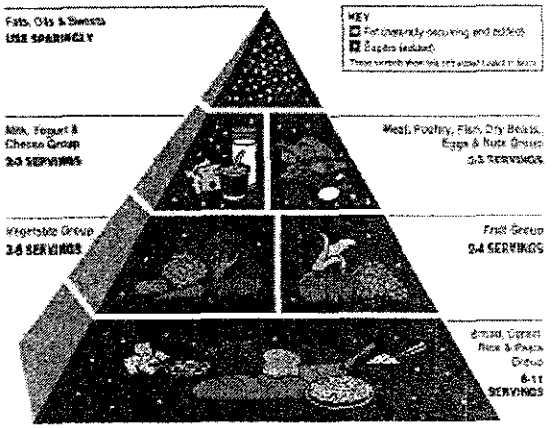
The Health Claim

- The health claim specifies the role of a nutrient, food products etc. in preventing, treatment and curing a disease and is highly controversial. Such a claim falls into the category of a drug.

Food and Health

- You are what you eat
- Traditional Chinese Concept:
  - Foods and medicines share the same origin

Food Pyramid



Food Pyramid promotes low fat diets rich in: fiber, grain products fruits and vegetables

Links with diet/lifestyle

- Studies have associated dietary factors or secondary lifestyle with 20-35% of cardiovascular deaths, 20-60% of fatal cancers, and 50-80% of diabetes mellitus cases, and 30% diabetes deaths

Diseases Most Affected by Diet

- Atherosclerosis & cardiovascular diseases
- Cancers
- Hypertension
- Diabetes (type II)
- Intestinal conditions
- Coronary heart diseases
- Stroke
- Osteoporosis

Major Markets for NFF

- Europe $15.0- 20.0 billion
- US $10.5- 37.0 billion
- Japan $10.5-14.0 billion
- Canada $ 1.0- 2.0 billion

- Other major markets:
  China, Hong Kong, Taiwan, Singapore, Thailand, Malaysia

Classification of Nutraceuticals

- By food source
- By mechanism of action
- By chemical nature

Food source-examples

- Plants
  - Ascorbic acid
  - Lutein
  - Daidzein
  - Capsaicin
  - Lycopene
  - Quercetin
- Animals
  - CLA
  - DHA
  - Ubiquinone (coenzyme Q10)
  - lecithin
- Microbial
  - Bifidobacterium bifidum
  - Saccharomyces boulardii (yeast)
  - Lactobacillus acidophilus (LCI)

Foods and Specific Nutraceutical Contents

Allyl sulfur compounds	Onion, garlic
Isoflavones	Soybeans and other legumes
capscicinoids	peppers
Lycopene	Tomatoes & products, water melon
Isothiocyanates	crucifers
Resveratrol	Grapes and wines
Catechins	Tea & berries
Anthocyanins	Red grape, berries, their juices & wines
Lutein/zeaxanthin	Squashes, corn, spinach
Quercetin	Onion, grapes, fruits broccoli

Mechanisms-examples

Anticancer	Cholesterol lowering	Antioxidant	Anti-inflammatory	Bone protection
genestein	B-glucan	Polyphenols	EPA	CLA
CLA	quercetin	Lutein	Capscicin	Soy protein
Tocopherols	Resveratrol	Ellagic acid	Curcumin	genestein
lutein	saponins	Chlorogenic acid	quercetin	calcium

Chemical Nature

- Isoprenoid derivatives
- Phenolic substances
- Fatty acids and structural lipids
- Carbohydrates and derivatives
- Amino acid-based substances
- Microbes
- Minerals

Mechanisms-examples

Isoprenoids (terpenoids)	Phenolics	Protein/ Amino acids	carbohy drates	Fatty acids etc	minerals	microbials
Carotenoids	Coumarines	Amino acids	Ascorbic aci	n-3 PUFA	Ca	probiotics
saponins	Tannins	Allyl-S compounds	aligosacc harides	CLA	Se	prebiotics
Tocotrienols	Lignin	Capsaisinoids	Non-starch PS	MUFA	Zn	
tocopherols	Anthocyanins	Isothiocyanates		sphingolipids		
Simple terpenes	Isoflavones	Indols		lecithin		
	Flavonones	Folate				
	flavonols	choline				

Nutraceuticals Chemistry

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What does it cover?

- Natural products chemistry
- Phytochemistry
- Nutritional chemistry
  - Absorption
  - Metabolism
- Antioxidants chemistry
- Toxicology / biochemistry

Antioxidants Chemistry

- What is an antioxidant?
- Types of antioxidants
  - By function
    - Primary
    - Secondary
  - By source
    - Plants (fruits, vegetables, herbs..)
    - Animals (fish, CLA...)
  - By chemical group
    - Phenolics (phenolic acids, flavonoids, isoflavones etc)
    - Carotenoids
    - Vitamins
    - Minerals (Se etc)

Antioxidants Chemistry Cont.

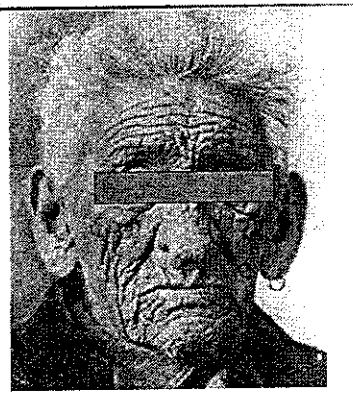
- Mechanisms of antioxidants
  - Free radical scavenging
  - Metal chelating
  - Enzymatic
  - Etc.
- Free radicals
  - What are they?
  - Why are they harmful?
  - Why do we need antioxidants?

Oxidation

- Oxidation is the incorporation of oxygen into a substance. But more precisely it is defined as the conversion of a chemical substance into another having fewer electrons; therefore, it is the loss of one or more electrons to another substance.

Larson, R. A., Naturally Occurring Antioxidants, 1997.

Natural Oxidative Processes



Wrinkles/Aging

Rust

Antioxidants

- Antioxidants are substances that when present in foods or in the body at low concentrations compared with that of an oxidizable substrate markedly delay or prevent the oxidation of that substrate.

Halliwell et al. *Food Chem. Toxicol.*, 33, 601, 1995

Our Body's Defense

Oxidative Stress (Free Radicals)

Endogenous — Exogenous

Defense System (Antioxidants)

Enzymatic — Nonenzymatic

Free Radicals

- Any chemical substances in which one (or, uncommonly, more than one) electron is unpaired or alone in its orbital.

Larson, R. A., Naturally Occurring Antioxidants, 1997.

Free Radical Chain Reactions

Chain Initiation $In\cdot + RH \longrightarrow R\cdot + InH$

Chain Propagation $R\cdot + O_2 \longrightarrow ROO\cdot$

$ROO\cdot + R'H \longrightarrow ROOH + R'\cdot$

Chain Termination

$ROO\cdot + ROO\cdot$

$ROO\cdot + R'\cdot$

$R\cdot + R'\cdot$

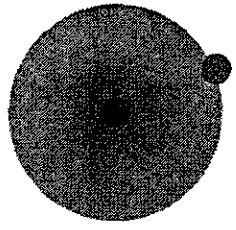
$R\cdot + R'\cdot$

$R\cdot + R'\cdot$

} Non-radical products

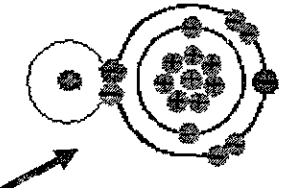
What are Free Radicals?

Molecules with a lone or unpaired electron

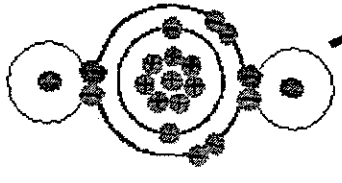


Free Radicals are not Stable

Hydroxyl radical



$H\text{-}O^{\bullet}$



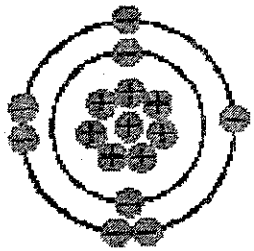
$H\text{-}O\text{-}H$ (H_2O, water)

Free Radical Chain Reaction

$A^{\bullet} + B \Rightarrow A + B^{\bullet}$

$B^{\bullet} + C \Rightarrow B + C^{\bullet}$

Reactive Oxygen Species



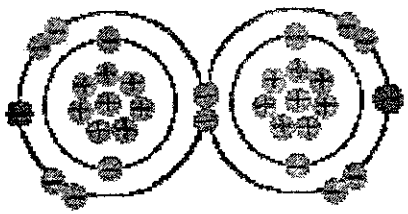
Inner shell
(2 electrons)

Outer shell
(6 electrons)

8 Protons

Oxygen Atom

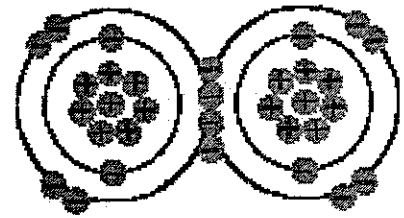
Singlet Oxygen



•O—O•

2 Unpaired electrons
Di-radical

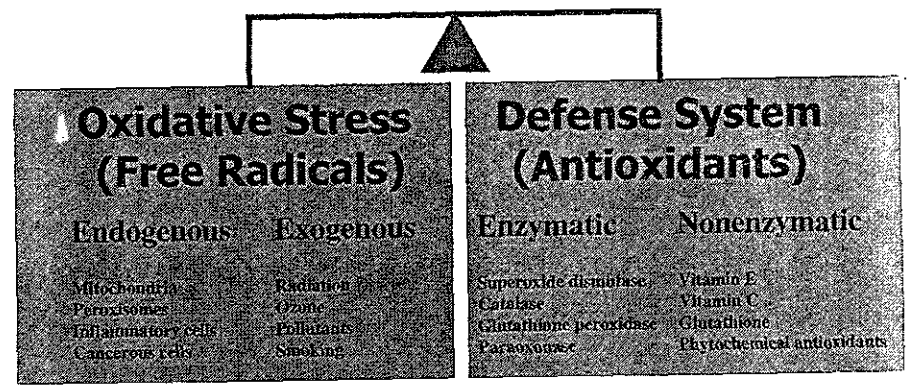
Oxygen Molecule (O_2)



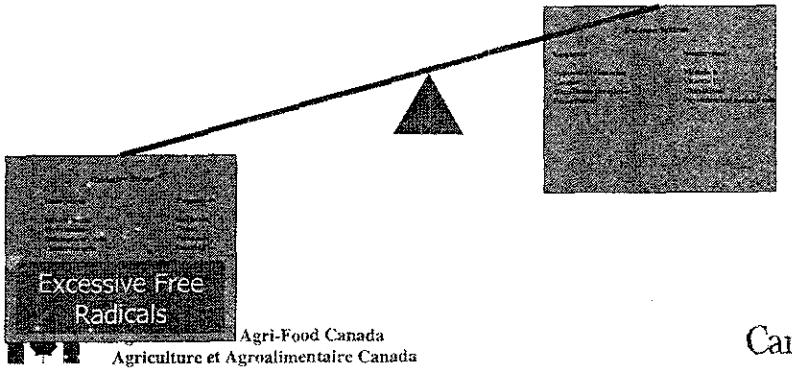
O=O

Filled outer shell
(triplet oxygen, inert)

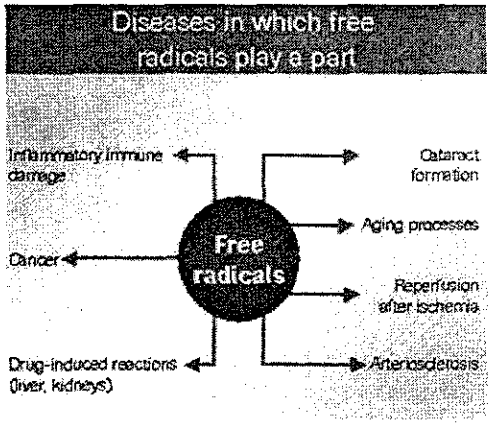
Our Body's Defense



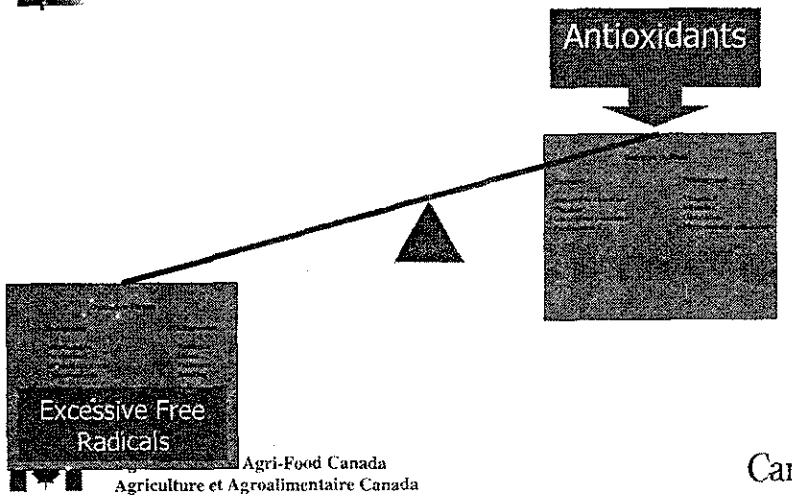
Excessive Free Radicals Brake the Balance



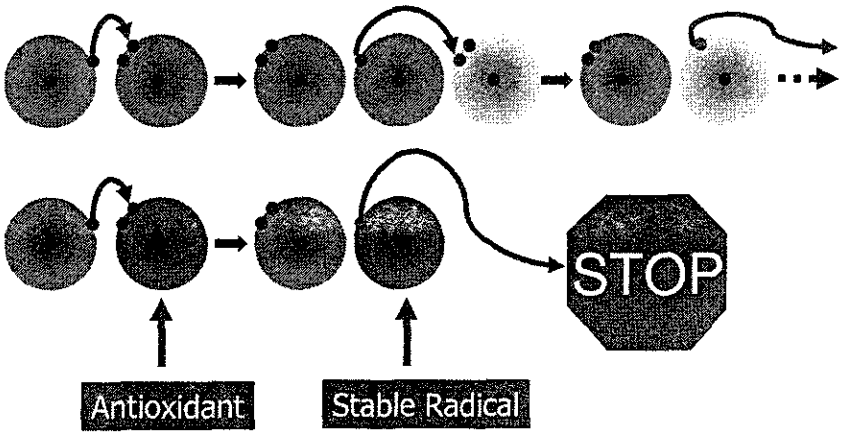
The Unbalance Causes Illnesses and Diseases



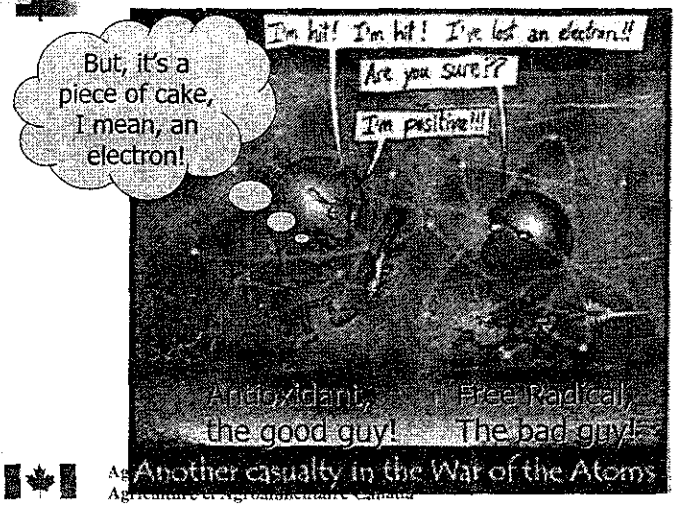
Antioxidants Restore the Balance



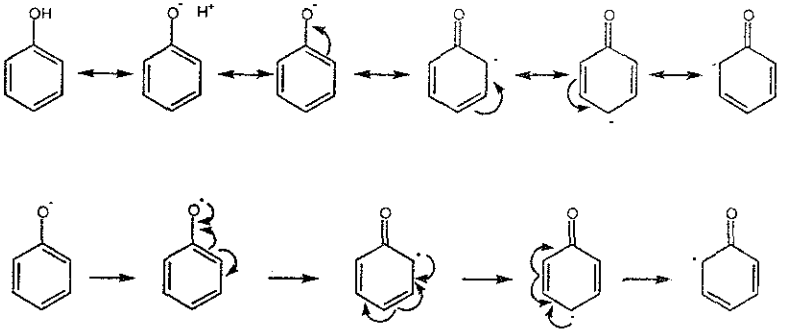
Antioxidants Stop the Chain



Battle between the GOOD & the BAD



Delocolization of Phenolics



Free Radical Damages

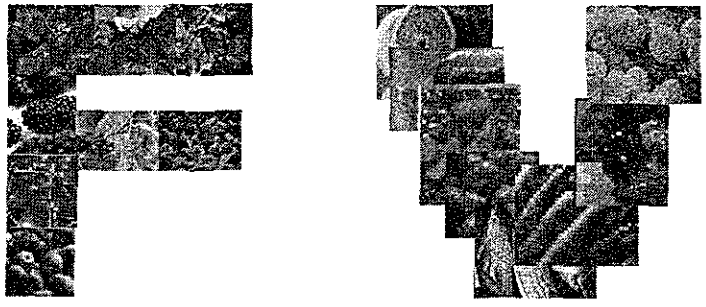
- **Lipid peroxidation**—cause fat to turn rancid and release more free radicals.
- **Cross-linking**—cause proteins and/or DNA to fuse together.
- **Membrane damage**—destroy the integrity of the cell membrane, thus affect nutrients intake and wastes discharge.
- **Lysosome damage**—rupture lysosome cell membranes; these then spill into the cell and digest critical cell compounds.
- **Accumulation of the age pigment** (lipofuscin), which may interfere with cell chemistry.

The Nutrition SuperLook, The Antioxidants, ed. by J. Barilla, M.S., Keats Publishing, Inc., 1995.

The Imbalance Causes Illnesses and Diseases

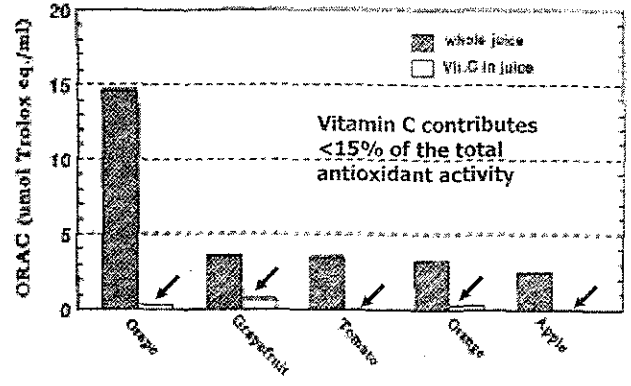
- Aging
- Arteriosclerosis
- Cardiovascular diseases
- Cancer
- Diabetes
- Inflammatory immune damage
- Cataract formation

Phytochemical Antioxidants



Phytochemicals v.s. Vitamin C

Wang et al., J. Agric. Food Chem. 1996, 44, 701-705



Phenolics

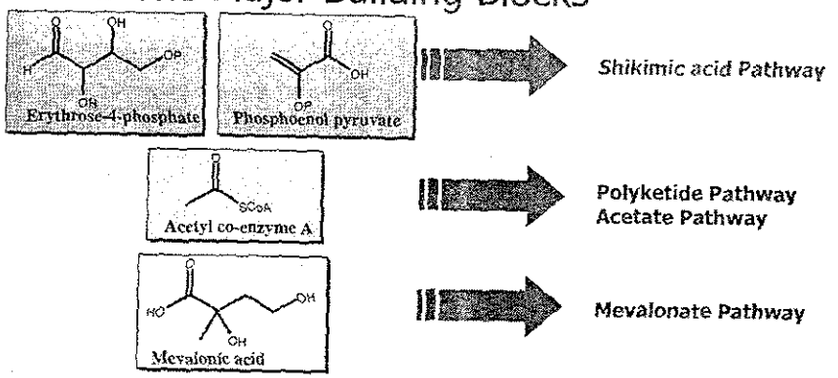
- Phenolic acids
- Lignans
- Coumarins
- Stilbenes
- Flavonoids
- Isoflavonoids
- Anthocyanidins

Phenolics

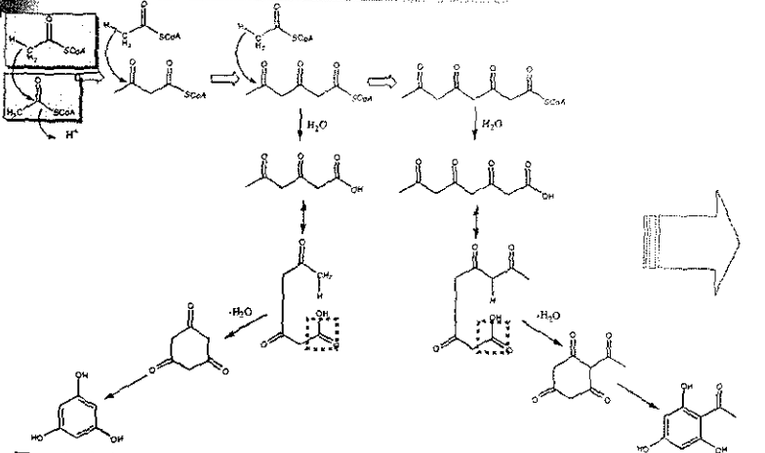
- Phenols
  - Gallic and procatechuic acids
  - Gallo- and ellagitannins
- Phenylpropanoid
- Polyphenols

Biosynthesis of Phenolics

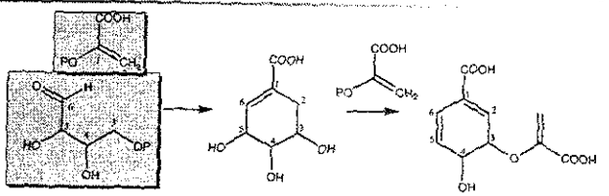
- The Major Building Blocks

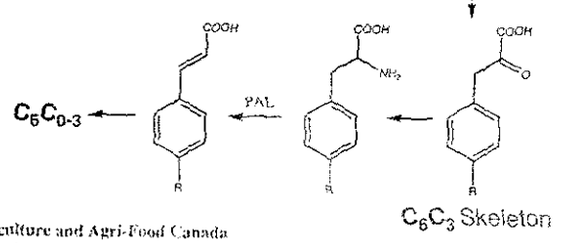


The Polyketide Pathway

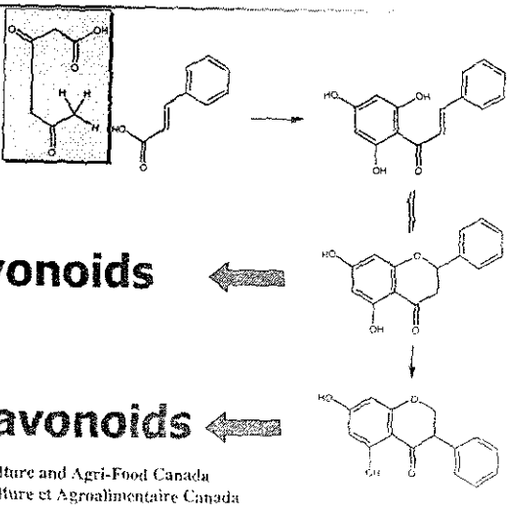


The Shikimic Acid Pathway

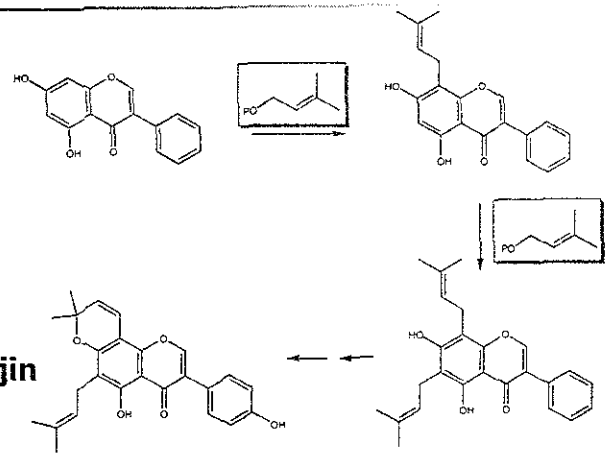




Combination of Polyketide & Shikimate Precursors

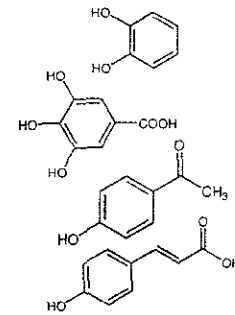


Addition of the Mevalonic Pathway



Classes of Phenolics

- Single Ring Phenolics
  - C_6C_0
  - C_6C_1
  - C_6C_2
  - C_6C_3



Catechol

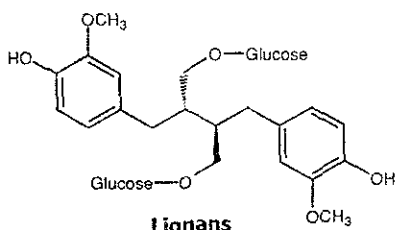
Gallic acid

p-OH-Phenylacetic acid

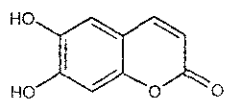
p-OH-cinnamic acid

C_6C_3

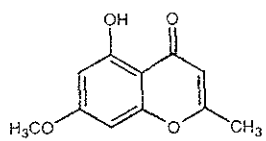
- Lignans (C_6C_3 dimers)
- Coumarins
- Chromones



Lignans
e.g. Secoisolariciresinol diglucoside



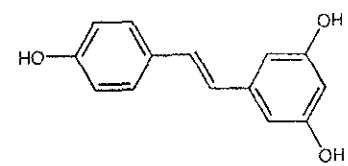
Coumarins
e.g. Aesculetin



Chromones
e.g. Eugenin

$C_6C_{0\text{-}2}C_6$

- Resveratrol



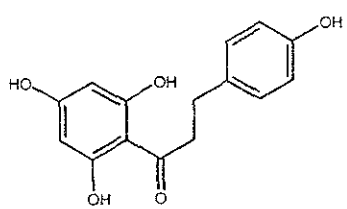
$C_6C_3C_6$

- Flavonoids
- Neoflavonoids
- Isoflavonoids

Flavonoids

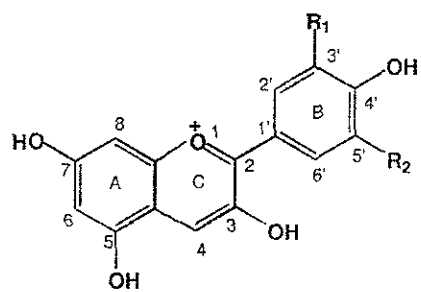
- Chalcones
- Anthocyanidins
- Flavanones
- Flavanols (Catechins)
- Flavones
- Flavonols

Chalcones



Phloretin

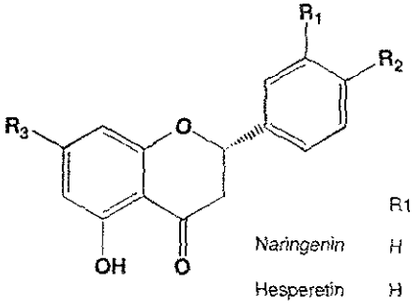
Anthocyanidins



	R1	R2
Pelargonidin	H	H
Cyanidin	H	OH

Anthocyanidins

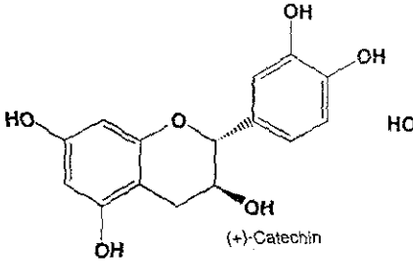
Flavanones

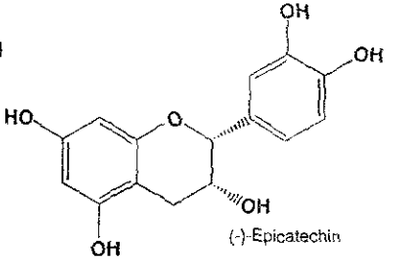


	R1	R2	R3
Naringenin	*H*	*OH*	*OH*
Hesperetin	H	OMe	OH

Flavanones

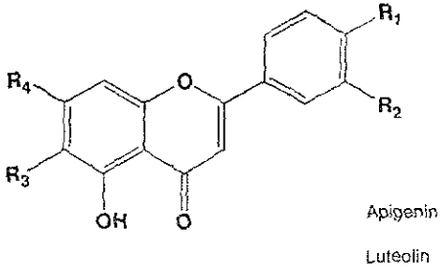
Flavanols





Flavanols (Catechins)

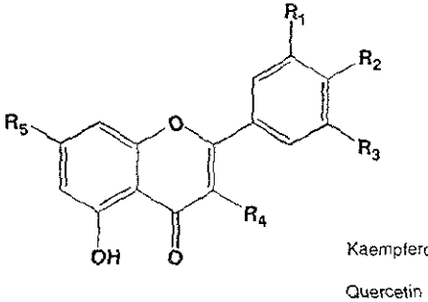
Flavones



	R1	R2	R3	R4
Apigenin	OH	H	H	OH
Luteolin	OH	OH	H	OH

Flavones

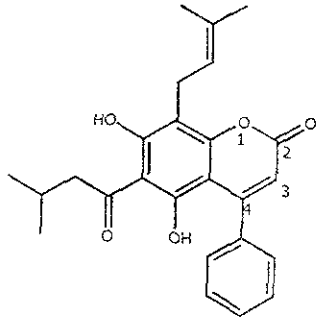
Flavonols



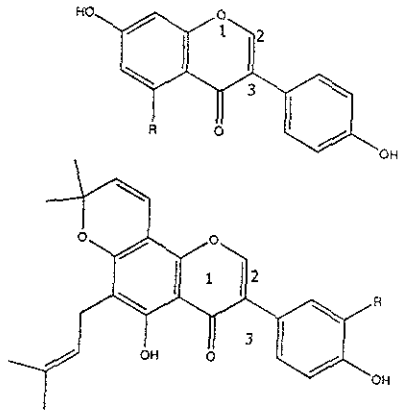
	R1	R2	R3	R4	R5
Kaempferol	H	OH	H	OH	OH
Quercetin	OH	OH	H	OH	OH
Myricetin	OH	OH	OH	OH	OH

Flavonols

Neoflavonoids



Isoflavonoids



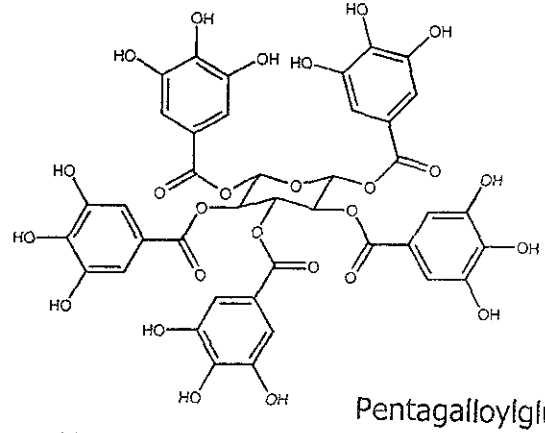
Daidzein: R=H
Genistein: R=OH

Osajin: R=H
Pomiferin: R=OH

Tannins

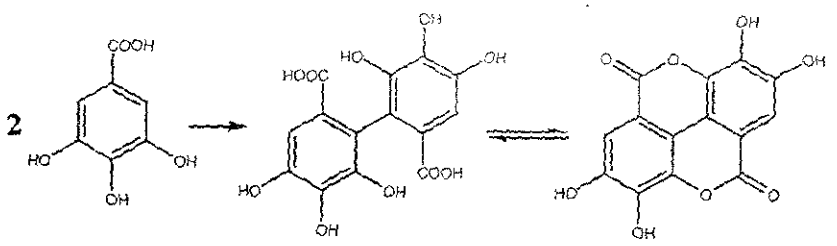
- Hydrolysable tannins
- Condensed tannins

Hydrolysable Tannins



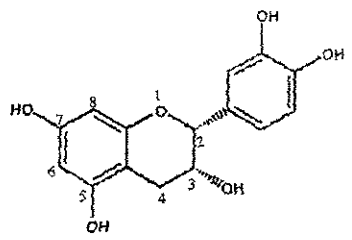
Pentagalloylglucose

Hydrolysable Tannins – Cont.

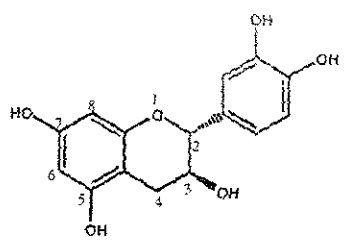


Gallic acid | Gallic acid dimer | Ellagic acid

Condensed Tannins-Monomers

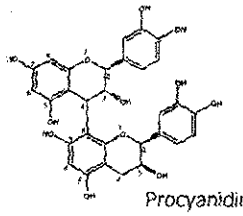


(-)-Epicatechin

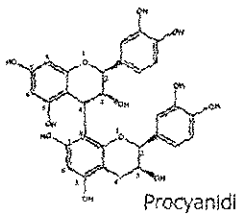


(+)-Catechin

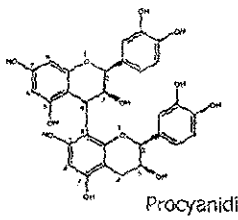
Procyanidins-Dimers



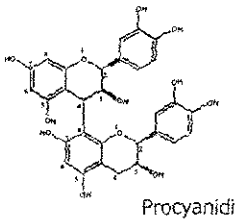
Procyanidin B1



Procyanidin B2

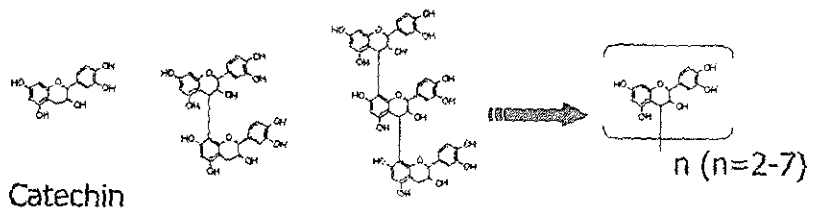


Procyanidin B3



Procyanidin B4

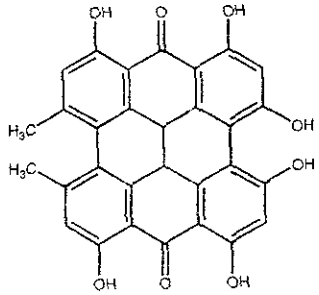
Oligomeric Procyanidins (OPC)



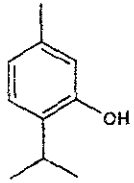
Catechin (monomer) | Dimer | Trimer | n (n=2-7) **Oligomers**

ProCyanidins **OPC**

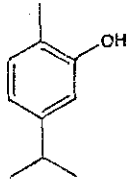
Other Phenolics



Hypericin



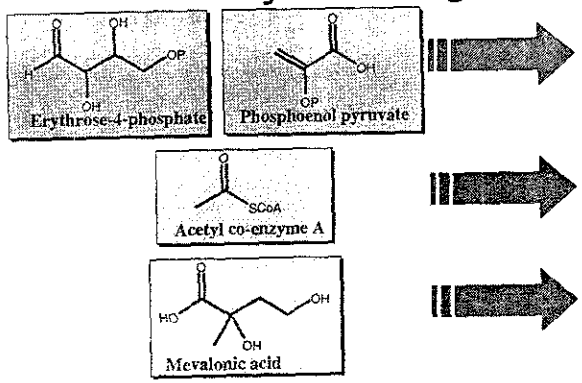
Thymol



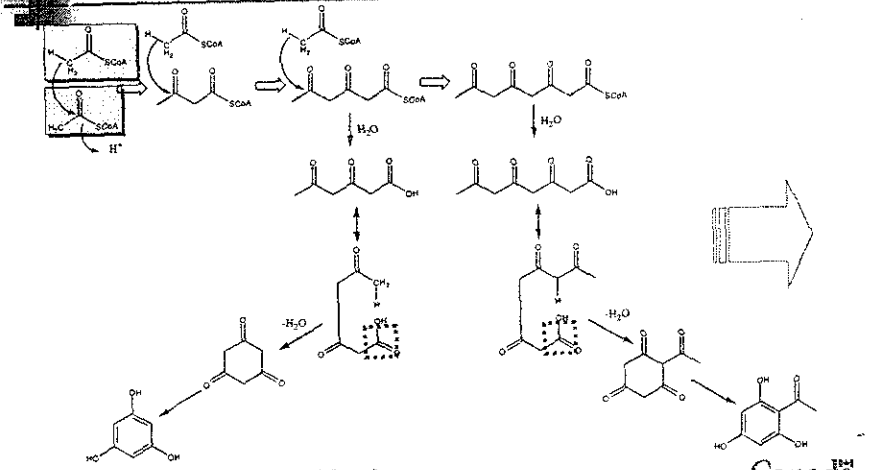
Carvacrol

Biosynthesis of Phenolics

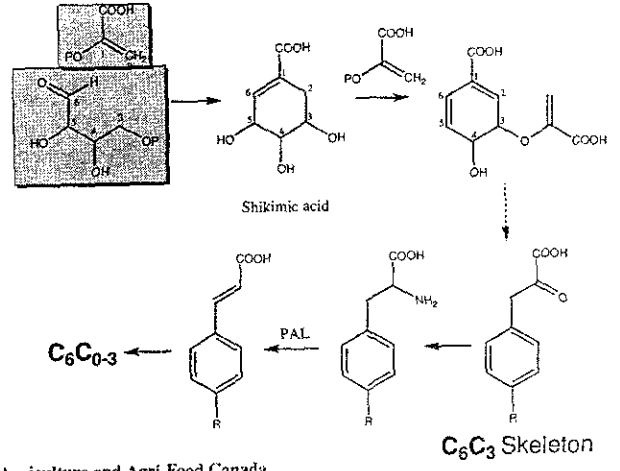
- The Major Building Blocks



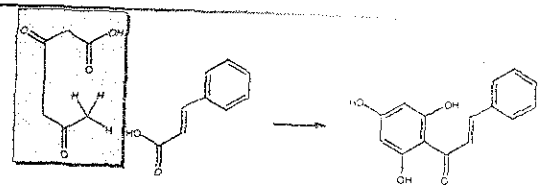
The Polyketide Pathway



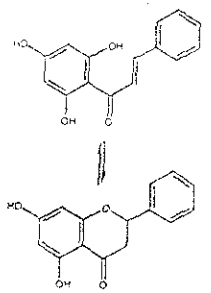
The Shikimic Acid Pathway



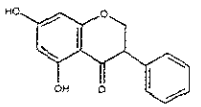
Combination of Polyketide & Shikimate Precursors



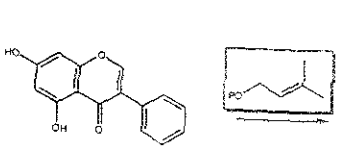
Flavonoids

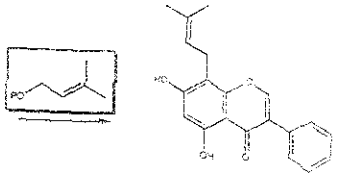


Isoflavonoids

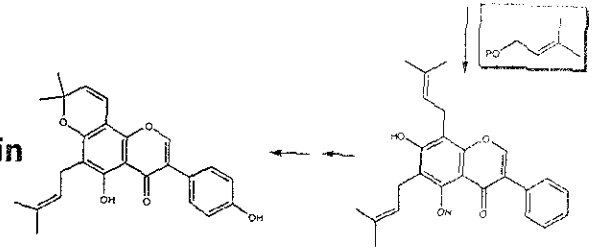


Addition of the Mevalonic Pathway





Osajin



Carotenoids

- Biosynthesis
  - The isoprenoid pathway
  - The formation of phytoene
  - The desaturation of phytoene
  - Cyclization
  - Introduction of oxygen

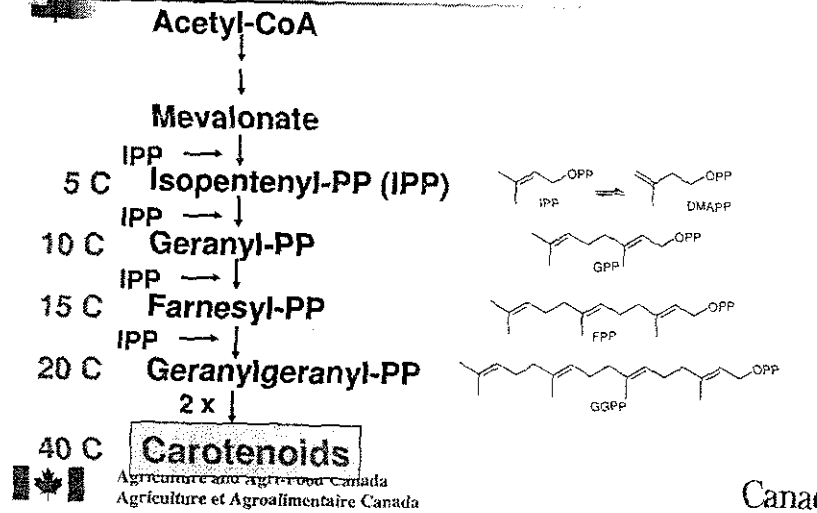
Carotenoids cont.

- Location of Carotenoids in the chloroplast
  - Distribution of carotenoids
  - Regulation of the biosynthesis and distribution of carotenoids in the chloroplast
- Function
  - General photochemistry
  - Light harvesting
  - Photoprotection
  - Scavenging of singlet oxygen and radical oxygen species
  - Quenching of triplet state chlorophyll
  - The xanthophyll cycle

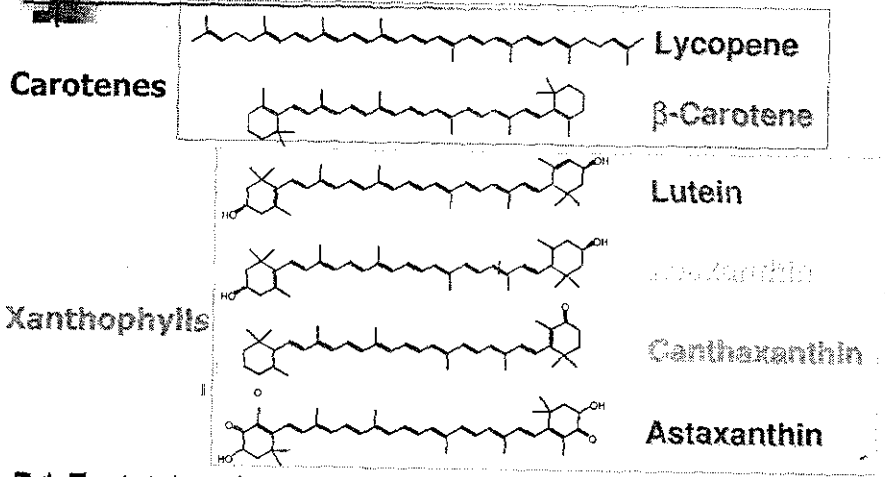
Carotenoids cont.

- Effect of photobleaching
  - Photoinhibition
    - Formation of b-carotene-5,6-epoxide
  - Effects of herbicides
    - *Inhibition of carotenoid biosynthesis*
    - *Interference with electron transport processes*
  - Other photooxidative processes
    - *Atmospheric pollutants*
    - *Exogenous sources of singlet oxigen*
    - *Formation of xanthophyll aculesters*
- Adaptation to environmental stress

Biosynthesis of Carotenoids

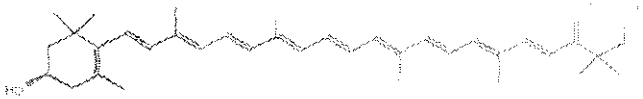


Typical Carotenoids



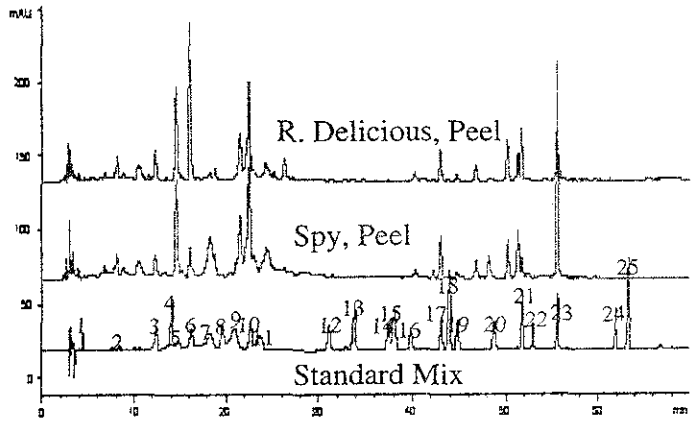
Lutein & Zeaxanthin

Lutein



Zeaxanthin

Analysis of Phenolics



Methodology

- Chemistry
  - Extraction
  - Separation
  - Purification
  - Structure ID
  - QSAR
- Bioassays
  - In vitro
  - In vivo

Extraction

- Liquid-liquid partitioning
- Solid-liquid extraction
  - Soaking
  - Soxhlet
- Supercritical
- Counter current
- etc

Separation and Purification

- Chromatographic techniques
  - Paper chromatography
  - Column chromatiography
  - Thin layer chromatography
  - Liquid chromatography
  - Gas chromatography
  - Counter current chromatography
  - Ion exchange
  - Size exclusion
  - Gel permeation
- Distillation
- Capillary electrophoresis

Analytical Methods

- IR
- UV
- MS
- NMR
- GC
- HPLC
- CE
- Etc.

In vitro tests

- Chemical
- Cell culture (cell model)

In vivo test

- Animal model
- Human trial

Reduction Potentials – Important Redox Couples

Couple	E°/mV
$HO^{\bullet}$, H^{+}/H2O	2310[a]
$RO^{\bullet}$, H^{+}/ROH	1600[a]
$ROO^{\bullet}$, H^{+}/ROOH	1000[a]
α-tocopheroxyl$^{\bullet}$, H^{+}/α-tocopherol	500[a]
quercetin$^{\bullet}$, $H^{\bullet}$/quercetin	330[b]
ascorbate$^{\bullet-}$, H^{+}/ascorbate^{-}	282[a]

[a] Buettner GR. (1993) The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. *Archives of Biochemistry & Biophysics*. 300(2):535-543.

[b] Pietta PG. (2000) Flavonoids as antioxidants. *J. Nat. Prod.* 63(7):1035-1042.

ROS

Reactive Oxygen Species

Species	Common name	Half-life (37°C)
$HO^{\bullet}$	hydroxyl radical	1 nanosecond
$HO_2^{\bullet}$	hydroperoxyl radical	unstable
$O_2^{\bullet-}$	superoxide anion radical	enzymatic
$^1O_2$	singlet oxygen	1 microsecond
$RO^{\bullet}$	alkoxyl radical	1 microsecond
$ROO^{\bullet}$	peroxyl radical	7 seconds
$NO^{\bullet}$	nitric oxide radical	1-10 seconds
H_2O_2	hydrogen peroxide	stable
HOCl	hypochlorous acid	stable

R=lipid, for example, linoleate

Antioxidant sources

Components of Antioxidant Protection

Endogenous Antioxidants	Dietary Antioxidants	Metal Binding Proteins
NADPH and NADH	Vitamin C (Ascorbic acid)	Ceruloplasmin (copper)
Glutathione and thiols (-SH)	Vitamin E (Tocopherols)	Metallothionein (copper)
Ubiquinol (coenzyme Q)	Carotenoids	Albumin (copper)
Uric acid		Transferrin (iron)
Bilirubin		Ferritin (iron)
Metalloenzymes		Myoglobin (iron)

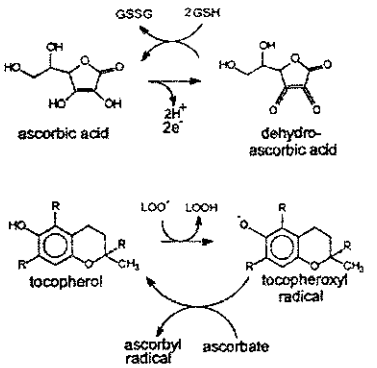
Enzymes

Free Radical Scavenging Enzymes

Enzymes	Reaction
Superoxide dismutase (SOD)	$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$
Glutathione peroxidase (GPx)	$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$

GSH = reduced glutathione, GSSG = oxidized glutathione

Recycling of antioxidant vitamins C and E.



LOO': a lipid peroxyl radical.

"Doctor, I have an Ear Ache."

- **2000 BC - "Here, eat this root"**
- **1000 BC - "That root is heathen. Say this prayer"**
- **1850 AD - "That prayer is a superstition. Drink this potion"**
- **1940 AD - "That potion is snake oil. Swallow this pill"**
- **1985 AD - "That pill is ineffective. Take this antibiotic"**
- **2000 AD - "That antibiotic is ineffective. Here, eat this root"**

Why Plants?

- Available in large quantity
- 250,000 - 500,000 species
- Only 10% has been examined chemically
- Plants are the major food source of human
- Plants are the richest source of vitamins and phytochemicals

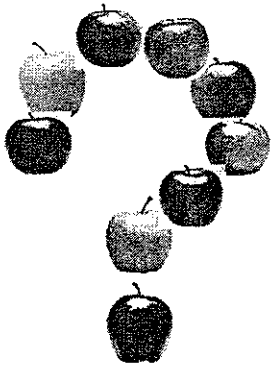
Chemicals in Plants (Phytochemicals)

- Phytochemicals are the non-essential nutrients that give the food or plant extra functions
- Plants' chemical defense against invading insects, microorganisms...
- Medicinal uses for human and animal diseases

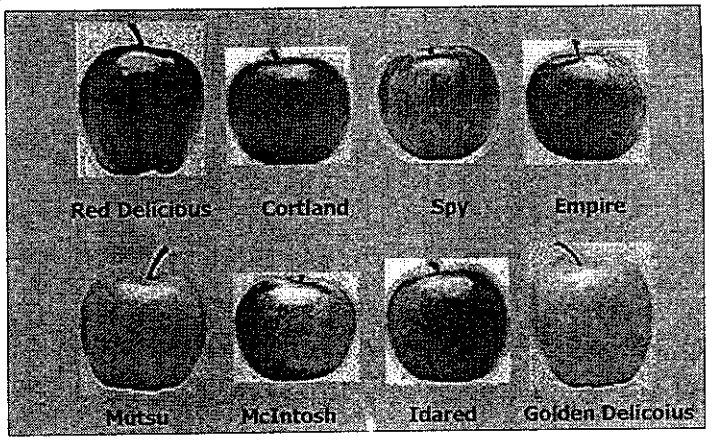
Examples-Apple

An Apple a Day.....

- But which one?



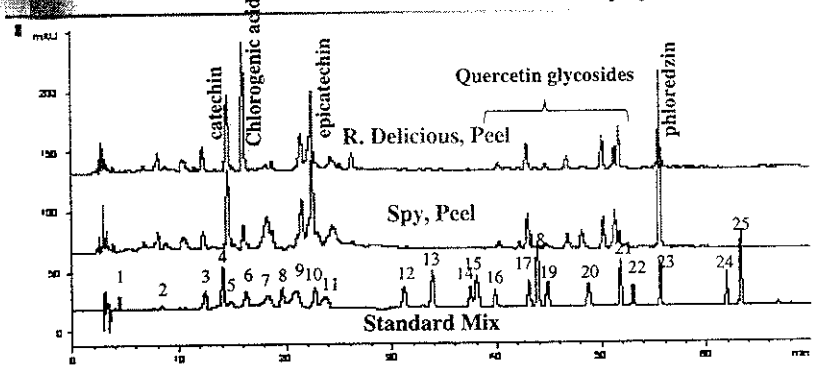
Popular Varieties in Ontario



Why Apple?

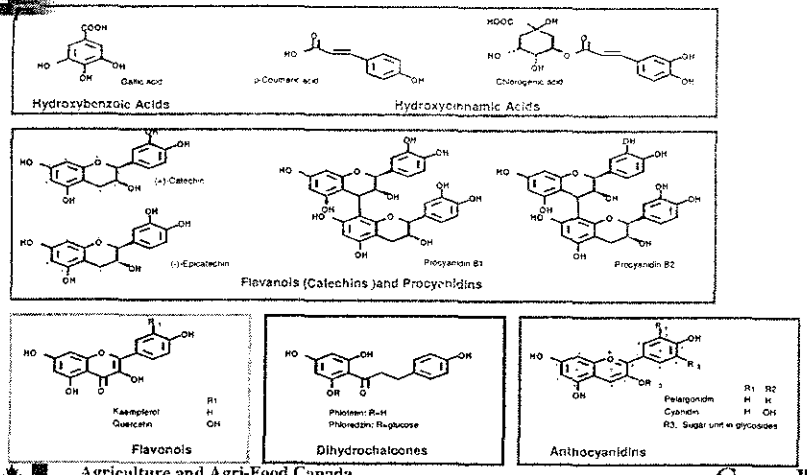
- Health Benefits
  - Cancers (colon, liver, prostate...)
  - Cardiovascular disease (LDL lowering)
  - Better lung function
  - ... ...

Phenolic Profiles of Apple

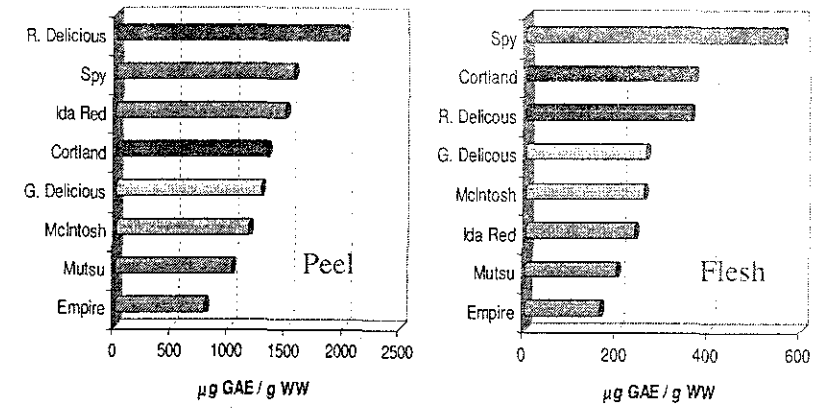


1, gallic acid; 2, procyanidin B_1; 3, procyanidin B_2; 4, 4-hydroxybenzoic acid; 5, catechin; 6, chlorogenic acid; 7, cyanidin-3-galactoside; 8, caffeic acid; 9, cyanidin-3-glucoside; 10, epicatechin; 11, cyanidin-3-rutinoside; 12, *p*-coumaric acid; 13, cyanidin chloride; 14, quercetin-3-xyloside; 15, ferulic acid; 16, quercetin-3-arabinoglucoside; 17, quercetin-3-galactoside; 18, quercetin-3-glucoside; 19, quercetin-3-rutinoside; 20, *o*-coumaric acid; 21, quercetin-3- rhamnoside; 22, quercetin-4-glucoside; 23, phloridzin; 24, quercetin; 25, cinnamic acid.

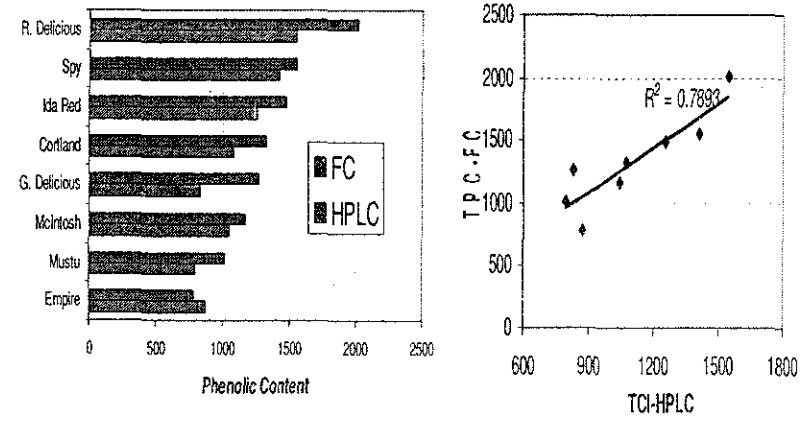
Phenolics in Apple



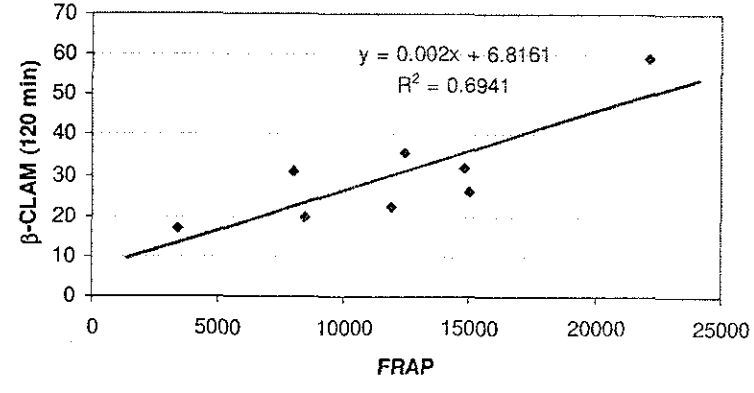
Total Phenolics in Peel & Flesh



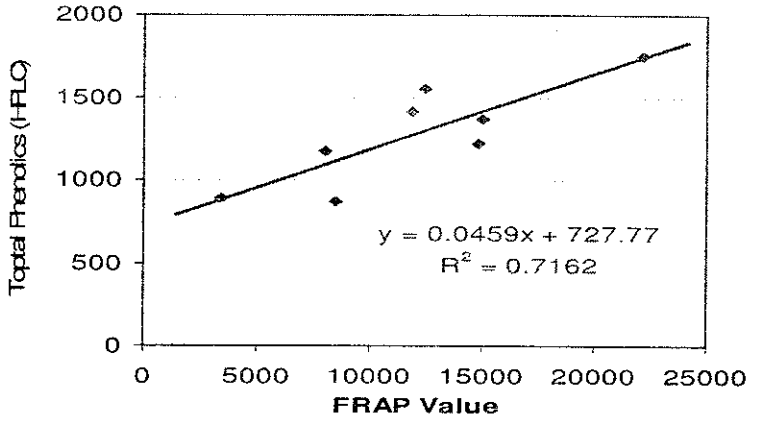
Phenolics by FC and HPLC



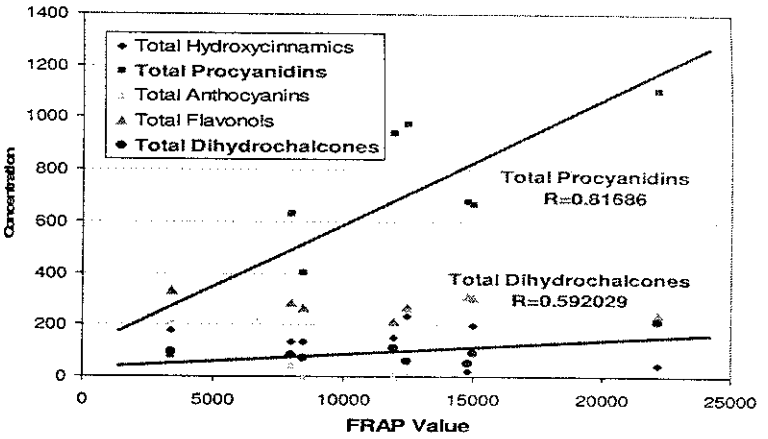
Comparison: FRAP / β-CLAMS



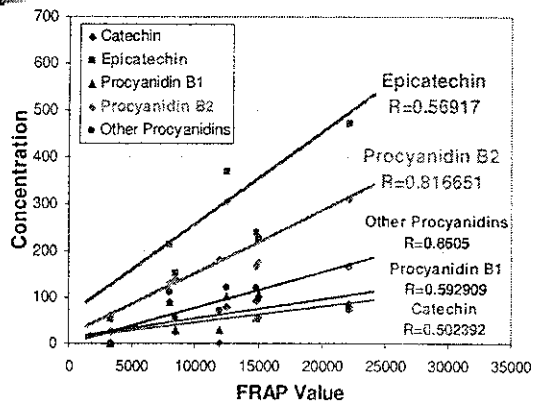
Peel Phenolics – HPLC / FRAP

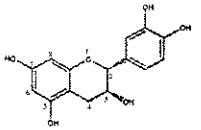


Contributing Groups - FRAP

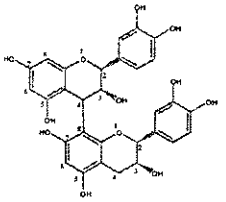


Procyanidins





(-)-Epicatechin



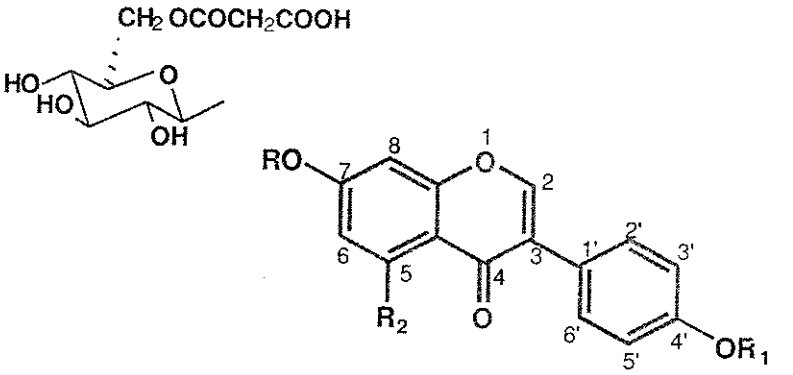
Procyanidin B2

Which

- Red delicious had the highest total phenolic content
- Antioxidant activities correlated with phenolic content
- Majority of antioxidants were in the skin
- Procyanidins, especially epicatechin and procyanidin B2 were the largest contributors

Isoflavones

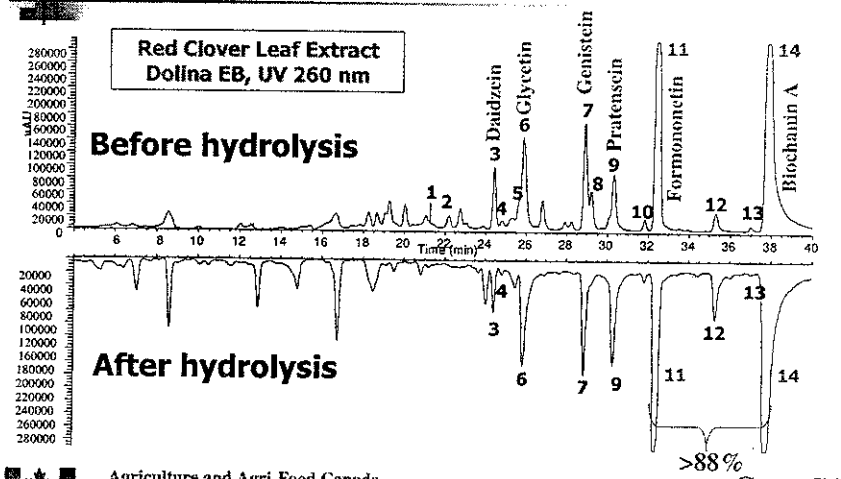
Aglycons vs. Glucosides



Isoflavones in Red Clovers and Soybean (mg/g)

Red Clover	Isoflavones
Chrisatie, Leaf	22.73 ± 4.65
Dolina, Leaf	25.95 ± 1.70
CRS 15, Leaf	31.64 ± 5.90
CRS 35, Leaf	31.61 ± 6.42
OAC Vision, Soy Seed	1.99 ± 0.00

HPLC Profiles of Red Clover

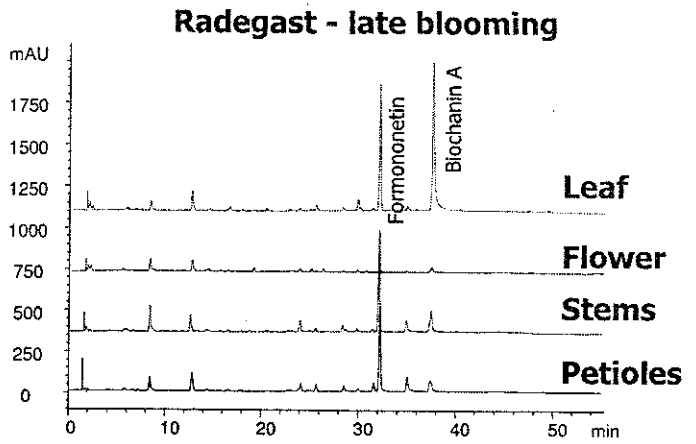


Red Clover

Agriculture and Agri-Food Canada
Agriculture et Agroalimentaire Canada

Canada

Isoflavone Profiles in Red Clover Parts



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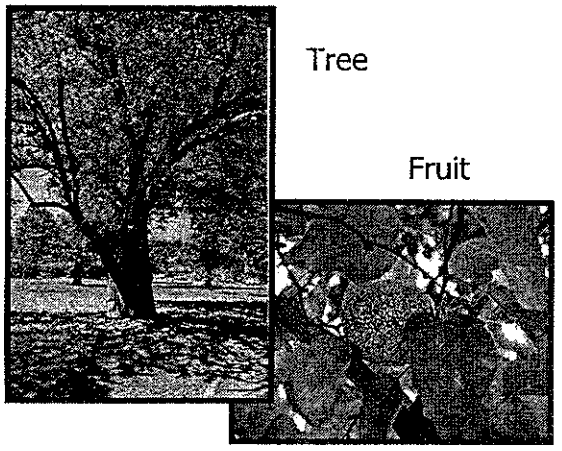

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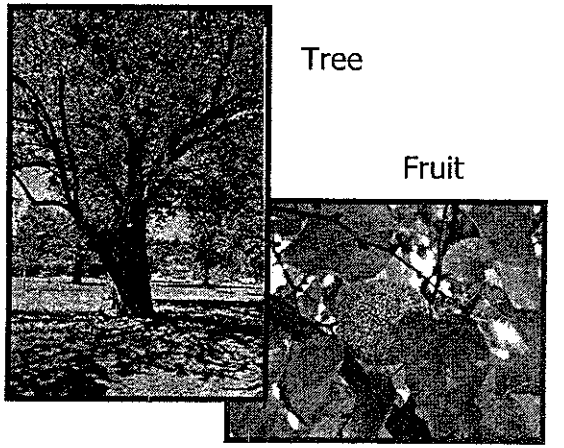
Osage Orange



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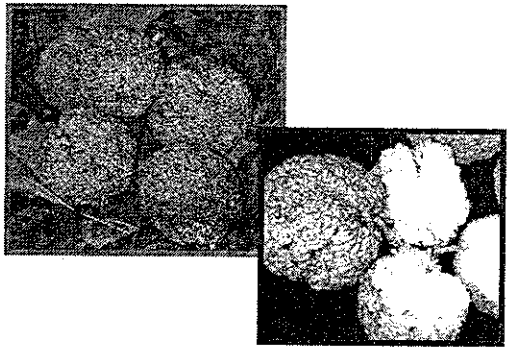
Folklore

Traditional Uses

Also Called: Hedge Apple

SE Iowa

The Fruits

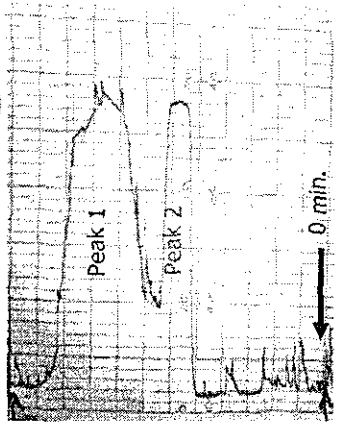


HSCCC

High-Speed Counter-Current Chromatography

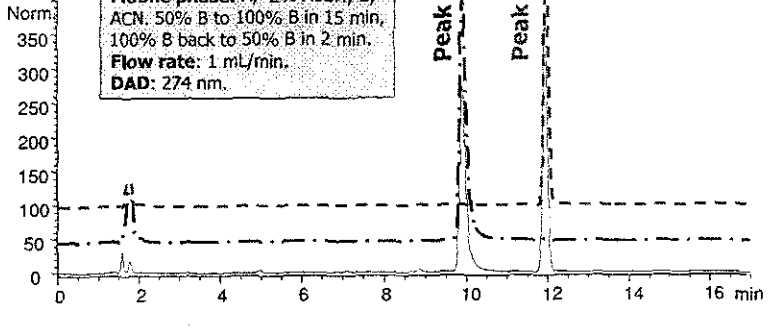
Crude Extract (ethyl acetate)
1.5% wet mass

Solvent system:
Hex/EtoAc/MeOH/H2O
=7.5/2.5/7.5/2.5
Detection: 254 nm
Flow rate: 1.5 mL/min
Recording: 1 mm/min

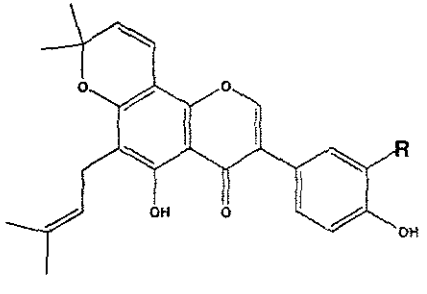


HPLC Separation and Quantification

HPLC: Agilent 1100,
Column: ODS-2 RP- C18.
Mobile phase: A, 2% AcOH; B, ACN. 50% B to 100% B in 15 min, 100% B back to 50% B in 2 min.
Flow rate: 1 mL/min.
DAD: 274 nm.

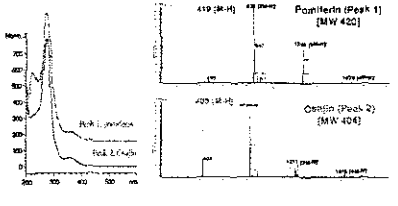


Structures



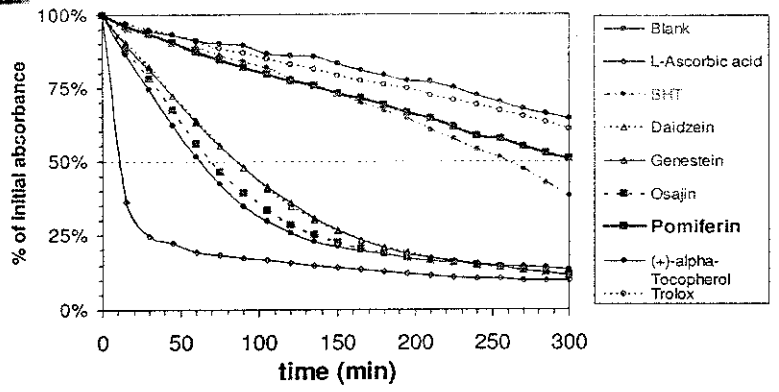
Pomiferin: R=OH
Osajin: R=H

	% of fruit (wet mass)	% in crude extract
Osajin	0.39	25.7
Pomiferin	0.55	36.2



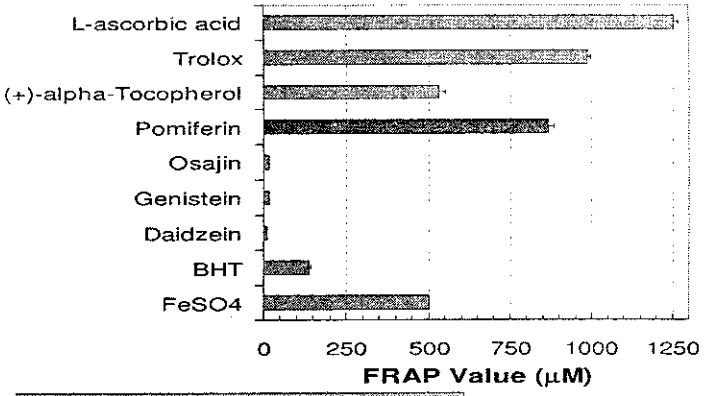
HPLC-ESI-MS, negative mode

Antioxidant Activity by β-CLAMS



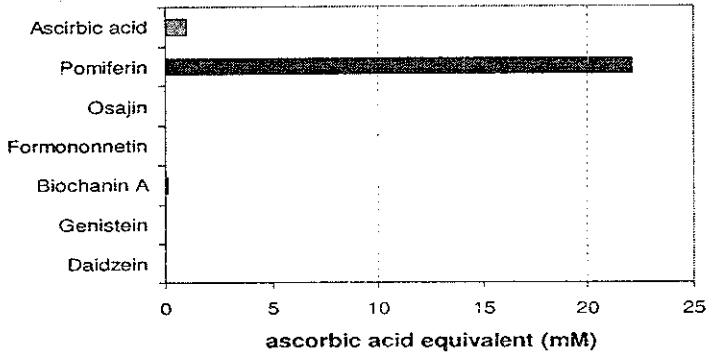
Protection against peroxidation L-OO•

Antioxidant Activity by FRAP



Strong Reducing Power (Redox Reaction)

Antioxidant Activity by Photo-chemiluminescence (**PCL**)

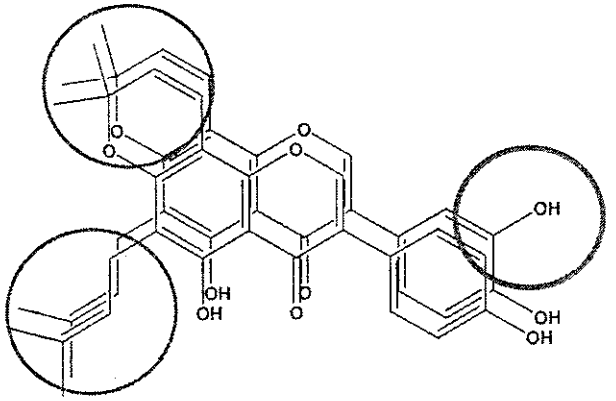


Protection Against Superoxide Radical $O_2^{\bullet}$

Effect on cell proliferation

Compound	IC_{50} (μM) MCF-7 (Human mammary carcinoma cell)	IC_{50}(μM) MCF-10A (Human breast epithelial cell)
Crude Extract	0.079 ± 0.06	0.392 ± 0.34
Osajin	~10	~10
Pomiferin	5.78±5.16	>10

Structure vs. Activity

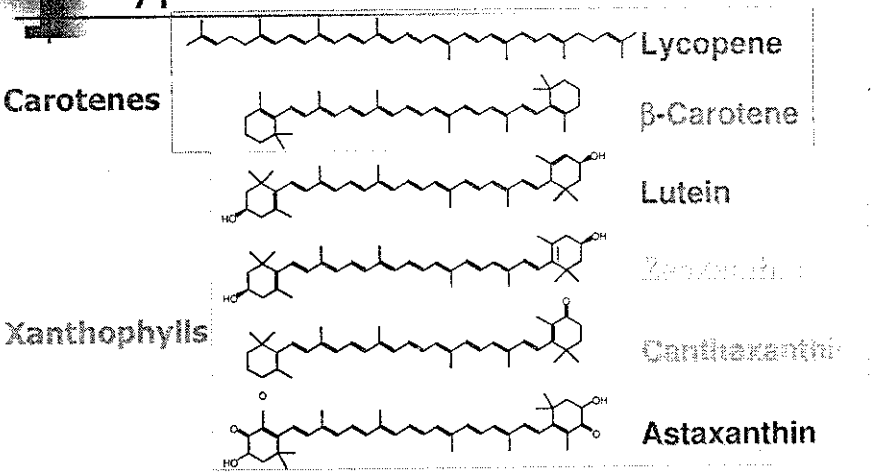


Conclusions

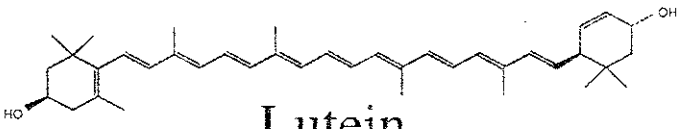
- Osage orange may be a good source of isoflavones
- Prenylated isoflavones in Osage orange had better in vitro antioxidant activity than those in dietary sources
- Structural difference may result in different antioxidant activity
- Potential anti-breast cancer agent?

Carotenoids-lutein

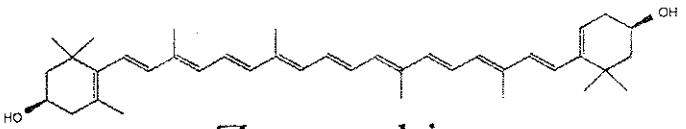
Typical Carotenoids



Lutein & Zeaxanthin

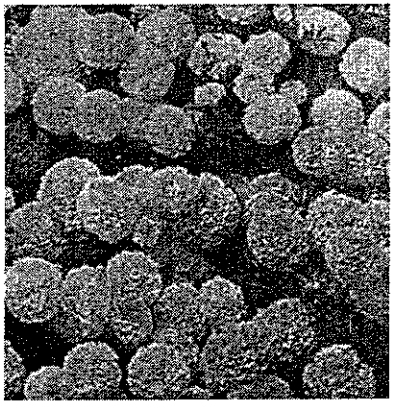


Lutein



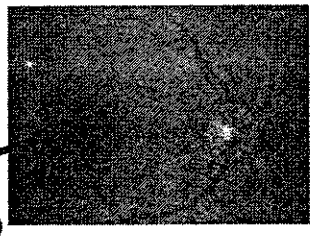
Zeaxanthin

Marigold, Lutein & AMD

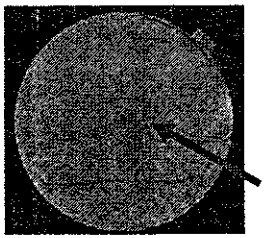


- *Tagetes erecta*
  - Richest source of xanthophylls (0.6-2.5%)
- Lutein **(83-87%)**
- Zeaxanthin **(<5%)**
- Found in the lens & macula of the eye
- Age-related Macula Degeneration

Age-related Macula Degeneration (AMD)



Macula (pigment)

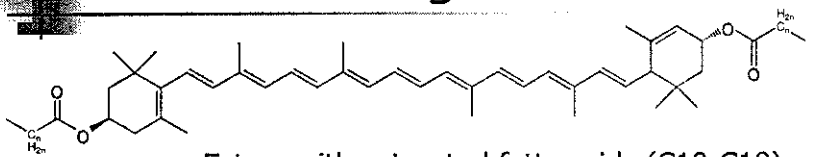


Macula (No pigment, bleeding)

Healthy Retina & Macula **Retina with AMD**

13 million in the USA alone?

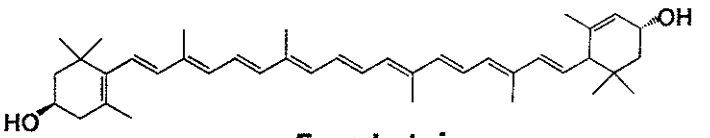
Lutein in Marigold



n = 8-16

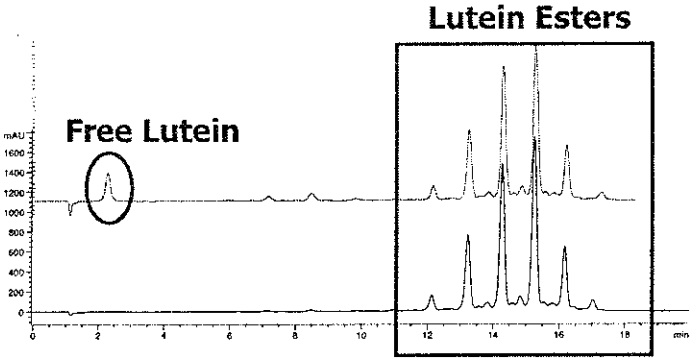
Esters with saturated fatty acids (C10-C18)

Saponification **OH^-**

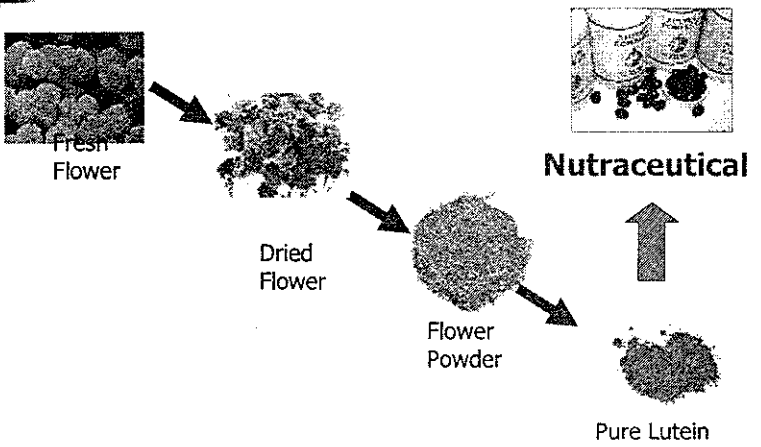


Free Lutein

Saponification



Lutein Nutraceutical Product Developed with Industrial Partner



Anti-mutagenicity of Lutein

Ames Test

strain		Controls			Treatments		
		Blank control	Positive control	Solvent control	1.335 mg/plate	0.668 mg/plate	0.334 mg/plate
TA_{98}	-S9	45±2	130±12	30±3	48±9*	52±6*	67±7*
	+S9	36±8	120±17	37±7	55±8*	67±5*	73±8
TA_{100}	-S9	153±23	1734±204	141±12	700±86*	830±108*	1090±141
	+S9	160±22	1380±207	168±18	688±91*	886±96	948±127

*compared with positive control, P<0.01

Anti-teratogenic activity – chromosomal aberration test / CHO

Test compound	Concentration (mg/L)	S9	Duration (h)	Rate of teratogenicity (%)
Lutein + mutagen	267.0	-	24	29
		-	48	16
		+	6	28
	133.5	-	24	22
		-	48	24
		+	6	13
	66.8	-	24	19
		-	48	14
		+	6	28
DMSO	50.0	-	24	26*
		-	48	14*
		+	6	28*
MMC	0.5	-	24	66**
		-	48	94**
CP	125.0	+	6	44**

*compared with test groups P<0.05; **compared with test groups P<0.05

Symposium in 2005-Hawaii

PACIFICHEM 2005
Honolulu, Hawaii, USA, December 15-20, 2005

http://www.pacifichem.org/symposia/

Area 1 - Agrochemistry

Chemistry and Biochemistry of Antioxidative Phytochemicals (#44)

Rong Tsao | Ping Li | Liangli (Lucy) Yu

Email: caor@agr.gc.ca

Development of Nutraceuticals and Functional Foods

Agriculture and Agri-Food Canada
Agriculture et Agroalimentaire Canada
Canada

Marketing Issues

- Good taste necessary
  - Taste more important than convenience and nutritional advantages
- Brand name connects to functional advantage
  - E.g. Psyllium cereal: Kellog's Heartwise vs. General Mills' Benefit
- Consumer education required
  - Commercial, public media, academic publication
- Avoid information overload
  - Simpler is better

Agriculture and Agri-Food Canada
Agriculture et Agroalimentaire Canada
Canada

Marketing Issues – Cont.

- Competitive set determined by health issue
  - Address contents (e.g. lycopene, oatmeal) rather than health condition (e.g. cholesterol-lowering) to avoid competitive response from the pharmaceutical industry
- Non-verbal messages important
  - Pictures or movies showing: e.g. natural, good feeling, etc
- Usage occasion
  - Encourage consumers to incorporate into a daily routine
- Avoid negative advertising
  - Emphasize on the better and good-for you sides, not "fear of disease" side

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Canada

Marketing Issues – Cont.

- Niche markets
  - Specific bioactives may start with small market, but can grow. Emphasize on "special", "different", superior etc.
- Exploit corporate heritage
  - Means trust and quality: E.g. Quaker Oats oatmeal, Tropicana's Pure Premium Plus; General Mills' Cheerios;
- Dosage and standardization
  - Consumers and even doctors will ask, so it needs to be done more in the future

Potential Product Positioning

- Health conscious consumers
  - Therapy
  - Prevention
  - Performance
  - Weight loss
- From "food-nutrition-health" to "food-nutrition-health-wellness-well being"

Important components for NFF Development

- Physical components
  - Products that emphasize exercise and health
- Emotional components
  - Products that nurtures and protects self and family
- Well-being components
  - Emphasize on "natural" and "good feeling"
- Social components
  - Products that are necessary socially and daily
- Financial components
  - Good food-good health-good retirement-good independence

Regulatory issues

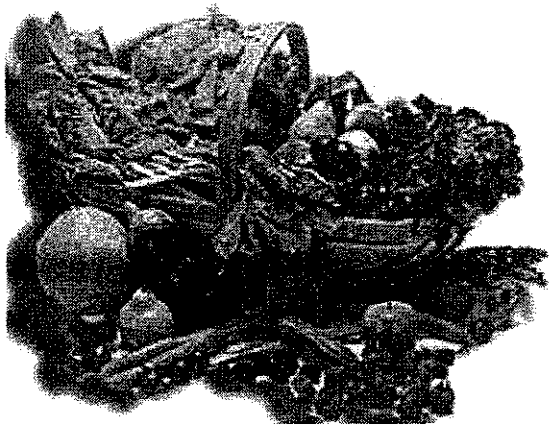
Health Claims Allowed by US-FDA

- Calcium and osteoporosis
- Sodium and high blood pressure
- Potassium and high blood pressure
- Plant stanol and sterol esters and coronary heart disease
- Dietary fat and cancer
- Dietary saturated fat and cholesterol and coronary heart disease
- Soy protein and coronary heart disease
- Fiber-containing grain products, fruits, and vegetables and cancer
- **Fruits, vegetables** and grain products that contain fiber, particularly soluble fiber, and **coronary heart disease**
- **Fruits and vegetables** and **cancer**
- Folate and neural tube birth defects
- Dietary sugar alcohols (sorbitol) and dental cavities
- Dietary soluble fiber, such as that found in whole oats and psyllium seed husk, and coronary heart disease

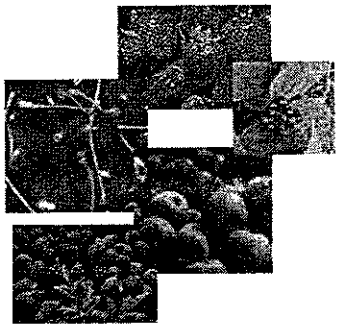
Health Claims-Canada

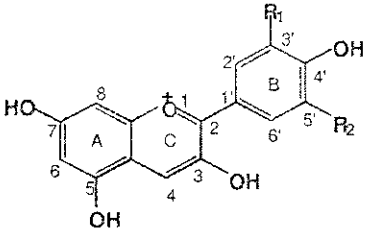
- sodium and hypertension
- calcium and osteoporosis
- saturated and *trans*- fat and cholesterol and coronary heart disease
- **Fruits and vegetable and Cancer**
- sugar alcohols and dental caries was apparent.

What in Fruits & Vegetables that are Good for Health?



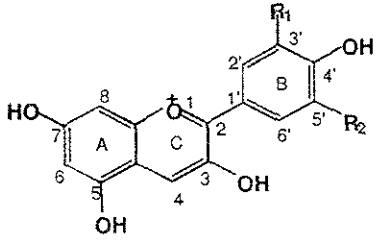
Red





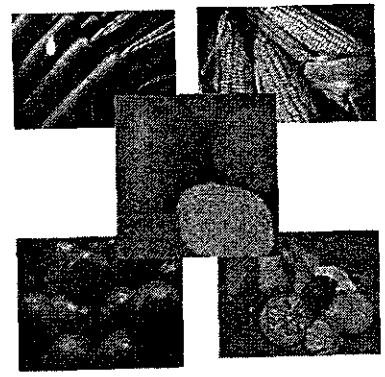
Anthocyanidins

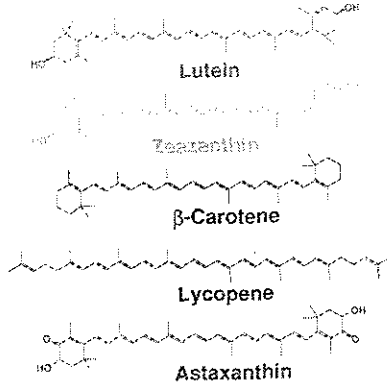
Blue/Purple



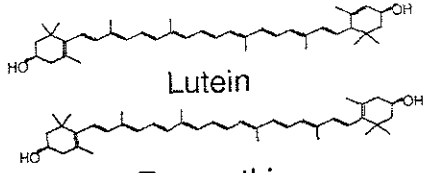
Anthocyanidins

Yellow/Orange





Green



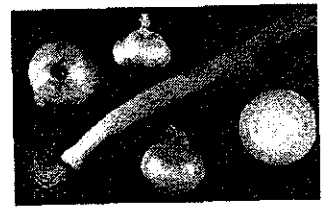
Lutein

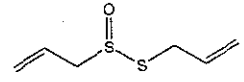
Zeaxanthin

Sinigrin AITC

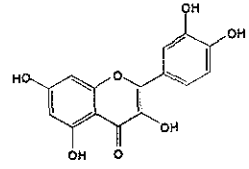
Glucosinolates

Lots of colourless compounds





Allicin



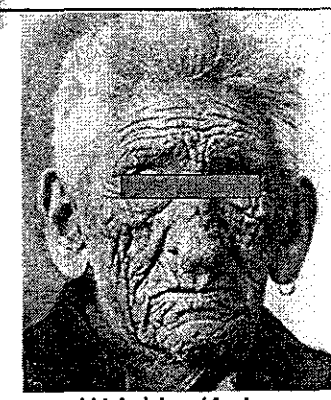
Quercetin

What is in Common for these Phytochemicals?

- Polyphenols
  - Phenolics
  - Flavanols
  - Flavonols
  - Anthocyanins
  - Procyanidins
- Carotenoids
  - Carotenes
  - Xanthophylls

Antioxidants!

Natural Oxidative Processes



Wrinkles/Aging

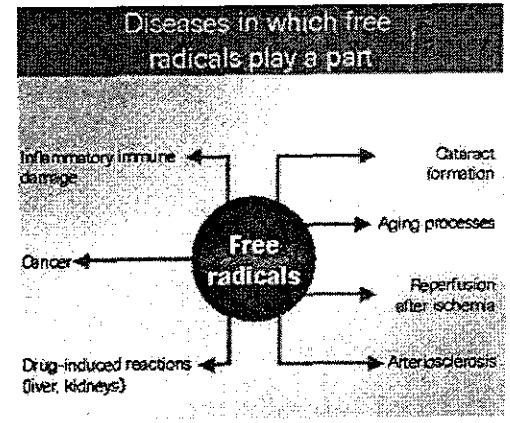
Rust

Free Radical Damages

- **Lipid peroxidation**—cause fat to turn rancid and release more free radicals.
- **Cross-linking**—cause proteins and/or DNA to fuse together.
- **Membrane damage**—destroy the integrity of the cell membrane, thus affect nutrients intake and wastes discharge.
- **Lysosome damage**—rupture lysosome cell membranes; these then spill into the cell and digest critical cell compounds.
- **Accumulation of the age pigment** (lipofuscin), which may interfere with cell chemistry.

The Nutrition Superbook, The Antioxidants, ed. by J. Barilla, M.S., Keats Publishing, Inc., 1995.

Free Radicals and Diseases



Our Body's Defense

Oxidative Stress (Free Radicals)

Endogenous
- Mitochondria
- Peroxisomes
- Inflammatory cells
- Cancerous cells

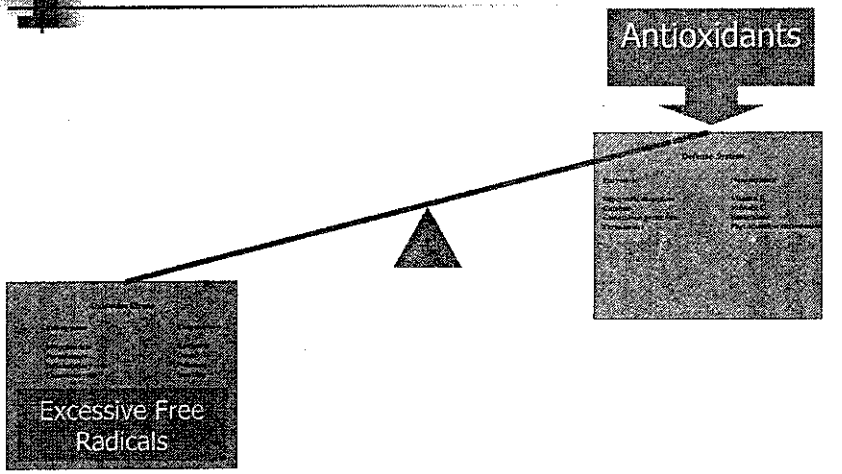
Exogenous
- Radiation
- Ozone
- Pollutants
- Smoking

Defense System (Antioxidants)

Enzymatic
- Superoxide dismutase
- Catalase
- Glutathione peroxidase
- Paraoxonase

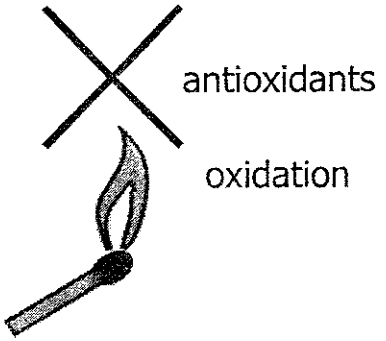
Nonenzymatic
- Vitamin E
- Vitamin C
- Glutathione
- Phytochemical antioxidants

Antioxidants Restore the Balance



Confucius Say.....

- **Suppress the seed of fire within you before it gets fully burning...**

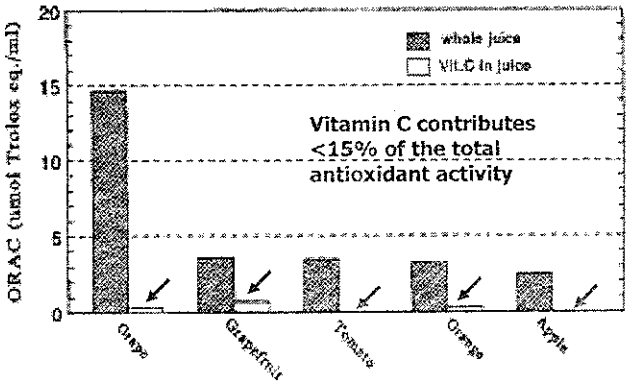


Pills vs. Whole Foods

- Pills/Tablets
  - Adequate dosage
  - Easy to take
  - Highly bioavailable
  - Over dose
  - Prooxidant
  - Toxicity
- Whole Foods
  - Full spectrum
  - Natural
  - Part of diet
  - Low cost
  - Short shelflife
  - Bulky
  - Slow absorption

Phytochemicals v.s. Vitamin C

Wang et al., J. Agric. Food Chem. 1996, 44, 701-705



Non-essential Phytochemical Antioxidants

- Phenolic compounds are the most strongest and versatile antioxidants
- Carotenoids
  - Xanthophylls
  - Carotenes

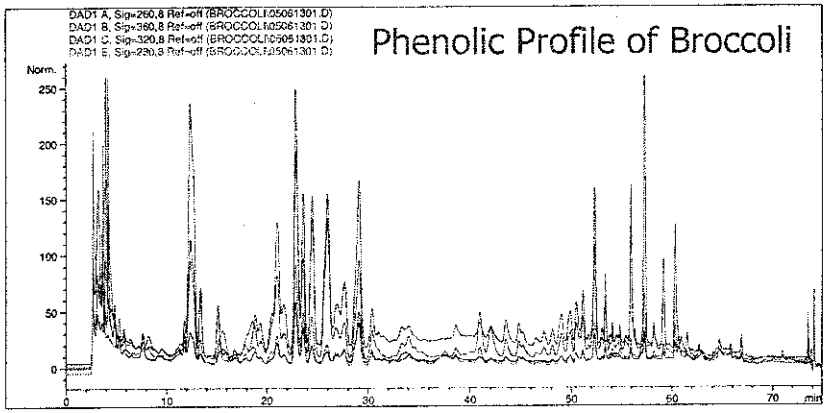
Phenolics

- Phenolic acids
- Lignans
- Coumarins
- Stilbenes
- Flavonoids
- Isoflavonoids

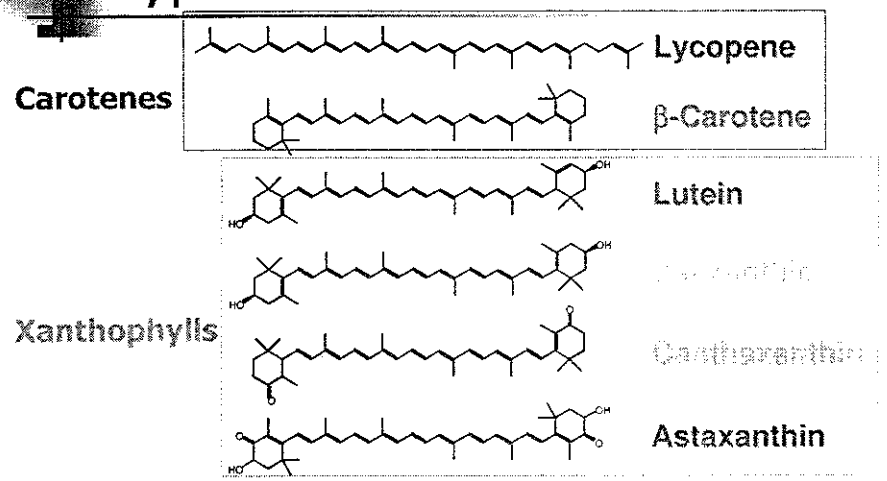
Flavonoids

- Chalcones
- Anthocyanidins
- Flavanones
- Flavanols (Catechins)
- Flavones
- Flavonols

Complex Chemistry



Typical Carotenoids



Factors Affecting Antioxidant Phytochemicals in F&V

- Agronomic Practice
  - Organic vs. Conventional Farming
- Genetics
- Environmental
  - Geographic location
  - Season
- Food Processing

Agronomic Practice

Organic Farming

Vs.

Conventional Farming

Organic vs. Conventional

Strawberry

Sample	Kaempferol	Quercetin	Ellagic acid	*p*-Coumaric acid	Total
Polka	0.5±0.08a	0.4±0.02b	51.7±6.9b	1.6±0.27a	54.2±6.5b
Polka-OR	0.5±0.11a	0.3±0.00a,b	51.3±0.0b	2.1±0.08a,c	54.2±0.3b
Jonsok	0.2±0.02b	0.3±0.01a	52.2±2.2b	1.7±0.01a	54.4±2.2b
Jonsok-OR	0.5±0.10a	0.3±0.08a,b	58.6±0.4c	1.6±0.10a	61.1±0.1c
Honeoye	0.9±0.13c	0.5±0.04b,c	46.7±3.7b	4.1±0.96d	52.2±2.5b
Honeoye-OR	0.9±0.06c	0.5±0.06b,c	48.3±0.2b	3.7±0.31d	53.4±0.5b

Hakkinen and Torronen, *Food Research International* 33 (2000) 517-524

Organic vs. Conventional

Peach & Pear

Table 1. PPO Activity and Total Polyphenol Content of Conventional and Organic Peach and Pear Samples[a]

sample	PPO activity (unit min^{-1}/100 g f.w.)[b] caffeic acid	chlorogenic acid	catechol	total polyphenols (mg tannic acid/100 g f.w.)
		Peach		Polyphenols
conventional	2451.9 ± 126.4	2053.2 ± 145.0	nd[c]	21.3 ± 1.6
organic ←	2174.5 ± 198.2	2655.3 ± 171.2*	nd	28.0 ± 1.2** ←
		Pear		
conventional	674.2 ± 50.5	959.1 ± 100.9	557.1 ± 143.2	58.4 ± 2.0
organic ←	955.1 ± 43.8*	3020.7 ± 235.4***	401.4 ± 110.3	64.5 ± 1.5* ←

[a] Values are the average of at least six determinations ± SD. [b] Conventional vs organic: significantly different; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. [c] nd, not detectable.

Carbonaro et al., *J. Agric. Food Chem.* **2002,** *50,* 5458-5462

Organic vs. Conventional

Grape

Table 7. Resveratrol Conent of Conventional and Organic Wine Grape Varieties Grown in Western Switzerland (Vintage 1997)

Variety - Location	Resveratrol Content (ppm) Conventional	Organic ←	Organic as a Percent of Conventional
Pinot Noir - Neuchatel	13.9	12.7	91%
Pinot Noir - Morges	13.5	17.6	130%
Pinot Noir - Peissy	8	11.0	138%
Pinot Noir - Ligerz	8	14.9	186%
Gamay - Morges	23.6	32.8	139%
Chardonnay - Neuchatel	0.2	0.3	150%
Chasselas - Aubonne	0.1	0.1	130%
Pinot Gris - Neuchatel	0.9	0.8	89%
Eight Comparisons			132%

Note: Data on Chasselas grown in Stere not include (0.0 ppm in conventional; 5.3 ppm in organic).

Source: Table 1 (Levite et al., 2002)

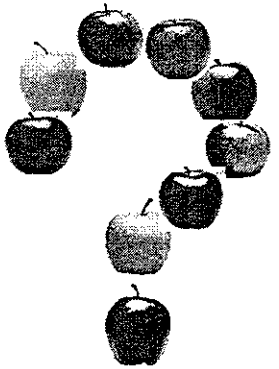
Resveretrol % ↑

Genetic Variation

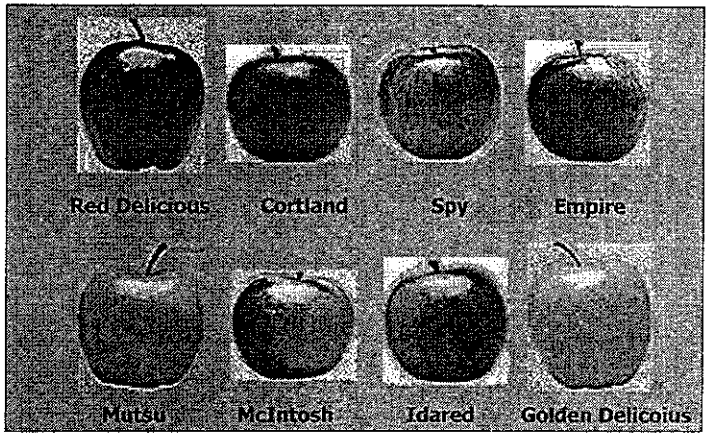
Not Every Apple was Created Equally

An Apple a Day.....

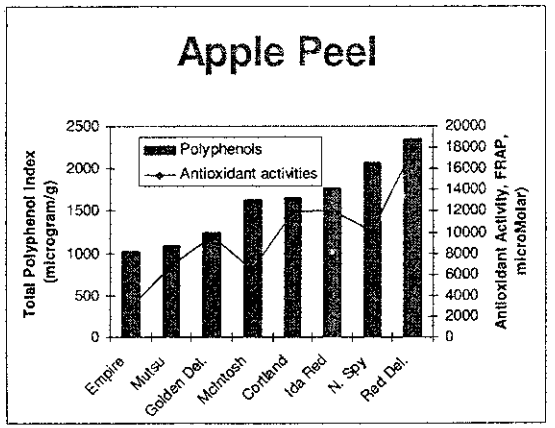
- But which one?



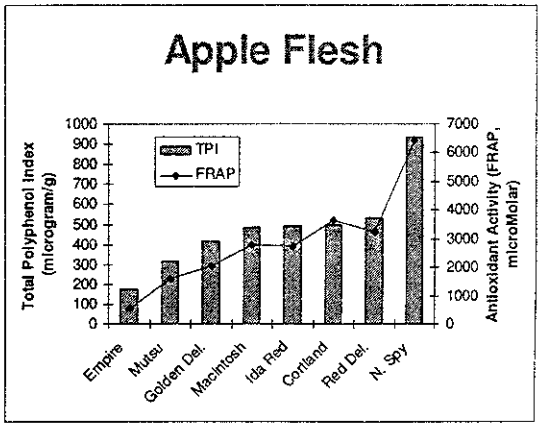
Popular Varieties in Ontario



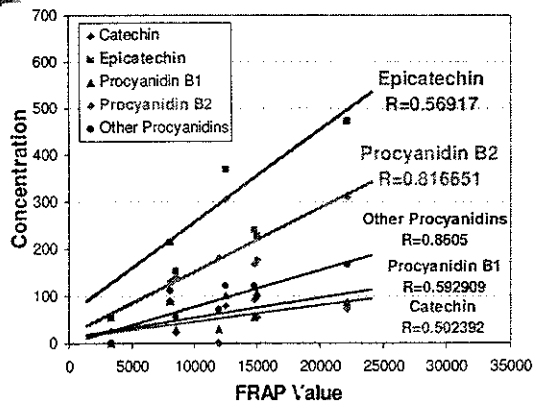
Correlation between Total Phenolics and Antioxidant Activity

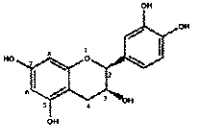


Correlation between Total Phenolics and Antioxidant Activity

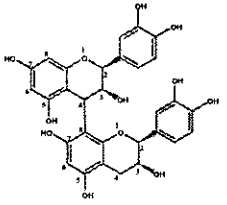


Procyanidins





(-)-Epicatechin



Procyanidin B2

J. Agric. Food Chem, June 29, 2005 issue)

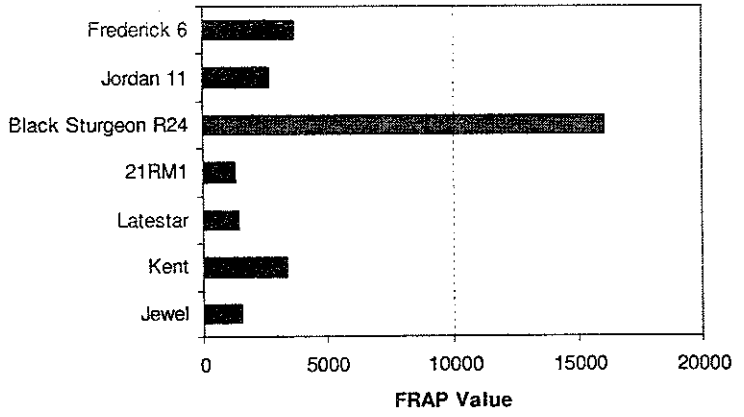
Total Phenolics & Anthocyanins-Strawberries

	Total Phenolic Content (± SD) (μg GAE[1]/g WW[2])		Total Anthocyanin Content (± SD) (μg PGE[3]/g WW)	
	Cultivated	Wild	Cultivated	Wild
2000	2023 ± 160[4]	-	543 ± 65	-
2001	1994 ± 525	2613 ± 394	471 ± 19	477 ± 67
2002	2010 ± 411	2630 ± 640	316 ± 13	662 ± 24

n=26, 34, and 71 for cultivated strawberries in year 2000, 2001 and 2002, respectively;
n=26 and 43 for year 2001 and 2002, respectively

Tsao et al., *Acta Horticulturae*, 2003 Acta Hort. (ISHS) 626:25-35.

Antioxidant Activity-FRAP



Cranberry

Total Phenolic Content

cultivar	initial
Ben Lear	25.0 ± 1.3[a]
Cropper	19.8 ± 1.3
Crowley	65.6 ± 2.0
Early Black	63.4 ± 1.5
Franklin	54.1 ± 3.6
Howes	23.5 ± 1.1
Pilgrim	20.7 ± 1.1
Stevens	22.8 ± 1.3
Wilcox	24.3 ± 1.2
#35	28.6 ± 1.7
mean[e]	34.8a[e]
significance[f]	
cultivar [*C*]	**
temp [*T*]	**
C x *T*	**

Total Anthocyanin Content

cultivar	initial
Ben Lear	137.5 ± 2.5[a]
Cropper	121.5 ± 2.0
Crowley	159.0 ± 7.3
Early Black	176.5 ± 5.2
Franklin	165.8 ± 2.8
Howes	128.3 ± 2.1
Pilgrim	120.0 ± 1.7
Stevens	126.0 ± 3.2
Wilcox	149.5 ± 3.6
#35	127.8 ± 2.5
mean[e]	141.2a[e]
significance[f]	
cultivar [*C*]	**
temp [*T*]	**
C x *T*	**

Wang and Stretch *J. Agric. Food Chem.* **2001,** *49,* 969-974

Correlation

cultivar (cv.)	correlation coefficient (temperature effect) ORAC vs total phenolics	ORAC vs anthocyanin
Ben Lear	0.902	0.895
Cropper	0.945	0.929
Crowley	0.941	0.899
Early Black	0.953	0.904
Franklin	0.950	0.902
Howes	0.935	0.925
Pilgrim	0.952	0.879
Stevens	0.917	0.893
Wilcox	0.934	0.887
#35	0.946	0.869

Wang and Stretch *J. Agric. Food Chem.* **2001,** *49,* 969-974

Plums

Table 1. Levels of Total Phenolics and Total Flavonoids, Vitamin C Equivalent Antioxidant Capacity (VCEAC), and IC_{50} from 11 Cultivars of Plums[a]

cultivar	total phenolics (mg of GAE/100 g)	total flavonoids (mg of CE/100 g)	VCEAC (mg of VCE/100 g)	IC_{50} (mg of VCE/100 g)
Autumn Sweet	228.5 ± 3.8 B	92.3 ± 4.0 E	333.2 ± 22.8 B	20.7
Bellville Elite B70197	372.6 ± 7.5 A	257.5 ± 16.1 A	567.0 ± 17.6 A	31.0
Castleton	176.4 ± 1.8 F	108.7 ± 3.6 D	264.3 ± 10.3 C	13.4
Early Magic	148.1 ± 2.0 H	67.9 ± 1.9 F	204.5 ± 16.7 E	15.5
Empress	187.0 ± 2.1 D	133.4 ± 4.5 C	269.8 ± 11.8 C	18.4
Longjohn	216.2 ± 1.8 C	150.3 ± 4.4 B	289.8 ± 38.4 C	17.0
Mirabelle	136.8 ± 2.3 I	95.8 ± 3.4 E	211.6 ± 20.2 E	31.7
NY 6	162.8 ± 1.6 G	109.3 ± 3.5 D	262.3 ± 23.7 CD	24.1
NY 9	175.9 ± 2.3 I	64.8 ± 1.5 G	239.1 ± 21.2 CD	45.7
NY 101	181.9 ± 2.2 E	145.2 ± 6.6 B	289.8 ± 17.9 C	28.2
Starley	181.3 ± 4.6 DE	110.0 ± 2.3 D	249.8 ± 12.5 CD	19.2

[a] The data except for IC_{50} are presented with mean ± standard deviation of six replications. GAE, gallic acid equivalent; CE, catechin equivalent; VCE, vitamin C equivalent. Values with the same letter in each column are not significantly different at the level of $p < 0.01$.

Kim et al., *J. Agric. Food Chem.* **2003,** *51,* 6509-6515

Blackberries

Table 7. TSS, TA, Maturity Index, Polyphenolic Composition, and Antioxidant Properties of Selected Blackberry Cultivars[a]

cultivar	TSS (°Brix)	TA (citric acid, g/100 g)	maturity index (°Brix/acid)	total phenolics (mg GAE/100 g)	total anthocyanins (mg Cyd-3-glu/100 g)	ORAC[c] (μmol TE/g)	FRAP[c] (μmol TE/g)
Marion	12.9 ± 5.49	1.28 ± 0.12	10.2 ± 5.24	1005 ± 54.2	245 ± 33.9	56.2 ± 4.64	91.7 ± 0.47
Waldo	13.6 ± 9.67	1.83 ± 0.13	7.43 ± 0.85	940 ± 28.1	158 ± 30.1	54.1 ± 1.38	81.7 ± 7.15
Evergreen[b]	11.5 ± 2.32	0.91 ± 0.06	12.6 ± 3.28	959 ± 27.0	131 ± 6.25	75.5 ± 20.7	91.5 ± 3.61
Chester[b]	8.20 ± 1.05	0.84 ± 0.08	9.75 ± 1.88	697 ± 94.5	152 ± 39.6	49.8 ± 3.91	69.2 ± 0.14
Silvan	12.8 ± 1.44	1.13 ± 0.15	11.3 ± 2.90	779 ± 14.2	161 ± 8.22	37.8 ± 2.81	67.4 ± 1.19
NZ 8126R-1	12.1 ± 1.15	1.13 ± 0.04	10.7 ± 1.40	758 ± 46.6	176 ± 11.4	39.0 ± 1.58	63.5 ± 1.87
NZ 9351-4	12.4 ± 0.89	1.93 ± 0.13	6.42 ± 0.86	682 ± 14.6	196 ± 8.68	46.3 ± 1.13	57.8 ± 1.31
ORUS 1643-3	9.64 ± 0.76	2.02 ± 0.07	4.81 ± 0.49	954 ± 40.7	256 ± 35.2	48.4 ± 1.85	84.8 ± 0.28
ORUS 1380-1	11.1 ± 1.52	1.38 ± 0.41	8.04 ± 3.27	1028 ± 194	197 ± 83.2	42.8 ± 1.21	75.2 ± 0.40
ORUS 1469-1	11.1 ± 0.86	2.62 ± 0.28	4.24 ± 0.48	1056 ± 31.5	235 ± 47.2	54.4 ± 6.80	97.3 ± 1.59
ORUS 1368-3	11.4 ± 0.52	1.30 ± 0.16	8.77 ± 1.44	1049 ± 252	246 ± 31.1	51.8 ± 3.00	77.8 ± 7.02
mean ± SD	11.5 ± 2.24	1.49 ± 0.56	8.58 ± 3.37	903 ± 157	198 ± 48.8	50.2 ± 11.7	77.5 ± 13.1

[a] Single combined sample from three plots. [b] Sample from one plot; data expressed as means ± standard deviations ($n = 3$ subsamples) on a FW basis. [c] Data expressed as means ± standard deviations ($n = 1$ subsample).

Siriwoharn et al., J. Agric. Food Chem. **2004,** 52, 8021-8030

Broccoli

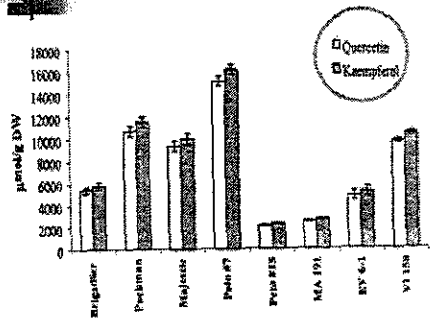


Figure 1. Quercetin and kaempferol contents of hydrophilic broccoli extracts.

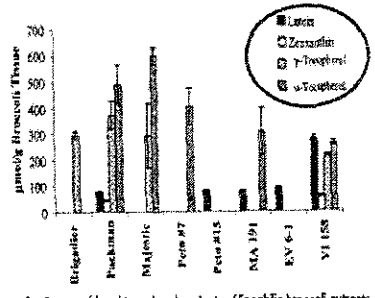
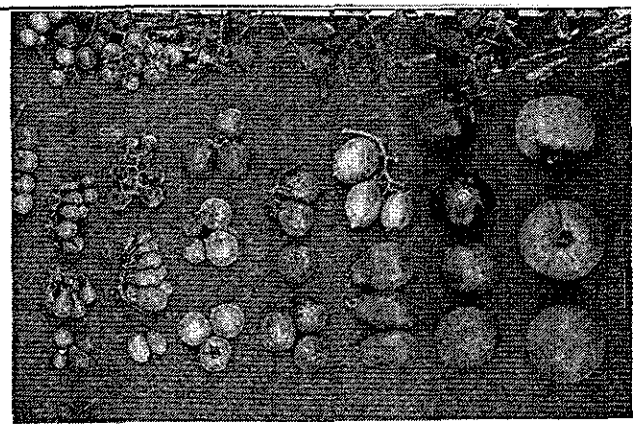


Figure 2. Carotenoid and tocopherol contents of lipophilic broccoli extracts.

Kurilich et al., *J. Agric. Food Chem.* **2002,** *50,* 5053-5057

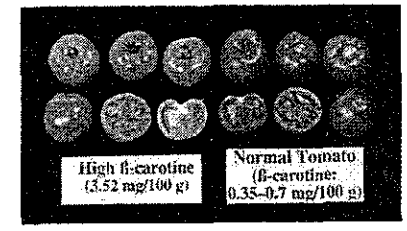
Building Better Tomatoes

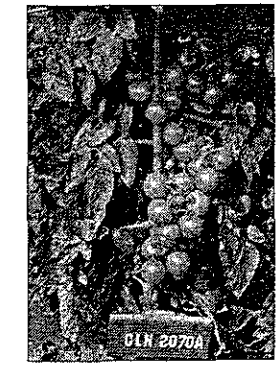


Courtesy: Asian Vegetable Research and Development Center

High beta-Carotene Tomato

- Beta-carotene precursor of Vitamin A
- Contain **3-12x more beta-carotene** than red tomato

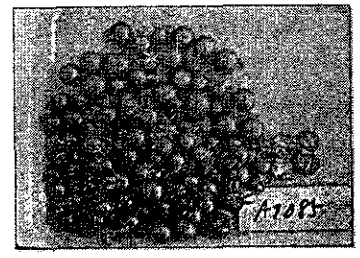




Courtesy: Asian Vegetable Research and Development Center

High Antioxidant Tomato

- Antioxidant activity most closely associated with **total phenolics**
- *L. pimpinellifolium* potential source of genes to improve tomato for specific antioxidants **(lycopene, total phenolics, beta-carotene**) and antioxidant activity.



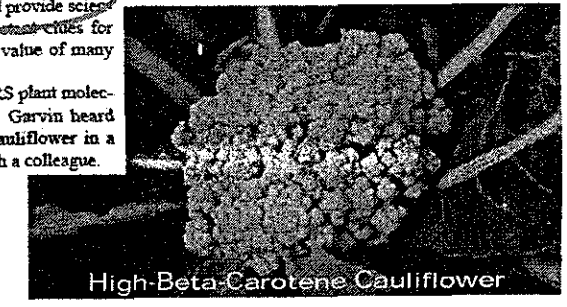
L. pimpinellifolium
LA1593

Courtesy: Asian Vegetable Research and Development Center

High β-Carotene Cauliflower

An orange cauliflower plant found growing spontaneously in a Canadian field nearly 30 years ago could provide scientists with important clues for boosting the nutritional value of many different crop species.

Several years ago, ARS plant molecular biologist David F. Garvin heard about the anomalous cauliflower in a chance conversation with a colleague.



Lutein in Vegetables

	Free Lutein	β-Carotene	Other Carotenoids	Total
Squash, Sweet Momma Peel	25.42±4.47	13.59±3.58		39.01
Squash, Buttercup Peel	18.48±0.35	2.09±0.02		20.58
Squash, Pepper Peel	30.18±2.66	1.73±0.00		31.92
Squash, Sweet Momma Flesh	1.86±0.02	0.37±0.00		2.23
Squash, Butternut Flesh	1.19±0.01	0.45±0.00		1.64
Squash, Pepper Flesh	0.87±0.01	0.10±0.02		0.97
Spinach	3.74±0.01			3.74
Kale	12.31±0.16	2.38±0.34		14.69
Potato, Yukon Gold Peel [b]	0.39±0.01			0.39
Potato, Yukon Gold Flesh [b]	**0.41±0.02**			**0.41**
Potato, Yukon Gold Flesh [c]	0.25±0.01		0.35±0.01 [d]	0.60

Environmental Factors

Geographic Locations, Growing Seasons...

Agriculture and Agri-Food Canada
Agriculture et Agroalimentaire Canada
Canada

Environmental Factors

- Geographic location-Strawberries

Table 2
Contents of flavonols and phenolic acids (mg/100 g fresh weight, mean±S.D. of duplicate assays) in 'Senga Sengana' strawberries cultivated in Poland and in Finland in 1997[a]

Sample	Kaempferol	Quercetin	Ellagic acid	p-Coumaric acid	Total
Poland					
'Senga'-PP[b]	0.7±0.13a	0.3±0.03a	35.5±4.1a	0.7±0.08a	37.2±4.3a
'Senga'-VP[b]	0.8±0.13a	0.3±0.10a	34.4±2.3a	0.7±0.02a	36.1±2.1a
Finland					
'Senga'-SW[c]	0.9±0.18a	0.4±0.06a	48.0±1.0b	1.4±0.02b	50.7±1.1b
'Senga'-E1[d]	0.7±0.13a	0.3±0.08a	46.5±1.2b	1.8±0.22b	49.3±1.1b
'Senga'-E2[c]	0.6±0.00a	0.3±0.01a	39.6±2.3a,b	1.7±0.03b	42.1±2.2a,b

Hakkinen and Torronen, *Food Research International* 33 (2000) 517-524

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Spinach-genetics, season

Table 1. Content of Phenolics and Oxygen Radical Absorbing Capacity (ORAC) of Spinach Cultivars Harvested in Spring and Fall 2000

cultivar (breeding line	Spring 2000 ORAC[a]	Spring 2000 phenolics[b]	Fall 2000 ORAC	Fall 2000 phenolics
		Advanced Breeding Lines		
86-70	12.1 ± 0.1[c]	3469.3 ± 16.9	13.4 ± 0.8	1895.6 ± 26.2[c]
86-130	20.2 ± 0.2	4815.3 ± 87.0	13.4 ± 1.1[c]	2032.8 ± 56.4[c]
86-212	14.5 ± 0.3	2596.3 ± 61.4	12.2 ± 1.0	1822.3 ± 34.8[c]
86-254	21.3 ± 0.3	4663.3 ± 29.5	10.4 ± 1.2	2308.4 ± 46.3[c]
90-168	13.2 ± 0.1	3637.8 ± 14.0	10.0 ± 0.2[c]	2290.8 ± 47.6[c]
90-252	19.3 ± 0.2	3682.7 ± 40.0	15.9 ± 0.3[c]	2100.1 ± 43.0[c]
90-276	23.9 ± 0.3	4552.2 ± 36.0	11.8 ± 1.1[c]	1842.8 ± 32.2[c]
91-227	20.5 ± 0.2	[illegible] 55.7	[illegible] ± 0.9[c]	2192.7 ± 35.7[c]
91-248	16.3 ± 0.2	3[illegible]	[illegible]	2241.6 ± 60.0[c]
91-415	11.1 ± 0.3	3351.7 ± [illegible]	13.5[illegible]	1725.9 ± 89.8[c]
97-139	22.4 ± 0.1	[illegible]	[illegible]	1704.0 ± 63.0[c]
97-152	18.0 ± 0.5	[illegible] 53.6	[illegible] ± 0.1	2268.9 ± 82.9[c]
97-154	17.3 ± 0.2	[illegible] ± 20.1	11.3 ± 0.6[c]	1947.1 ± 68.8[c]
97-165	12.3 ± 0.2	3489.0 ± 5.0	9.9 ± 1.5	1715.1 ± 57.9[c]
97-173	18.2 ± 0.1	3532.3 ± 21.9	12.4 ± 0.6[c]	1809.7 ± 42.2[c]
means advanced breeding lines	17.8 ± 0.2	3917.8 ± 36.4	11.4 ± 0.9[c]	2018.2 ± 52.6[c]
		Commercial Cultivars		
Avon	11.2 ± 0.3	2464.9 ± 25.0	10.8 ± 1.2	1888.5 ± 45.4[c]
Bolero	13.4 ± 0.1	2291.8 ± 76.6	10.9 ± 1.5	1774.1 ± 85.7[c]
Cono	12.2 ± 0.1	2564.6 ± 17.3	12.2 ± 0.4	1848.9 ± 90.9[c]
DMC 5408	20.4 ± 0.1	3388.2 ± 16.5	10.8 ± 0.4[c]	1820.8 ± 69.1[c]
F380	25.0 ± 0.2	3832.8 ± 26.1	13.6 ± 0.6[c]	2051.3 ± 76.0[c]
Falcynea	20.8 ± 0.4	3409.8 ± 33.6	12.6 ± 1.0[c]	1642.4 ± 43.9[c]
Osiris II	14.4 ± 0.3	4544.4 ± 12.1	14.8 ± 0.1	1940.8 ± 105.8[c]
Samish	10.7 ± 0.1	3406.6 ± 16.5	13.0 ± 0.1[c]	1599.6 ± 59.2[c]
San Juan	16.4 ± 0.2	3026.8 ± 27.8	14.2 ± 0.4[c]	1908.6 ± 82.2[c]
St. Helens	11.0 ± 0.3	3162.7 ± 8.5	12.8 ± 0.1[c]	1811.4 ± 52.8[c]
Winterpree	13.8 ± 0.3	4000.8 ± 8.2	9.6 ± 1.0[c]	1529.1 ± 34.2[c]
means commercial cultivars	14.1 ± 0.2	3187.8 ± 23.9	12.5 ± 0.7[c]	1813.4 ± 62.1[c]
overall means	17.1 ± 0.2	3633.6 ± 32.0	11.5 ± 0.8[c]	1922.1 ± 58.7[c]

[a] Micromoles of Trolox equivalents per gram of fresh weight. [b] Milligrams of chlorogenic acid equivalents per gram of fresh weight. [c] Standard error of the mean (N = 3). [c] Indicates significant difference between growing seasons, Student's *t* test ($P < 0.05$).

Howard et al., *J. Agric. Food Chem.* **2002**, *50*, 5891-5896

Advanced Breeding Lines

Commercial Cultivars

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Postharvest Factors

Storage,

Processing...

Changes in Blueberry, 5 °C, Air/High O_2 Atmosphere

treatment	total phenolics[A] (mg/100g)	total anthocyanins[B] (mg/100 g)	ORAC[C] (umol of TE/g)
day 0	315 ± 8	182 ± 9	13.1 ± 0.9
day 7, air	323 ± 28	204 ± 8	15.4 ± 0.8
40% O_2	351 ± 15	203 ± 11	15.5 ± 1.3
60% O_2	330 ± 7	360 ± 5	15.2 ± 0.4
80% O_2	338 ± 15	192 ± 11	15.7 ± 0.5
100% O_2			
day 14, air	331 ± 15	199 ± 11	15.5 ± 0.6
40% O_2	329 ± 22	193 ± 38	14.9 ± 0.4
60% O_2	354 ± 17	217 ± 16	17.5 ± 1.5
80% O_2	357 ± 24	218 ± 20	17.3 ± 2.2
100% O_2	348 ± 15	213 ± 8	17.6 ± 1.8
day 21, air	324 ± 15	189 ± 23	13.4 ± 0.2
40% O_2	330 ± 9	181 ± 9	14.5 ± 0.6
60% O_2	357 ± 13	204 ± 12	18.5 ± 1.1
80% O_2	359 ± 26	206 ± 7	18.5 ± 1.3
100% O_2	360 ± 17	207 ± 13	18.3 ± 0.8
day 28, air	323 ± 4	185 ± 13	13.8 ± 1.9
40% O_2	344 ± 8	183 ± 13	14.2 ± 2.5
60% O_2	383 ± 24	224 ± 22	18.0 ± 2.6
80% O_2	378 ± 21	216 ± 13	20.6 ± 3.8
100% O_2	386 ± 49	228 ± 45	19.5 ± 2.4
day 35, air	316 ± 18	168 ± 20	11.9 ± 1.5
40% O_2	343 ± 2	180 ± 25	12.1 ± 3.1
60% O_2	362 ± 14	200 ± 9	19.7 ± 3.0
80% O_2	382 ± 27	206 ± 12	19.8 ± 1.4
100% O_2	390 ± 12	217 ± 9	20.8 ± 1.2
LSD[D]	18	15	3.91
significance[E]			
treatment (T)	sig	sig	sig
duration (D)	ns	ns	ns
T × D	sig	sig	sig

Zheng et al., *J. Agric. Food Chem.* **2003**, *51*, 7162-7169

Cooking on Lutein

	Lutein
Sweet Momma Peel	25.42±4.47
Sweet Momma Peel (cooked)	42.04±2.37
Pepper Peel	30.18±2.66
Pepper Peel (cooked)	36.95±1.61

Outline

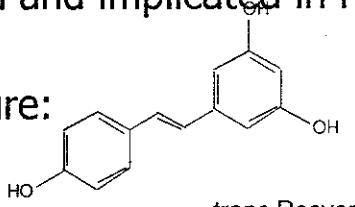
- What are stilbenes?
- Why look at them?
- Where are they found?
- Stilbenes as phytoalexins
- Eliciting stilbene production
- Other considerations

What are Stilbenes?

- Phenolic compounds, made via phenylalanine ammonia lyase and stilbene synthase
- Antimicrobial and implicated in natural resistance

What are Stilbenes?

- Phenolic compounds, made via phenylalanine ammonia lyase and stilbene synthase
- Antimicrobial and implicated in natural resistance
- Basic structure:



trans-Resveratrol

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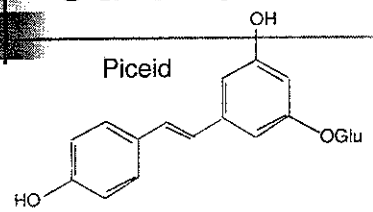
Other Stilbenes in Grapevine



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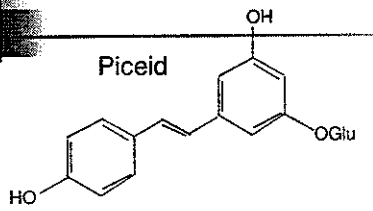
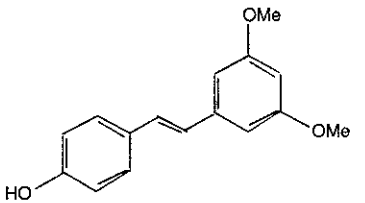
Other Stilbenes in Grapevine





Pterostilbene

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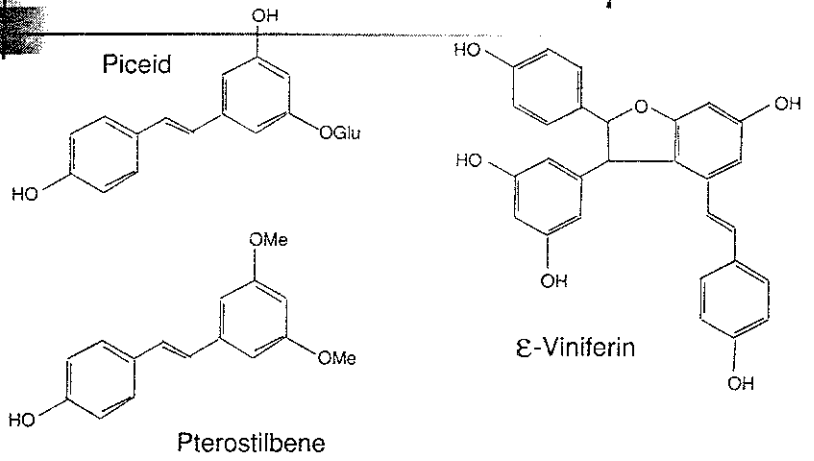
Other Stilbenes in Grapevine



Why Look at Stilbenes?

- Their antimicrobial properties
- Botrytis a problem in many growing areas
  - resistance to fungicides a growing concern

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Why Look at Stilbenes?

- Their antimicrobial properties
- Botrytis a problem in many growing areas
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- "Riches of a Clean Green Land"

Agriculture and Agri-Food Canada
Agriculture et Agroalimentaire Canada

New Zealand Wine
the riches of a clean green land

Canada

Why Look at Stilbenes?

- Their antimicrobial properties
- Botrytis a problem in many growing areas
  - resistance to fungicides a growing concern
- "Riches of a Clean Green Land"
- Healthful aspects of moderate wine consumption
  - "french paradox"
  - also found in grape juices and table grapes

New Zealand Wine
the riches of a clean green land
Agriculture and Agri-Food Canada
Agriculture et Agroalimentaire Canada

Where are Stilbenes Found?

- Present in many plant species
  - *e.g.* spruce pine, peanut, fescue grass, eucalyptus, *etc.*
- In grapevine, constitutive part of woody tissues
- Can be considered phytoalexins
  - low molecular weight, antimicrobial compounds that are both synthesised and accumulated in plants after exposure to microorganisms (Paxton, 1980)

Conclusion

- Phenolics and carotenoids are major antioxidants in fruits & vegetables
- Many factors can affect the level of these phytochemical antioxidants, but genetic variation is most important
- Designer fruits & vegetables with enriched phytochemical antioxidants can be developed through breeding
- Diets containing such designer fruits & vegetables have higher antioxidant activities, therefore potentially lead to better health for humans

Challenges ahead

- Phytochemistry of fruits & vegetables is very complicated
- Factors affecting phytochemicals are multi-facet
- Important to collaborate among breeders/agronomists/food scientists/nutritionists/chemists...

Phytochemical Antimicrobials and Biopreservatives

Health Risks from Foods

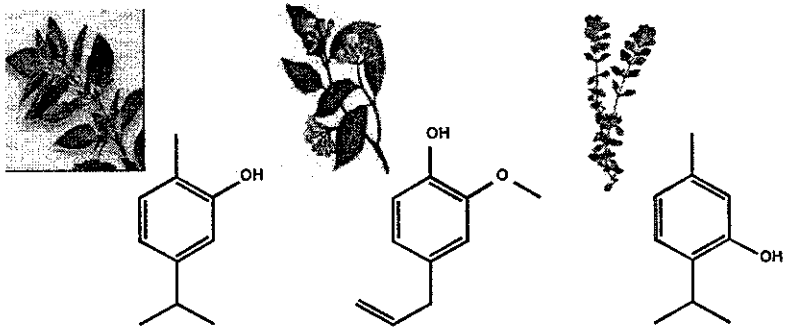
Acute		Chronic
▪ **Microbiological** ▪ Phycotoxins ▪ Some phytotoxins ▪ **Mycotoxins** ▪ Anthropogenic contaminants ▪ Food additives ▪ Pesticide residues	High Low	▪ **Mycotoxins** ▪ Anthropogenic contaminants ▪ Some phytotoxins ▪ Unbalanced diet ▪ Phycotoxins ▪ Microbiological ▪ Food additives ▪ Pesticide residues

T. Kuiper-Goodman, 1998

Use of spices in food preservation

- Traditional food preservation using spices and herbs
- Mostly to control microorganisms, bacteria and fungi (moulds)

Phenolics in Spices & Herbs

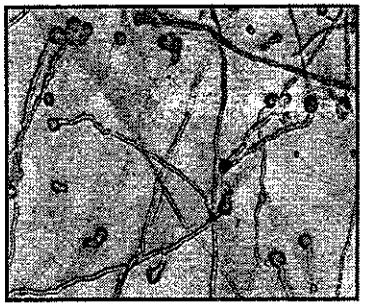


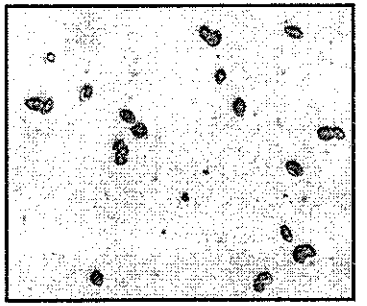
Oregano & Carvacrol | Clove & Eugenol | Thyme & Thymol

Antifungal Activity-Thymol

Monilinia fructicola

Germination on agar, 18 h at 22 °C



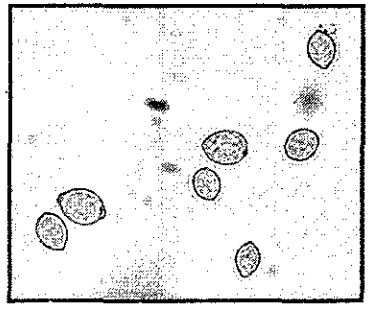


▲ fumigated with thymol at 8 mg/L

◀ Untreated CK

Antifungal Activity-Thymol

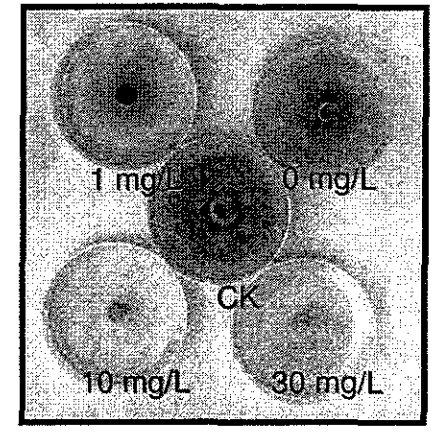
Monilinia fructicola
Conidia from plum

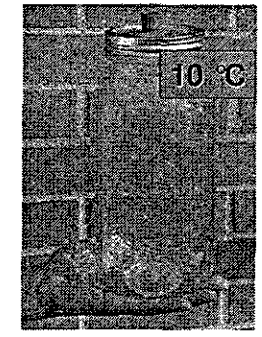


▲ fumigated with thymol at 8 mg/L

◀ Untreated CK

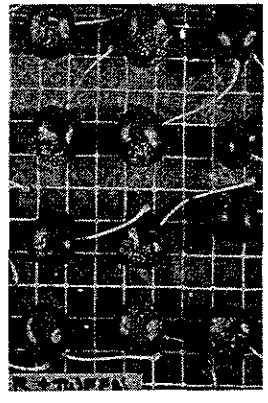
Antifungal Activity - Thymol



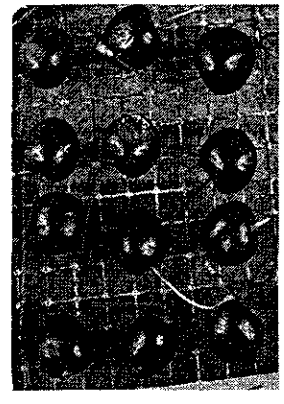


Fumigation - *M. fructicola*

Dipping - Cherry

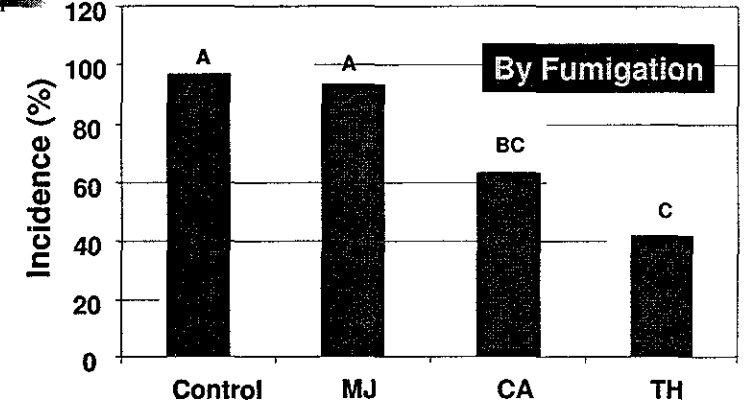


Control

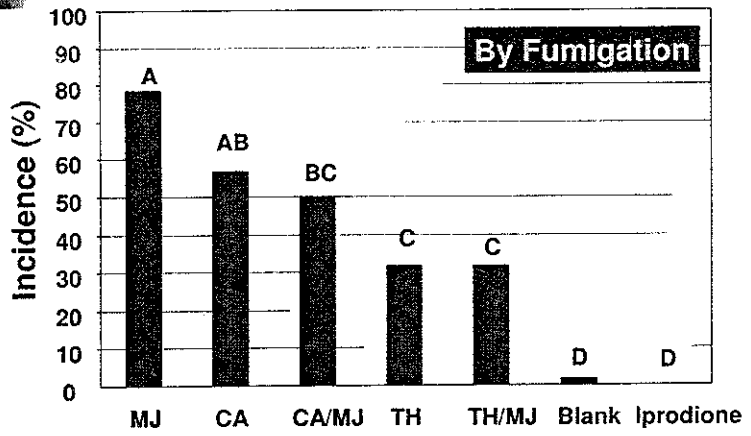


Thymol

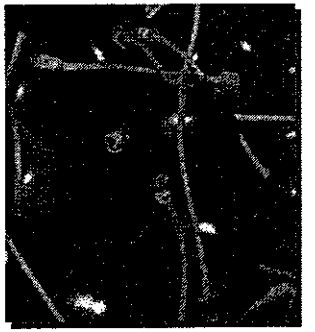
Fumigation / Brown Rot (%)



MJ & Thymol/Carvacrol on Brown Rot Incidence (%)



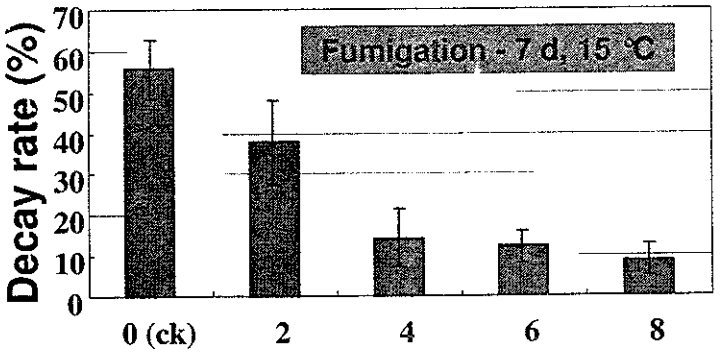
Phytotoxicity - Cherry



Thymol + MJ

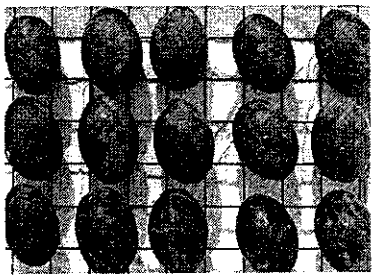
Thymol only

Brown Rot of Plum - Thymol



Brown Rot of Plum - Thymol

Fumigation - 7 d, 15 °C



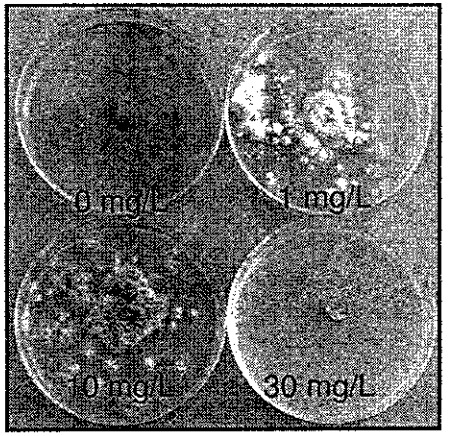
▲ Thymol at 8 mg/L

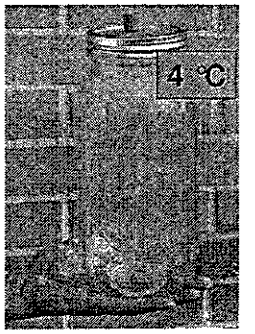
◀ Untreated CK

Conclusions

- Monoterpenoids, as the major components of essential oils, are effective against postharvest diseases
- Phenol-type monoterpenoids had the highest antifungal activity
- Phytotoxicity can be reduced
- Odors is insignificant at lower concentration

Antifungal Activity - Thymol

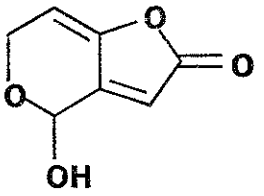




Fumigation - *P. expansum*

Patulin / *P. expansum* /apple

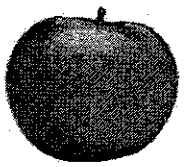
- Patulin is a secondary metabolite produced by several soil-borne fungal species in the genera *Penicillium, Aspergillus, Byssochlamys*
- *P. expansum* is the most commonly encountered species

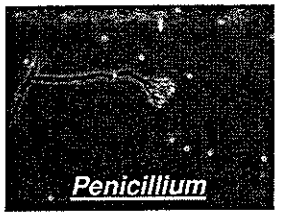


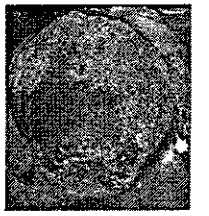
LD_{50}: 29 mg/kg (oral, mice)
LD_{50}: 10-15 mg/kg (S. C., mice)

Food Sources of Patulin

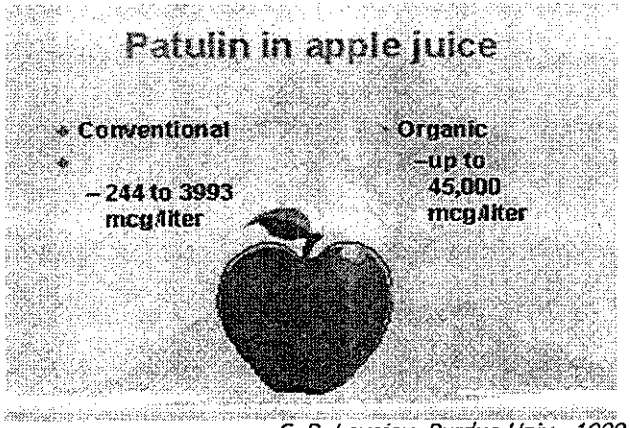
- It has been found in moldy fruits, vegetables, cereals peanut, cooked meats, cheese ...
- The major sources of contamination are apples and apple products.







Organic Vs. Conventional



S. B. Lovejoy, Purdue Univ., 1999

Anti-bacterial activity

- Essential oils from spices

Prebiotic activity of phenolics

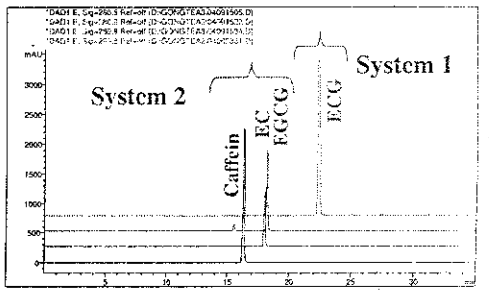
Oil/component	ppm	Inhibition (%)							
		E. coli 0157 H7	*E. coli* K88	*S. typhimurium* DT 104	*L. plantarum* 9OL11	*L. plantarum* 62E11a	*L. acidophilus* RFP	*B. breve* FRP 67	*B. longum* FRP 63
Carvacrol	200	49±3	101±1	99±0	5± 4	31±2	7±3	3.±7[c]	22±7[d]
Eugenol	300	100±0	92±6	58±9	3±1	3±1	6±7	2±1[c]	-3±4[c]
Thymol	200	100±0	102±0	68±4	15±7	20±9	88±15	16±26[cd]	8±12[d]
2-*tert*-Butyl-4-Methylphenol	200	45±7	102±0	38±4	15±3	13±1	75±16	54±4[d]	65±31[d]

Tea - A source of anti-bacterial polyphenols

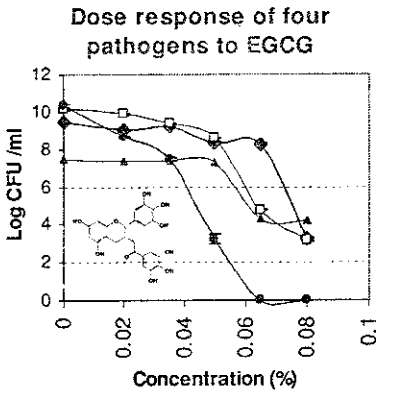
- Bioassay guided separation using high speed counter current chromatography

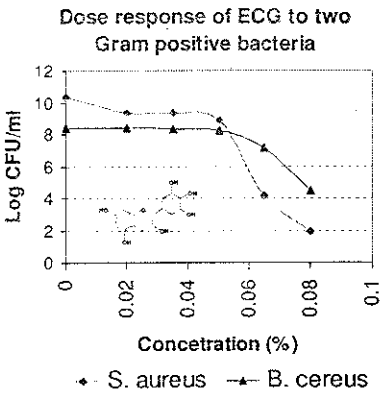
HSCCC - System 1:
Hex: EtOAc: MeOH: H_2O
= (2:8:2:8, v/v/v/v)

HSCCC - System 2:
Hex: EtOAc: MeOH: H_2O
=(1:9:1:9, v/v/v/v)



Dose Responses





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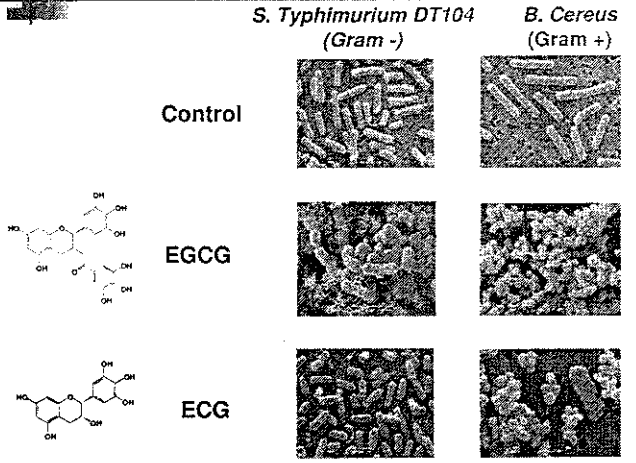

Thank you!

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Review

Separation procedures for naturally occurring antioxidant phytochemicals

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Abstract

Phytochemicals in fruits, vegetables, spices and traditional herbal medicinal plants have been found to play protective roles against many human chronic diseases including cancer and cardiovascular diseases (CVD). These diseases are associated with oxidative stresses caused by excess free radicals and other reactive oxygen species. Antioxidant phytochemicals exert their effect by neutralizing these highly reactive radicals. Among the tens of thousands of phytochemicals found in our diets or traditional medicines, polyphenols and carotenoids stand out as the two most important groups of natural antioxidants. However, although collectively these phytochemicals are good antioxidants, the roles and effect of individual compounds are often not well known. Hundreds of carotenoids and thousands of polyphenols have been identified so far from various plants. A single plant could contain highly complex profiles of these compounds, which sometimes are labile to heat, air and light, and they may exist at very low concentrations in the plants. This makes the separation and detection of these antioxidant phytochemicals a challenging task. The present review focuses on the antioxidant activity, chemical types, sampling and sample processing procedures, and separation using various chromatographic and electrophoretic techniques. Detection and quantification using ultraviolet–visible–diode array and mass spectrometry will be discussed.

Keywords: Review; Antioxidant phytochemicals; Separation; Phytochemicals; Antioxidants; Carotenoids; Polyphenols; Flavonoids; HPLC; HSCCC; LC–MS; SFE; SFC

Contents

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1. Introduction

1.1. Antioxidant phytochemicals and human health

In recent years, many studies have shown that diets containing high content of phytochemicals can provide protection against various diseases. Approximately 90% of all cancer cases correlate with environmental factors, including one's dietary habits, and one-third of all cancer deaths in the United States are avoidable by changing dietary habits only [1,2]. These discoveries have rapidly amplified the consumer awareness of the potential benefits of naturally occurring compounds from plants in health promotion and maintenance, and researches in nutraceuticals and functional foods (NFF) and natural health products (NHP) have been hot topics in recent years [3–5]. The protective effects of fruits, vegetables and spices and herbs were found not only for cancer [5–9], but also other chronic diseases such as cardiovascular diseases (CVD) [10–18].

Among the causes of the major chronic health problems, harmful free radicals and reactive oxygen species (ROS) have been found to play an important role [19,20]. Radicals and ROS such as the superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and peroxy radical ($ROO^{\bullet}$) have been implicated as mediators of degenerative and chronic deteriorative, inflammatory, and autoimmune diseases [4,21], diabetes, vascular disease and hypertension [22–24], cancer and hyperplastic diseases [11,25], cataract formation [11,26], emphysema [27], arthritis, malaria, multiple sclerosis, myocardial ischemia-reperfusion injury [4], immune system decline, and brain dysfunction as well as the aging process [11].

Antioxidants such as Vitamins C and E are essential for the protection against ROS. However, the majority of the antioxidant activity of a fruit or vegetable may be from compounds such as phenolic acids and flavonoids, rather than from Vitamin C, E or β-carotene [28–32]. Intake of controlled diets rich in fruits and vegetables increased significantly the antioxidant capacity of plasma. This increase could not be explained by the increase in the plasma α-tocopherol or carotenoid concentration [33].

Antioxidant phytochemicals such as flavonoids are therefore the focus of many recent studies. The antioxidant activity of these compounds is predominantly determined by their structures, in particular the electron delocalization over an aromatic nucleus, in those based on a phenolic structure. When these compounds react with a free radical, it is the delocalization of the gained electron over the phenolic antioxidant, and the stabilization by the resonance effect of the aromatic nucleus, that prevents the continuation of the free radical chain reaction. This is often called radical scavenging, but polyphenolic compounds inhibit oxidation through a variety of mechanisms [34–37]. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) only tend to have one mode of action, i.e. via free radical scavenging, and are not able to sequester metal ions through the metal catalyzed route [4]. The anticancer activity of flavonoids has been attributed to a large variety of different mechanisms [38].

1.2. Chemical types and sources of antioxidant phytochemicals

Among the different groups of naturally occurring antioxidants from plants, carotenoids and polyphenolics are perhaps the two most important [39,40]. This review therefore will focus on the techniques used in the separation of these two major groups of antioxidants. Other antioxidant phytochemicals such as alkylamides in pepper and Echinacea will also be mentioned. Fig. 1 shows the chemical structures of typical polyphenolics, carotenoids and amides that are known to be antioxidants.

Carotenoids, including xanthophylls (oxygen-containing carotenoids) are naturally occurring coloured compounds that are abundant as pigments in plants. To date, about 500 and 600 specific carotenoids have been identified, mostly from plants and algae [41]. Carotenoids have the capacity to trap not only lipid peroxyl radicals, but also singlet oxygen species [42]. The essential role of carotenoids as a major dietary source of Vitamin A has been known for many years. Although all carotenoids contain extensive conjugated double bonds, individual carotenoids differ in their antioxidant potential in humans [43]. Some have no measurable antioxidant potential in vitro. The true antioxidant capacity of the most prevalent carotenoids in vivo is still in question. The antioxidant capacity of carotenoids may also be related to the structure. Larger conjugated system such as astaxanthin is known to have a higher antioxidant activity [44].

Polyphenolics is a highly inclusive term that covers many different subgroups of phenolic acids and flavonoids. More than 5000 polyphenolics, including over 2000 flavonoids have been identified, and the number is still growing [45]. Polyphenolics vary in structures: hydroxybenzoic acids and hydroxycinnamic acids have a single-ring structure, while

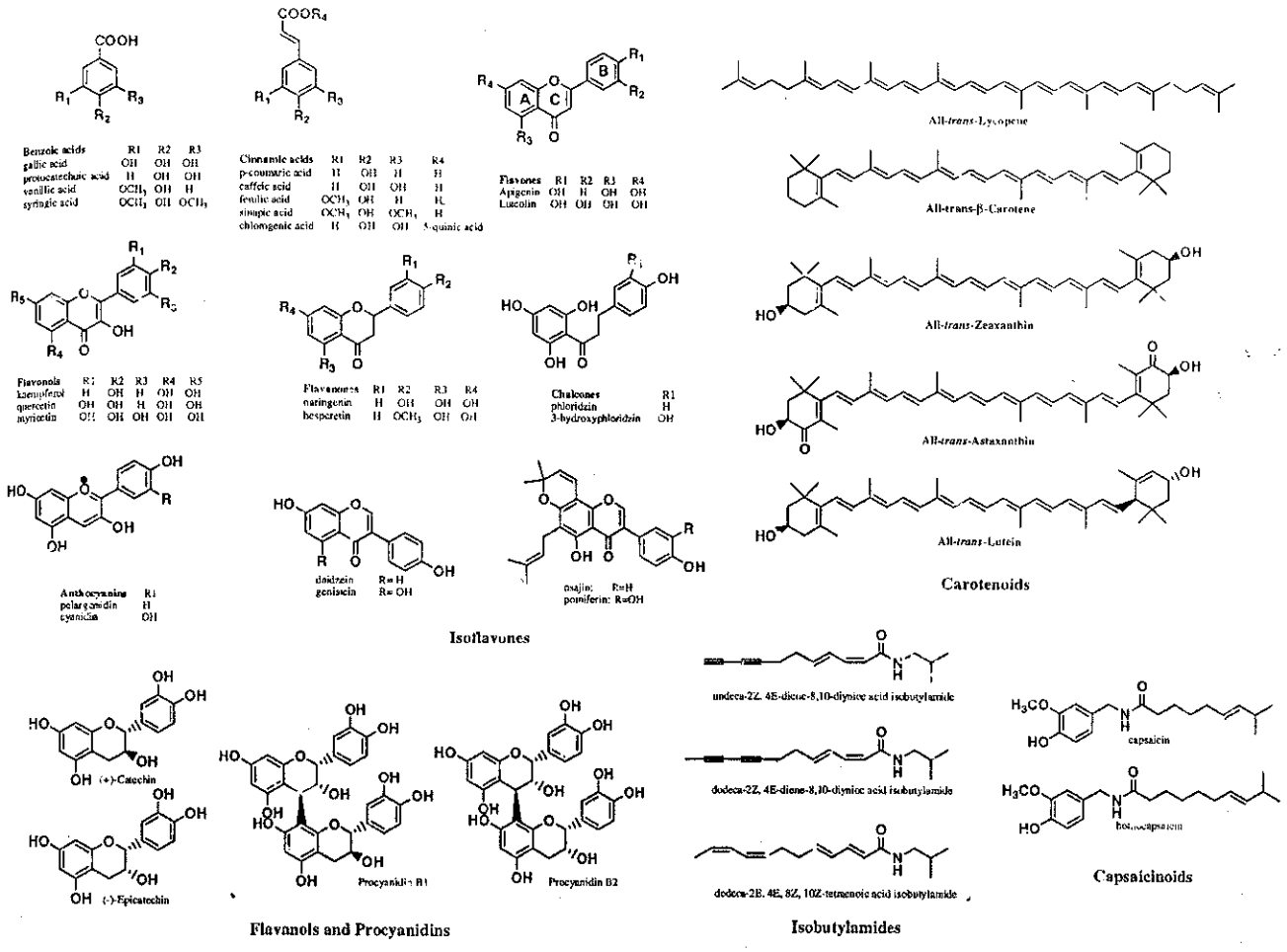

Fig. 1. Chemical structures of major antioxidant groups and representative individual antioxidant phytochemicals.

flavonoids can be further classified into anthocyanins, flavan-3-ols, flavones, flavanones and flavonols. Some of the flavonoids such as flavan-3-ols can be found in dimmers, trimers and polymers (Fig. 1). Many of the phenolics are often associated with sugar moieties that further complicate the phenolic profiles of plants [46]. Polyphenols are especially important antioxidants, because of their high redox potentials, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers [47]. In addition, they have a metal chelating potential [48]. The antioxidant activity of the dietary polyphenolics is considered to be much greater than that of the essential vitamins, therefore contributing significantly to the health benefits of fruits [30].

Flavonoids and related polyphenols are ubiquitous in land plants, and have the general structure as shown in Fig. 1. Flavonoids generally consist of two benzene rings (rings A and B, Fig. 1) linked by an oxygen-containing heterocycle (ring C, Fig. 1). It should be noted that the chalcones are considered by many authorities to be members of the flavonoid family, despite lacking the heterocyclic ring C. The fused A and C rings are often collectively termed the flavonoid nucleus.

1.3. Evaluation of antioxidant activity

Many in vitro models and in vivo methods have been developed for the evaluation of antioxidant activity. However, the interpretation of results obtained from these model systems has to be dealt with caution due to the different methods being based on different mechanisms, resulting in considerably varied antioxidant activity. There is no perfect system available to help us know about the "true" antioxidant power or capacity of a single antioxidant or a complex medium of antioxidant phytochemicals [49,50].

The following are examples of the most frequently used simple in vitro models for the evaluation of total antioxidant activity.

Ferric reducing/antioxidant power (FRAP) assay: The FRAP assay was first introduced by Benzie and Strain [51] for measuring the total antioxidant activity. More recently this method has been modified for the 96-well microplate reader [52], giving better reproducibility and higher throughput of samples. The assay is based on the reducing power of a compound (antioxidant). A potential antioxidant will reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}); the latter forms

a blue complex (Fe^{2+}/2,4,6-tripyridyl-*s*-triazine (TPTZ), which increases the absorption at 593 nm. Stronger absorption at this wavelength therefore indicates higher reducing power of the phytochemical, thus, higher antioxidant activity.

β-Carotene–linoleic acid model system (β-CLAMS): The β-CLAMS method is based on the decolouration of β-carotene by the peroxides generated during the oxidation of linoleic acid at an elevated temperature [53,54]. This method has also been adapted for the 96-well microplate reader recently [52]. Readings are taken at 490 nm immediately after and typically at 15 min time intervals for 100–300 min. Flatter decaying curves indicate the presence of stronger antioxidants.

Oxygen radical absorption capacity (ORAC) method: The ORAC assay was developed by Cao et al. [55,56] and has been used to evaluate the antioxidant capacity of water-soluble phytochemicals. A fluorescent protein, R-phycoerythrin (R-PE) and a peroxyl radical generator, AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride were used in the assay. The excitation and emission wavelengths were set at 540 and 565 nm, respectively.

Thiobarbituric acid reactive substance (TBARS) method: During lipid peroxidation, lipid peroxides are formed, with a subsequent formation of peroxyl radicals, followed by a decomposition phase to yield aldehydes such as hexanal, malondialdehyde and 4-hydroxynonenal. This assay is based on the detection of a stable product, which is formed between aldehydes and thiobarbituric acid (TBA) in the aqueous phase. The production of TBARS was measured spectrophotometrically at 535 nm after an incubation period of 20 min at 80 °C [57].

Trolox equivalent antioxidant capacity (TEAC) method: This assay is based on the relative ability of antioxidants to scavenge the radical cation 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^+$). The radical is generated by the interaction of ABTS with the ferrylmyoglobin radical species, generated by the activation of metmyoglobin with H_2O_2. The extent of quenching of the ABTS radical is measured spectrophotometrically at 734 nm and compared with Trolox, a water-soluble Vitamin E analogue. Results are expressed as Trolox equivalents [58]. Other free radicals such as 2,2-diphenyl-1-picrylhydrazyl ($DPPH^•$) have also been used to measure antioxidant activities [59]. $DPPH^•$ shows an absorbance maximum at 515 nm which disappears upon reduction by an antioxidant phytochemical that has anti-radical property.

Photochemiluminescence (PCL) method [60]: PCL is based on an approximately 1000-fold acceleration of the oxidative reaction in vitro compared to normal conditions. This effect is achieved by optical excitation of a suitable photosensitizer, which exclusively results in the generation of the superoxide radical $O_2^{•-}$. The radicals are visualized with a chemiluminescent detection reagent. A synthetic fluorescent compound luminol is used in this assay. This compound plays a double role acting as both the photosensitizer and the radical reaction agent. A commercial instrument designed specifically for PCL is now available, and the author has found it quite a useful tool. Fig. 2 shows some typical curves of selected polyphenols and plant extracts.

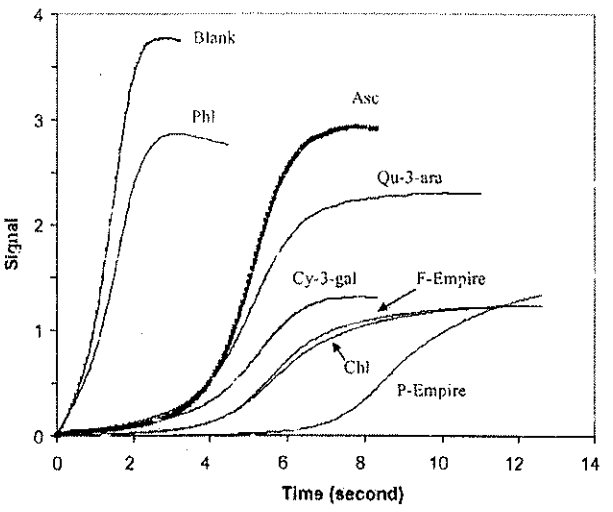

Fig. 2. Antioxidant activities of selected polyphenols and plant extracts measured by PCL method. The longer the lag phase, the stronger the antioxidant activity. The blank had the shortest lag phase. Phl: phloridzin; Asc: ascorbic acid; Qu-3-ara: quercetin-3-arabinoside; Cy-3-gal: cyanidin-3-galactoside; Chl: chlorogenic acid; F-Empire: extract of the Empire apple flesh; P-Empire: extract of the Empire apple peel. Apples were extracted with 70% aqueous methanol (1:1, w/v). Data for the extracts were obtained from a 50-fold dilution of the peel extract, and 10-fold dilution of the flesh extract. All other standards were in 10 μM concentration (R. Tsao et al., unpublished).

1.4. Sample collection, storage and extraction

Due to the vast reservoir of plants, the variation of different parts in a plant, and the diverse chemical structures and physicochemical properties of the antioxidant phytochemicals, it is nearly impossible to have any definitive procedure or protocol for the collection and storage of all plant materials. However, this is perhaps the most important step in the separation of antioxidant phytochemicals, because the aforementioned factors, and many others such as plant variety, growing location and season, may significantly affect the quantity and quality of the phytochemicals. Most traditional oriental medicines are harvested and dried for storage, while at other times, fresh or frozen plant materials have been used. The processing and storage conditions such as drying temperature and duration, thawing method, storage length and humidity therefore may also affect the outcome. In-depth discussion on this topic is beyond the scope of this review, however, it is the author's opinion that sample collection and storage conditions are essential and should be treated carefully.

Extraction method is also critical to the recovery of antioxidant phytochemicals. The nature of both plant materials and the bioactive components should be considered in order to achieve good extraction efficiency. Lipophilicity or hydrophilicity affects the solubility of a phytochemical in the ex-

racting solvent, and conversely, polarity of a solvent also has in impact on the extraction efficiency. Some compounds such is lignans and procyanidins are often in bound or polymerzed forms. Hydrolysis is therefore necessary before the extraction. Many different extraction methods exist for antioxdant phytochemicals, but most of them are based on solvent extraction using water, organic solvent or liquefied gas, or combinations of them under different temperature and pressure, although other methods such as physical press, filtration, steam distillation and solid adsorption (of liquid or head space) have also been used. Enzyme activity of the plants and he existence of oxygen and light during the extraction also mpact the efficiency, therefore extreme care must be taken o avoid hydrolysis, oxidation [61,62] and/or isomerization 63]. Often, due to the analytical difficulties in later separaion procedures, intentional hydrolysis for obtaining the aglyones of some flavonoids or derivatization of some fatty acids o esters may be incorporated into the extraction process.

The extraction procedure is determined by the types of anioxidants to be extracted and whether the objective is quantiative or qualitative. Polar antioxidants such as phenolic acids nd glycosides of many flavonoids are generally extracted usng water, alcohols or a mixture of water and alcohols. For ntioxidants such as aglycones of some flavonoids and most arotenoids, non-aqueous solvents are used.

Methanol is more frequently used than ethanol due to ts higher extraction efficiency. Aqueous methanol between 50 and 80% has been used for extracting hydroxycinnamic cids, and many subgroups of flavonoids. Higher water omposition in the solvent can aid in the extraction of glycoides of these compounds, although due to the complexity of eterosidic combinations, certain groups of flavonoids, such is flavones and flavanols, are not generally characterized as ntact compounds but in the form of their aglycones. For that eason, a hydrolysis procedure before or during extraction is equired [64–67].

Solvent extraction offers good recovery of antioxidant hytochemicals from various samples, however, the use of arge amount of organic solvents poses health and safety risks, nd is environmentally unfriendly. There are many alternaive methods that either eliminate or reduce significantly the se of organic solvents. Some of them offer identical, if not etter, extraction efficiency and cost effectiveness. Methods uch as solid-phase extraction (SPE) use solid absorbents to xtract phytochemicals from liquid matrix such as juices. It s easy, rapid and economical compared to solvent extraction. lowever, SPE is perhaps more often used in sample cleanup, urification or pre-concentration than in extraction because f the selectivity and saturation of the absorbents. The folowing alternative extraction methods, microwave-assisted xtraction (MAE), supercritical fluid extraction (SFE) and ressurized liquid extraction (PLE), due to their increasingly opular uses in the extraction of antioxidant phytochemicals, vill be briefly discussed.

MAE is a relatively new extraction technique that combines microwave and the use of traditional solvent. In MAE, it is important that the extraction solvent has a good polarity, because solvents with high dielectric constants (polar) can absorb more microwave energy, therefore result in better extraction efficiency [68,69]. Water or other polar solvent is therefore often added as modifiers in order to achieve an optimal dielectric constant of the extraction solvent. However, disagreeing opinions also exist: when solvents of low dielectric constants are used, all the microwave energy may be directed to the sample material, the moisture inside the cellular structure absorbs the energy so quickly that it erupts and breaks the cell wall, releasing the phytochemicals to the surrounding solvent. Nonetheless, compared with the conventional solvent extraction, MAE offers many advantages: (1) shorter extraction time, often few minutes instead of hours; (2) less solvent; and (3) higher extraction efficiency.

Certain gases such as carbon dioxide (CO_2) can be liquefied to a state called supercritical fluid when the pressure and temperature are right. Characteristics of a supercritical fluid resemble both a gas and a liquid, and SFE takes advantages of such fluids. The gas-like characteristics help the fluids diffuse to the matrix and access to the phytochemicals, and the liquid-like characteristics provides good solvitation power. SFE has been used in recent years in many applications, and supercritical CO_2 is the most widely used solvent for many antioxidant phytochemicals. The most obvious merit of SFE is the cleanness. CO_2 is chemically inactive, has a low toxicity, and poses virtually no harm to the environment. However, SFE is a highly expensive technology. Extraction efficiency in CO_2 SFE can be optimized by changing the density of CO_2, modifier (e.g. organic solvent), modifier percentage, temperature, time and other parameters. Due to the apolar property of CO_2, it is most suitable for the extraction of antioxidants such as carotenoids and other relatively lipophilic antioxidants [70–72]. For most polyphenolic antioxidants, unfortunately, even though a good recovery rate can be achieved by changing the above-mentioned parameters (often by adding polar solvent), it often significantly offsets the many advantages that SFE offers. For example, in extracting polyphenol antioxidants from green tea, the best extraction yield was found in a system using 95% methanol and 5% CO_2 [73]. Others also found that significant amount of polar organic modifiers has to be added to obtain high extraction yield [74–79]. These studies showed that high concentrations of organic modifiers lead to reduced selectivity. Antioxidants in rosemary have been extracted using SFE [80,81], and it was found that supercritical CO_2 gave higher recovery than typical organic solvents [80].

PLE or accelerated solvent extraction (ASE) has been used for the extraction of bound residues of pesticides and other environmental contaminants. This technology has only recently been used for the extraction of antioxidant phytochemicals [82,83]. In PLE, fast and efficient extraction is achieved by applying high pressure and elevated temperature. It was found that using higher temperatures resulted in higher recovery rates [82,83]. At higher temperatures, although most phenolic antioxidants were stable, others such as catechin

and epicatechin were degraded (ca. 14% at 150 °C) [82]. In extracting catechin and epicatechin from tea and grape seed, it was found that among water, methanol, ethanol and ethyl acetate, methanol had the highest yield [83]. PLE was also applied to extract procyanidins [84].

2. Separation methods

2.1. Conventional chromatography

Paper, packed column and thin-layer chromatographic methods have been used for the separation and purification of many antioxidant phytochemicals. However, due to the lack of good separation efficiency and resolution, and the difficulties in detection, quantification and sensitivity, these conventional chromatographic techniques, particularly the paper chromatography (PC), are not being used as often as before. PC was used to separate flavonoids, cinnamic acids and coumarines from the different tissues and traditional medicinal preparations of dandelions (*Taraxacum officinale*) [85]. In this study, separation was achieved by using multiple 2DPC (two-dimensional) techniques on 3 mm Whatman paper and a mobile phase consisting of *n*-butanol–acetic acid–water (4:1:5) and 15% acetic acid. Thin-layer chromatography (TLC) and open column chromatography (CC) are still being used as separation tools for many antioxidant phytochemicals due to the convenience, low cost, simultaneous separation and detection of considerable amount of samples and the availability of new stationary phases [86]. A considerable number of TLC stationary phases were examined and compared for the separation of carotenoids in paprika. Among the alumina, silica, silica–diatomaceous earth (1:1, m/m), diatomaceous earth, cellulose, polyamide, cyano, diol and amino silica stationary phases, and different combinations of solvent systems, the best separations have been found in adsorption alumina TLC with hexane–chloroform mixtures as mobile phase [87]. The crude extracts of plant materials contain highly complex profiles of phytochemical antioxidants, and often, isocratic separation cannot achieve satisfactory separation. Multiple mobile phases, in regular or 2D TLC are therefore useful for good separation of antioxidant carotenoids from complicated plant materials or extracts [88–91]. Despite these applications, disadvantages such as large requirement in sample amount may restrict the use of TLC and CC because such amount is not always available. Recovery of the antioxidant phytochemicals from the TLC plates or CC could also be challenging [92,93]. The majority of the TLC and CC applications are in the fractionation and preliminary separation of antioxidant phytochemicals before they are separated, quantified and identified by HPLC or other high-performance separation techniques. TLC often has an additional role as a monitoring tool for CC fractionation. Using TLC and CC, nine antioxidants were separated and purified from the aerial parts of St. John's wort (*Hypericum hyssopifolium* L.) [94]. TLC and CC are also used, often in combination, in bioassay-guided fractionation of antioxidant phytochemicals. TLC was used to separate and identify phenolic acids and flavonoids in the water extracts of Lamiaceae family aromatic plants [95]. A strong antioxidant, rosmarinic acid was separated and purified from Summer savory (*Satureja hortensis* L.) using normal phase silica gel and reversed-phase C18 CC [96]. Similarly, several antioxidant phenolic acids and polyphenolics were isolated from the root sample of a traditional medicine *Polygonum multiflorum* Thunb using silica gel and Sephadex LH-20 CC [97]. TLC is also one of the main methods for class fractionation and speciation of lipids [98,99], and is used increasingly to determine the botanical origin, potency, and flavour potential of plant materials (e.g. herbs and spices) [100–102]. Many core and new TLC technologies have been identified and developed in recent years, including: (1) methods to provide a constant and optimum mobile phase velocity (forced flow and electroosmotically-driven flow), (2) video densitometry for recording multidimensional chromatograms, (3) in situ scanning mass spectrometry, and (4) bioactivity monitoring for selective detection [103]. These technologies, in combination with 2D, multiple development and coupled column–layer separation techniques could dramatically increase the use of TLC for the characterization of complex mixtures such as plant extracts containing phytochemical antioxidants [103].

2.2. Gas chromatography

Despite the high resolution and sensitivity of GC, due to the lack of volatility of the majority of plant derived antioxidants, its use in the separation has not been as popular as the high-performance liquid chromatography (HPLC). Application of GC is also limited because of the difficulty of large-scale separation and purification. Separation of antioxidant phytochemicals by GC has mostly been attempted for compounds in the essential oils of herbs. Depending on the physicochemical property of the antioxidants, columns of different polarity and lengths have been used in the separation. GC with a capillary column and a MS detector is the predominant system. A column with medium polarity (e.g. DB-5, with 5% biphenyl and 95% dimethylpolysiloxane) was found to give the best results in the separation of antioxidants from *Crataegus oxyacantha*, *Hamamelis virginiana* and *Hydrastis canadensis* [104]. The essential oil of *H. virginiana* showed the strongest antioxidant activity, and its major active component was identified to be 1,2,3-trihydroxybenzene, a phenolic compound by GC. Other studies also indicated that phenolic components in essential oils are the major contributor of the antioxidant activity [105,106]. Among the phenolic compounds of essential oils, carvacrol and thymol are probably the two most recognized antioxidants, typically found in thyme and oregano, respectively [107,108]. GC and GC–MS were used to separate alkylamides in Echinacea [109].

2.3. High-performance liquid chromatography

There is increasing need to know the photochemical profiles of antioxidants in different plants, and among different

varieties of the same plant, but conventional chromatographic techniques (PC, TLC and CC) in general lack the sensitivity and resolution that are often required for trace amount of antioxidant phytochemicals. GC meets these requirements, but its use is somewhat limited due to the non-volatility of many antioxidants. As most researchers would agree, HPLC is perhaps the most popular and reliable system among all chromatographic separation techniques for the separation of antioxidant phytochemicals. The versatility of HPLC is also aided by the different separation modes and types of detection methods, among which is the diode array detector (DAD) coupled with mass spectrometer (MS).

2.3.1. HPLC for carotenoid antioxidants

The lipophilic characteristics of carotenoids have made normal phase HPLC a more favourable choice for the separation of these phytochemical antioxidants. The majority of adsorption HPLC techniques used for the analysis of carotenoids employed silica stationary phase [86]. Separation of saponified carotenoids was carried out on a silica column (250 mm × 4.6 mm i.d., 5 μm) using gradient elution from 95% of light petroleum to 95% acetone [110]. With reversed-phase HPLC, C8 and C18 columns have been proven well suited for routine separations of carotenoids [111–113]. Piccaglia et al. [114] used a C18 column and achieved relatively good separation of free lutein, three lutein monoesters and five lutein diesters. However, for more complex samples, particularly those high in esters, a C30-column seems to have better separation and selectivity than the conventional C8 and C18 materials. RP C30 column is particularly a good choice for the separation of geometric isomers of carotenoids [115–121]. In a method by Sander et al. [122] it was found that with monomeric C18 column, non-polar carotenoid isomers were poorly resolved, and lutein and zeaxanthin were not separated. Better separation of the hydrocarbon carotenoids was possible with the polymeric C30 column. In monomeric C18 or C30 columns, the silica was treated so the surface has thorough endcapping, whereas in polymeric C18 or C30 columns, the material was synthesized from polyfunctional silanes which produce crosslinking of the hydrophobic phase on the silica surface. In the same paper Sander et al. also found that the retention behavior of lycopene varies dramatically with stationary phase properties. With monomeric C18 columns, lycopene usually elutes before α- and β-carotene, whereas with polymeric C18 and C30 columns, lycopene is strongly retained and elutes after these carotenoids [122]. Most recently, using C30 LC–MS, Breithaupt et al. were able to identify eight regioisomeric monoesters in addition to known lutein mono and diesters [123]. Geometric isomers of free carotenoids have been separated using mainly C30 columns, however, we recently developed a method using RP C18 column in combination with DAD and MS detection, and for the first time, separated several *cis* isomers of lutein diesters [124]. Several good review papers have been published in recent years on the separation of carotenoids, and readers are referred to those for more detailed discussions [111,125,126].

2.3.2. HPLC for polyphenolic antioxidants

For the separation of phenolic acids and flavonoids, the chromatographic conditions of the HPLC methods include the use of, almost exclusively, a reversed-phase C18 column; UV–vis diode array detector (DAD), and a binary solvent system containing acidified water (solvent A) and a polar organic solvent (solvent B). The separation normally requires 1 h at a flow rate of 1.0–1.5 mL/min. Solvent A usually includes aqueous acids or additives such as phosphate. Solvent B is normally pure or acidified methanol or acetonitrile. Vast amount of literature exists. Antioxidant flavonoids including rutin and chlorogenic acid in Solidago plants were separated using HPLC-UV [127]. Among the numerous separation systems, only a few procedures were developed to specifically measure polyphenolic concentrations in several commonly consumed foods [46]. Most of these methods have been developed to measure different groups of polyphenolics in a single plant, or a single or a few groups in multiple plant sources. Van Sumere et al. [128] indeed developed a good method that separated nearly 50 phenolic compounds from the rose flower pedals. However, some important antioxidant polyphenols such as procyanidins, chlorogenic acid and phloretin-glycosides were not included in their method. A method by Paganga et al. [129] and two other recent HPLC methods by Schieber et al. [130] and Shui and Leong [131] were developed for the separation and measurement of prominent flavonoids that are members of the subgroups of flavonoids; anthocyanins and procyanidins, however, were not included in their methods. Some methods such as those developed by Escarpa and Gonzalez [132,133], separated multiple groups of the most prominent phenolics with a relatively short analysis time, an obvious advantage for those who are interested in analyzing the major phenolic components. In shortening the analysis time, however, some minor or unknown compounds may have been missed due to co-elution. The co-elution may also affect the quantification of known compounds. Obtaining good resolution is considered to be the main difficulty for a method that is targeted for separation of multiple polyphenolic groups [131]. A method with improved separation was recently developed in the author's group using a binary mobile phase consisting of 6% acetic acid in 2 mM sodium acetate aqueous solution (v/v, final pH 2.55) (solvent A) and acetonitrile (solvent B) and a RP C18 column. The use of sodium acetate was key to the near baseline separation of 25 phenolics commonly found in fruits [134]. Such improved separation is particularly informative in terms of phytochemical profiling and quantification. Detection in HPLC is routinely achieved by UV absorption, often using DAD, however, DAD has mostly been used as a convenient multiple wavelength detector, and its versatility often appears to have been neglected [135]. The hydrophilicity of polyphenols is relative and it spans over a wide range. Oligomeric procyanidins for example are relatively less hydrophilic. Although RP-HPLC has been the primary separation means for the procyanidins, past studies illustrates the difficulty in determining the degree of polymerization of these antioxi-

dants. Hammerstone et al. therefore have developed a normal phase (NP) HPLC method that utilized a series of linear gradients of methanol into dichloromethane with constant amount of acetic acid and water [136]. For the detailed separation method for polyphenols, readers are referred to a recent book by C. Santos-Buelga and G. Williamson [137].

2.3.3. HPLC for other antioxidant phytochemicals

Although carotenoids and polyphenols are the two major antioxidant phytochemicals, many other naturally occurring antioxidants are found in plants [138]. Among them, alkylamides from the chrysanthemum plants and capsaicinoids from the chili peppers are strong antioxidants of particular interest. Isobutylamides in Echinacea were separated using a C8 or C18 HPLC with UV and MS detectors [139–142]. Using HPLC–DAD and HPLC–MS-SIR (selected ion recording), Luo et al. [139] were able to simultaneously separate and identify 12 isobutylamides and other phytochemicals in *Echinacea purpurea*. Capsaicin and related compounds also belong to amide group of phytochemicals and they are good antioxidants as well. Separation of capsaicinoids have been carried out using a MetaSil Basic C2–C8 RP-HPLC column and detected and identified by UV and MS or MS–MS [143].

2.3.4. HPLC with non-adsorption columns

The most frequently used NP and some RP-HPLC techniques for antioxidant phytochemicals are based on an adsorption/desorption mechanism. However, other modes have been used for the separation of some antioxidant phytochemicals. Procyanidins, for example, were separated by size-exclusion chromatography (SEC). Using a TSK gel α-2500 column, and a mobile phase consisting of acetone and 8 M urea (pH 2) (6:4), procyanidins with various degrees of polymerization were separated in native forms from apple and other plant extracts [144,145]. Some other separation modes such as ion exchange chromatography (IEC) have been used for the separation of antioxidant phytochemicals such as anthocyanins. However, these techniques are often used in combination with conventional RP-HPLC (e.g. C18). For instance, ion exchange resins such as Amberlite XAD-7 are often used to separate anthocyanins from other highly water soluble interference like sugars. Anthocyanins separated by IEC are often further purified on a Sephadex LH-20 column before finally being analyzed on a RP C18 column [146].

2.4. High-speed counter-current chromatography

Separation by counter-current chromatography (CCC) is based on the partition coefficient (K) of a phytochemical. High-speed CCC (HSCCC) is a relatively new technology and it is the most advanced CCC form in terms of partition efficiency and separation time. The separation in HSCCC is aided by pressure and centrifugal force; the latter is generated from both rotational and synchronous planetary motion of coiled columns. The force provides vigorous mixing between the two immiscible liquid phases, and retention of a very large fraction of the stationary phase [147]. Unlike other chromatographic techniques, HSCCC does not use solid support as the stationary phase, therefore has many advantages over conventional chromatography: (1) the elimination of sample loss caused by irrecoverable adsorption to the solid support matrix; (2) easy scale-up to larger fractionation system by simply changing the Teflon tubing coil (column) to larger sizes; (3) low-cost because it does not use expensive absorbents and columns; and (4) it reflects the real distribution profile of phytochemicals in a sample [148,149]. The most important step in developing a good HSCCC method is perhaps the determination of the K-value of an analyte in different two-phase liquid systems. This is normally done by dissolving a small amount of analyte in the same volume of each phase of the pre-equilibrated two-phase solvent system. The two solutions were mixed, shaken vigorously for 10 min, centrifuged at 4000 × g for 5 min to obtain a thorough equilibrium. An aliquot of each phase was then analyzed by HPLC or a spectrophotometer. The K-value was expressed as the concentration or absorbance of the phytochemical of interest in the upper phase divided by that in the lower phase. It is generally recognized that the K-value of the target antioxidant phytochemical must be in the range of 0.2–5 in a given two-phase system in order to obtain good separation [150]. Low K-values will result in a poor peak resolution, while high K-values tend to produce excessive sample band broadening [151]. In addition to the K-value, a suitable two-phase system should also have a satisfactory retention of the stationary phase and short settling time of the two solvents (<30 s) [151]. For more in-depth information on HSCCC theories readers are directed to a general review by Conway [152].

Although the first CCC separation of antioxidant phytochemicals was done nearly two decades ago by Putman and Butler for the separation of condensed tannins [153], a great number of HSCCC applications have been reported in the past several years, particularly in the separation and preparation of active ingredients from traditional herbal medicines including antioxidants [147–166]. Procyanidins in apple, were successfully separated by using type-J multilayer coil planet centrifugation with a two-phase solvent system composed of *tert*-butylmethylether–acetonitrile–water and/or a system containing methyl acetate–water [154,155]. Chlorogenic acid, an antioxidant found in apple and other fruits was separated with high purity and recovery rate from a traditional Chinese medicine *Flos Lonicerae* using HSCCC with a two-phase solvent system containing *n*-butanol–acetic acid–water (4:1:5) [147]. HSCCC has also been applied to separate more lipophilic phytochemicals such as carotenoids. A two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (5:5:6.5:3) was successfully used to separate a strong antioxidant astaxanthin [148].

2.5. Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) is a new technology similar to HPLC, however, due to the use of super-

critical fluid such as carbon dioxide, it has several advantages over regular HPLC [167,168]: (1) the use of supercritical fluid such as carbon dioxide significantly reduces solvent waste, and makes it easier in removing the solvent when collecting fractions; (2) antioxidant phytochemicals have higher diffusivity in a supercritical fluid, because such liquid has low viscosity, therefore leads to more homogeneous diffusion of the antioxidant compounds into the packing materials, resulting in higher resolution and faster separation time; and (3) parameters such as temperature, pressure, and fluid composition of the mobile phase can be changed therefore give more venues for better separation [169].

Most of the SFC related literature is on the separation of relatively lipophilic antioxidants. This is not surprising because carbon dioxide, which is non-polar, is the most popular supercritical fluid. The simultaneous separation of *cis*- and *trans*-α- and β-carotenes was achieved using SFC [170,171]. Separation of geometric isomers of α- and β-carotene was also performed on a capillary column [172]. The results are very encouraging in terms of separation and gain in analysis time. Other antioxidants have also been separated by SFC. SFE extract of *Artemisia annua* L., was analyzed by supercritical fluid chromatography (SFC) using a capillary column, coupled with a flame ionization detector (FID). With optimized operating conditions, artemisinin and artemisinic acid were quantitatively extracted at a flow rate of 2 mL/min in less than 20 min. The supercritical fluid was composed of carbon dioxide and 3% methanol with temperature and pressure fixed at 50 °C and 15 MPa, respectively. Results were compared with two conventional liquid solvent extraction processes [173]. Natural α-tocopherol from γ- and δ-tocopherols were also separated by SFC, and the effects of pressure, temperature and the ethanol concentration in the mobile phase on the retention factor and resolution of tocopherols were studied comprehensively by Jiang et al. [174]. A method using normal-phase SFC with methanol as modifier has been developed for determination and quantification of the various indol-3-ylmethyl derivatives including ascorbigens formed from the glucobrassicin degradation product, indol-3-ylmethanol, under acidic conditions (pH 2–6) with and without the presence of ascorbic acid. The SFC method had detection limits in the 10–100 pmol range [175].

2.6. Capillary electrophoresis

Although HPLC stays as the most dominating separation technique for antioxidant phytochemicals, capillary electrophoresis (CE) is gaining popularity. Like HSCCC and SFC, CE is also a relatively new technique; however, it represents an alternative method for the analysis of different groups of antioxidant phytochemicals [176,177]. CE has several unique advantages compared to HPLC [178]: (1) it requires a very small sample size, (2) high efficiency due to non-parabolic fronting; (3) shorter analytical time; (4) low cost, particularly when use capillary zone electrophoresis (CZE) and fused-silica capillary; and (5) use no or only small amount of organic solvent therefore limits solvent waste [178–183]. Separation of antioxidant phytochemicals in capillary electrophoresis is based on the differences in mass to charge ratios of these compounds, and complex formation with tetraborate molecules when the phenolic compound has *ortho*-hydroxy groups. There are different modes in CE separations. CZE is the simplest mode and has been used to separate various types of antioxidant phytochemicals, particularly phenolic compounds [184–190]. Several CZE methods were developed for the separation of polyphenolic antioxidants such as epicatechin, catechin, quercetin, gentistic acid, caffeic acid, gallic acid and *trans*-resveratrol, myricetin and rutin in wine and grape samples [189,191]. Antioxidants in Ginkgo leaf infusates were also separated using a CZE system [192]. A recent CZE method was developed for the separation of anthocyanins in wine [193]. This method had comparable quantitative results with the HPLC method, but it significantly reduced the analysis time by nearly 75%. da Costa et al. separated anthocyanins from blackcurrant (*Ribes nigrum*) using CZE, and found that resolution and peak shapes of the anthocyanins were critically influenced by the pH of the running buffer and the presence of an organic solvent. Optimum qualitative separation was achieved on a fused-silica capillary with a phosphate running buffer containing 30% (v/v) acetonitrile at an apparent pH of 1.5 [194,195]. In CZE, modifiers such as organic solvents are often added to the running buffer to increase resolution of phytochemicals [182,196]. Addition of a modifier can reduce the viscosity, lower the zeta potential of the capillary wall, and increase selectivity and resolution. The major phenolic diterpenes responsible for the antioxidant properties of rosemary extracts, carnosol and carnosic acid, were separated by capillary zone electrophoresis (CZE) using a 56 cm long uncoated fused-silica capillary and a 50 mM disodium tetraborate buffer of pH 10.1. The CZE method had good reproducibility (relative standard deviation less than 5%) and the separation of carnosol and carnosic acid was accomplished in less than 11 min [197]. CZE was also used to separate puerarin, daidzein and rutin, antioxidants from the traditional Chinese medicinal plants, *Pueraria lobata* (Wild.) Ohwi and *Puerariae Radix* [198] and farrerol, quercetin, syringic acid, vanillic acid, 4-hydroxybenzoic acid, protocatechuic acid in *Rhododendron dauricum* L. [185].

Micellar electrokinetic chromatography (MEKC) uses surfactants such as sodium dodecyl sulfate (SDS) which form highly organized spherical micelles at concentrations above the critical micellar concentration (CMC) with the lipophilic tails toward the interior and the hydrophilic ends on the surface of the micelle. This creates an additional separation mechanism through partition. Compounds with different *K*-values can then partition differently between the micelles and the mobile phase (running buffer). This mode is particularly important for the separation of different neutral antioxidants. The CMC for SDS is typically 20 mM. The MEKC technique has been used for the separation of polyphenolic antioxidants [199–205]. Organic modifiers were also incorporated into the

MEKC system in some of these methods to increase the separation efficiency and resolution. Antioxidants from rosemary were determined using a new MEKC method, and found that MEKC had slightly lower reproducibility in peak area, but similar in retention time. However, the main advantage of MEKC is its much higher separation speed [206].

CE as a separation technology is still rapidly evolving and new modes of separation are being developed. Among them, a hybrid technique combining solid-phase matrix such as C18 and C30 polymers with high voltage electrophoresis has been found very useful. This new mode is called capillary electrochromatography (CEC). Sander et al. first reported the use of polymeric C30 stationary phases in CEC for the separation of carotenoid isomers [207,208]. This method was able to separate lycopene isomers, β-carotene isomers, α-carotene isomers, lutein isomers, zeaxanthin isomers and β-cryptoxanthin isomers in 35 min.

3. Quantification and online identification

The main purpose of this review is to give up-to-date information on the separation of antioxidants in plants. Methods for detection, quantification and online identification are inseparable from many of the above discussed separation techniques, however, they are ultimately a means for the confirmation of good separation. The above discussed separation modes (HPLC, CE, SFC, HSCCC) often share the same detection techniques, particularly UV–vis–DAD and MS detectors.

3.1. UV–vis and diode array detection

UV–vis spectrophotometry has been long used for quantitation of organic compounds that absorb light in the ultraviolet and visible region. Most antioxidant phytochemicals have highly conjugated double bond or aromatic systems that absorb light in this region. The application of UV–vis detector in separation technologies, particularly the combination of DAD and HPLC has gone beyond quantification by light absorbance. HPLC–DAD has played important roles in the identification of antioxidant phytochemicals, particularly polyphenolic compounds and carotenoids. In HPLC–DAD, the spectral information of known standards can be obtained online and saved as a library database. The UV–vis spectral data of all eluting peaks of a sample can be scanned, stored and later retrieved for comparison with the library data. A match of both UV–vis spectrum and retention time can lead to highly positive identification of an antioxidant phytochemical. As a detector, DAD is also capable of simultaneously detect and record chromatograms at different wavelengths. This feature significantly enhances the performance of the separation system, particularly when different groups of antioxidant phytochemicals are mixed in one sample. When proper wavelengths are chosen, e.g. at the maximum absorptions, all groups of antioxidant phytochemicals can be detected with the highest sensitivity [134]. An appropriate selection of the detection wavelength can also make possible the quantification of an unresolved or poorly resolved peak. DAD can also be used to examine the purity of a peak. In-depth discussion of HPLC–DAD and its use in identification of phytochemicals are beyond the scope of this paper, and there are several excellent reviews recently published if readers want to obtain further information [111,209].

Although UV–vis and DAD provide useful information for the identification of antioxidant phytochemicals, the use of conventional approaches based on spectra is often limited when samples contain very similar compounds. Unambiguous identification of structures cannot be done using UV–vis and DAD spectral data only [209]. For complete structural identification, other techniques such as MS and NMR are often necessary. Having said that, the combination of DAD and MS in HPLC has been a highly useful tool in the separation and determination of antioxidant phytochemicals. DAD is particularly useful in the selective detection of antioxidants with distinct UV–vis absorption patterns. Good examples of such compounds can be the carotenoids and anthocyanins. All carotenoids have the characteristic absorption pattern in the visible region between 410 and 470 nm. There are normally three absorption maxima at 410, 440 and 476 nm, although slight shifts may occur depending on the structural differences. A strong absorption at 330 nm indicates the *cis*-configuration [124]. In terms of polyphenolic antioxidants, certain subgroups can be separated by monitoring at different maximum UV–vis absorption (λ_{max}). The λ_{max} for the cinnamic acid and its derivatives is near 320 nm, and that for the benzoic acids, flavan-3-ols (including the dimers) and dihydrochalcones is about 280 nm. The λ_{max} for the flavonols is usually around 360 nm. Among the flavonoids, however, anthocyanins are the most unique subgroup because they absorb visible light near 520 nm when the molecules are in the flavylium cation status (when pH is low). This spectral characteristic gives anthocyanins advantages in being detected without the interference from other groups of phytochemicals. The maximum wavelength of absorption in the visible region for anthocyanins is found to be related to the substituent pattern in the B ring (Fig. 1).

3.2. Mass spectrometry and tandem mass spectrometry

Mass spectrometry (MS) can be carried out online coupled with chromatographic or electrophoretic techniques or offline as a stand-alone instrument. However, it is the former that provides unsurpassed opportunities in the identification and structure elucidation of antioxidant phytochemicals. There are two main types of ionization techniques for the antioxidant phytochemicals, the ion-spray techniques such as electrospray ionization (ESI), thermospray and atmospheric pressure chemical ionization (APCI), and the ion-desorption techniques which include fast atom bombardment (FAB), plasma desorption (PD), and matrix-assisted laser desorption ionization (MALDI). ESI and APCI are the two most widely used ionization methods for antioxidant phytochemi-

cals, and most commercial chromatography–mass spectrometry (LC–MS) instruments can accommodate both of these techniques. Although there is no clear line, ESI is more often used to ionize antioxidant molecules such as anthocyanins that are polar and exist as ions in aqueous solutions, and APCI is used for less polar and non-ionic antioxidants such as carotenoids [210]. APCI and ESI can be operated under both positive and negative ion modes (PI and NI). The most frequently used mass analyzers can also be separated into two main groups: analyzers based on ion beam transport such as magnetic field, time-of-flight (TOF), and quadruple mass filter; and those based on ion trapping technology. These analyzers vary in their capabilities with respect to resolution, accuracy and mass range. MS detector is critical for the identification of antioxidant phytochemicals because of the complex and diverse structures, and low concentrations in the plants. Sensitivity and selectivity of detection can be increased using tandem mass spectrometry, i.e. two (MS–MS) or more (MS^n) mass analyzers coupled in series. MS–MS and MS^n produce more fragmentation of the precursor and daughter ions, therefore, provide additional structural information for the identification of antioxidant phytochemicals. There are many excellent recent reviews on the application of LC–MS in quantitative and qualitative analyses of phytochemicals including antioxidants [121,135,195,211–215]. Flamini [216] has summarized the use of LC–MS in studies of polyphenols in grape extracts and wine. He specifically indicated that LC–MS techniques are the most effective tool in the study of the structure of anthocyanins, particularly the MS–MS approach which is a very powerful tool that permits anthocyanin aglycone and sugar moiety characterization. In the same review, other LC–MS techniques such as the matrix-assisted-laser-desorption-ionization–time-of-flight (MALDI–TOF) was also discussed by the author for the analysis of procyanidin oligomers.

Although PI-MS were used for the detection of various antioxidant phytochemicals, it was found that NI-MS methods, both APCI and ESI were excellent for flavonoid analysis, both in sensitivity and specific structural information [217]. Data reported in this paper showed that ESI was the method of choice for the analysis of low-molecular-mass phenols under NI mode, whereas flavan-3-ol compounds were well detected under both positive and negative ion. Negative LC–APCI–MS and low-energy collision induced dissociation (CID) MS–MS were used to provide molecular mass information and product-ion spectra of the flavonoid glycosides in some herbs [218]. Detection of phytochemicals including antioxidants has been subjected to many recent reviews [210,211].

4. Conclusion

The human health benefits of phytochemicals have been shown by many recent studies, and the roles of antioxidant phytochemicals as a whole have also been clearly demonstrated. However, despite the strong evidence that many groups of phytochemicals have good antioxidant activity both in vitro and in vivo, our knowledge about the biological function of individual antioxidant phytochemicals is lacking. Some of the biggest hurdles may include the low concentration, instability and difficulty in separation and detection of these bioactive compounds. In this review, the authors intend to condense some of the latest technologies that have been applied to the separation of antioxidant phytochemicals. The authors also provided some background information about the antioxidants chemistry and biochemistry, and their links to the health benefits. It is the authors' sincere wish that by emphasizing on the major antioxidant groups, i.e. carotenoids and polyphenolics, readers will be encouraged to carry out further studies on the development of new separation techniques and apply to these and other groups of antioxidant phytochemicals.

5. Nomenclature

2D	two-dimensional
AAPH	2,2′-azobis(2-amido-propane)dihydrochloride
ABTS	2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate
APCI	atmospheric pressure chemical ionization
ASE	accelerated solvent extraction
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
CC	column chromatography
CCC	counter current chromatography
CE	capillary electrophoresis
CEC	capillary electrochromatography
CID	collisionally-induced dissociation
β-CLAMS	β-carotene–linoleic acid model system
CMC	critical micellar concentration
CVD	cardiovascular disease
CZE	capillary zone electrophoresis
DAD	diode array detector (or diode array detection)
ESI	electrospray ionization
FAB	fast atom bombardment
FRAP	ferric reducing/antioxidant power
GC	gas chromatography
GC–MS	gas chromatography–mass spectrometry
HPLC	high-performance liquid chromatography
HSCCC	high-speed counter current chromatography
IEC	ion exchange chromatography
K-value	partition coefficient
LC	liquid chromatography
LC–MS	liquid chromatography–mass spectrometry
MAE	microwave-assisted extraction
MALDI	matrix-assisted laser desorption ionization
MEKC	micellar electrokinetic chromatography
MS	mass spectrometer (or mass spectrometry)
MS–MS	tandem mass spectrometry
NHP	natural health products
NFF	nutraceuticals and functional foods

NI	negative ion
NMR	nuclear magnetic resonance
NP	normal phase
$O_2^{\bullet -}$	superoxide anion
$^{\bullet}OH$	hydroxyl radical
ORAC	oxygen radical absorption capacity
PC	paper chromatography
PCL	photochemiluminescence
PD	plasma desorption
PI	positive ion
PLE	pressurized liquid extraction
P-PE	R-phycoerythrin
$ROO^{\bullet}$	peroxyl radical
ROS	reactive oxygen species
RP	reversed-phase
SDS	sodium dodecyl sulphate
SEC	size-exclusion chromatography
SFC	supercritical fluid chromatography
SFE	supercritical fluid extraction
SIR	selected ion recording
SPE	solid-phase extraction
TBARS	thiobarbituric acid reactive substance
TEAC	trolox equivalent antioxidant capacity
TLC	thin-layer chromatography
TOF	time of flight
UV	ultraviolet
vis	visible

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Detection of saponins in extract of *Panax notoginseng* by liquid chromatography–electrospray ionisation-mass spectrometry

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bstract

The liquid chromatography–electrospray ionisation-mass spectrometry (LC–ESI-MS) method was developed for the analyses and identifiion of saponins in plant extract from the root of *Panax notoginseng* (Burk.) F.H. Chen. The HPLC experiments were proceeded by means a reversed-phase C18 column and a binary mobile phase system consisting of 0.2% acetic acid and acetonitrile under gradient elution conlions. Eight major peaks were separated and detected using both evaporative light scattering and MS detectors. The mass spectrometer was erated in the negative ion mode using electrospray ionization. The molecular ions, $[M-H]^-$ and the adduct ions $[M+AcO]^-$ of saponins re observed, and from which the molecular weights were obtained. A collision-induced dissociation (CID) experiment was carried out aid the identification of the backbone and glycosidic linkage sites of the saponins. The identification of the saponins (peaks 1–7) in the tract of *P. notoginseng* was based on matching their retention times, the detection of the saponin molecular ions, and the fragment ions of e molecular ion obtained in the CID experiments with those of the authentic standards and data reported in the literature. The molecular ructure of peak 8 was elucidated according to the fragmentation patterns and the literature reports.
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ywords: *Panax notoginseng*; Saponins; LC–ESI-MS; CID

Introduction

Panax notoginseng (Burk.) F.H. Chen (Chinese name: inQi or TianQi) is a traditional Chinese medicine (TCM) at has been used to treat cardiovascular diseases, differt pains, bruise, and hemostasia [1–3] in China. The mar bioactive constituents of *P. notoginseng* are dammarane ponins such as ginsenosides and notoginsenosides [4,5], hich are usually obtained from the roots of this plant. The ructures of saponins from *P. notoginseng* studied in this vestigation are showed in Fig. 1.

The profile of the saponins in *P. notoginseng* was similar to those in *Panax ginseng* and *Panax quinquefolium* [4,5]; however, compared to the many reported methods on *P. ginseng* and *P. quinquefolium*, analytical methods using high performance liquid chromatography (LC) for *P. notoginseng* are scarce. UV has been the detector of choice for the detection of saponins in *P. notoginseng* in reported LC methods, however due to poor absorbance of these compounds in the UV region, the detectors are often set at 198–205 nm, which greatly increases the baseline noise and lowers the sensitivity of the detection [6–8]. Some methods using LC–evaporative light scattering detection (LC–ELSD) have been found to provide a stable baseline even with a gradient elution and have been successfully applied to the analysis of saponins in *P. ginseng* [9–11]. Only one paper has reported on the analysis of saponins from *P. notoginseng* using LC–ELSD [12].

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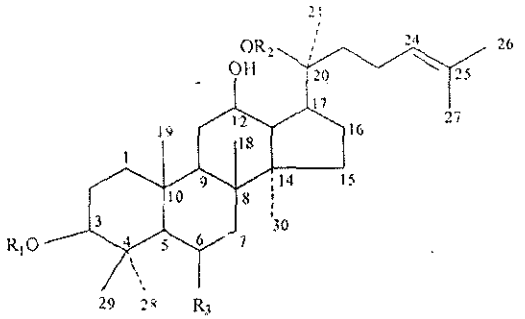

saponins	R1	R2	R3	Molecular mass
Notoginsenoside R1	-H	-Glc	-O-Glc$\overset{2-1}{—}$Xyl	932
Ginsenoside Rg1	-H	-Glc	-O-Glc	800
Ginsenoside Re	-H	-Glc	-O-Glc$\overset{2-1}{—}$Rha	946
Ginsenoside Rb1	-Glc$\overset{2-1}{—}$Glc	-Glc$\overset{6-1}{—}$Glc	-H	1108
Ginsenoside Rc	-Glc$\overset{2-1}{—}$Glc	-Glc$\overset{6-1}{—}$Ara(f)	-H	1078
Ginsenoside Rb2	-Glc$\overset{2-1}{—}$Glc	-Glc$\overset{6-1}{—}$Ara(p)	-H	1078
Ginsenoside Rd	-Glc$\overset{2-1}{—}$Glc	-Glc	-H	946
Notoginsenoside K	-Glc$\overset{6-1}{—}$Glc	-Glc	-H	946

Abbreviations: Glc, β-D-glucose; Ara(p), arabinose in pyranose form; Ara(f), arabinose in furanose form; Rha, α-L-rhamnose; Xyl, β-D-xylopyranosyl

Fig. 1. Structures of saponins in *P. notoginseng* identified in this study.

Among various methods that have been applied to the analysis and identification of ginsenosides from the extract of *P. ginseng* and *P. quinquefolium*, LC–MS appears to be most favorable and capable [10,13–15]. Only one paper reported the analysis the saponins in *P. notoginseng* using LC–MS in the positive ion mode, however, the method provided only limited structural information of saponins [6].

In the present paper, a simple, direct and reliable LC–ESI-MS method for the identification of saponins in the crude extract from the root of *P. notoginseng* was reported. Structural information of the saponins was obtained by using the collision-induced dissociation (CID) technique.

2. Experimental

2.1. Reagents and materials

Acetonitrile and methanol were of HPLC grade from Fisher Chemicals (USA), the other reagents were of analytical grade from Beijing Chemicals (China). Water was purified using a Milli-Q water purification system (Millipore, France). Ginsenosides Re, Rg1, Rb1, Rb2, Rc, Rd and notoginsenoside R1 were purchased from Jilin University (China). *P. notoginseng* root powder was purchased from Beijing TongRenTang Medicinal Store (China).

2.2. Preparation of samples

P. notoginseng root powder (1.0062 g) was immersed in 50 mL of methanol and extracted using ultrasonication for 1 h at room temperature. The mixture was filtered through a Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England), and the filtrate was evaporated to dryness using a rotary evaporator at <40 °C. The residue was then dissolved in 10 mL methanol and filtered through a 0.45 μm membrane before being used for the LC–ESI-MS analyses.

2.3. LC–ELSD analysis

An LC system consisting of a Waters 600 pump equipped with a gradient controller, an automatic sample injector (Agilent 1100 Series), and an ELSD (Alltech 2000) was used. The separation was performed on a Capcell Pak C_{18} column (250 mm × 4.6 mm, 5 μm) (Shiseido, Japan), and the column temperature was kept at 35 °C using a column heater–cooler (Agilent 1100 Series, USA). A binary mobile phase consisted of 0.2% acetic acid in water (A) and acetonitrile (B) was used for the separation. All solvents were filtered through a 0.45 μm filter prior to use. The flow-rate was kept constant at 0.5 mL/min for a total run time of 50 min. The system was run with a gradient program: 25% B to 25% B in 2 min, 25% B to 50% B in 50 min. The sample injection volume was 10 μL. In order to achieve the maximum sensitivity and minimum baseline noise, the following parameters were optimized for the ELSD: temperature of the nebulizer, 80 °C; nebulizing gas (N_2, 99.99% purity) flow rate, 2.0 L/min; gain, 1; impactor, off.

2.4. LC–ESI-MS analysis

A Finngan LCQ ion-trap mass spectrometer (MAT, San Jose, CA, USA) with an electrospray ion source was coupled to the HPLC system described in Section 2.3. The mass spectrometer conditions were optimized for notoginsenoside R1 and ginsenoside Rb1 prior to sample analysis in order to achieve maximum sensitivity. As a result, the following mass spectrometer conditions were chosen: sheath gas flow rate, 60 bar; auxiliary gas flow rate, 10 bar; electrospray voltage of the ion source, 5 kV; capillary voltage, 10 V; capillary temperature, 280 °C. Full scan of ions ranging from 400 to 2000 molecular weights in the negative ion mode was carried out. Source CID experiment was performed to obtain detailed structural information of the saponins.

3. Results and discussion

The LC–ELSD chromatogram of the crude extract from the root of *P. notoginseng* is given in Fig. 2. Eight major peaks were separated and detected. Tentative identification of the saponins was achieved by comparing their retention times with those of the authentic standards; consequently peaks 1–7 were identified to be notoginsenoside R1, ginsenosides Rg1, Re, Rb1, Rc, Rb2 and Rd, respectively, peak 8 was an unknown compound.

Further identification of the structures of these eight compounds by LC–ESI-MS, source CID experiments was attempted and the results are shown in Figs. 3–6 and Table 1. Cui et al. [14] reported that mass spectral data obtained in the negative ion mode gave more information on saponin structures than those obtained in the positive ion mode. Our preliminary direct infusion studies with notoginsenoside R1 and ginsenoside Rb1 also confirmed that negative ion mode was more sensitive, and provided straightforward structural information of saponins. The compounds related to peaks in Fig. 2 exhibited intense deprotonated molecular ions $[M-H]^-$ and their respective adduct ions $[M+AcO]^-$ in the negative ion mass spectra (NI-MS), which were in agreement with the

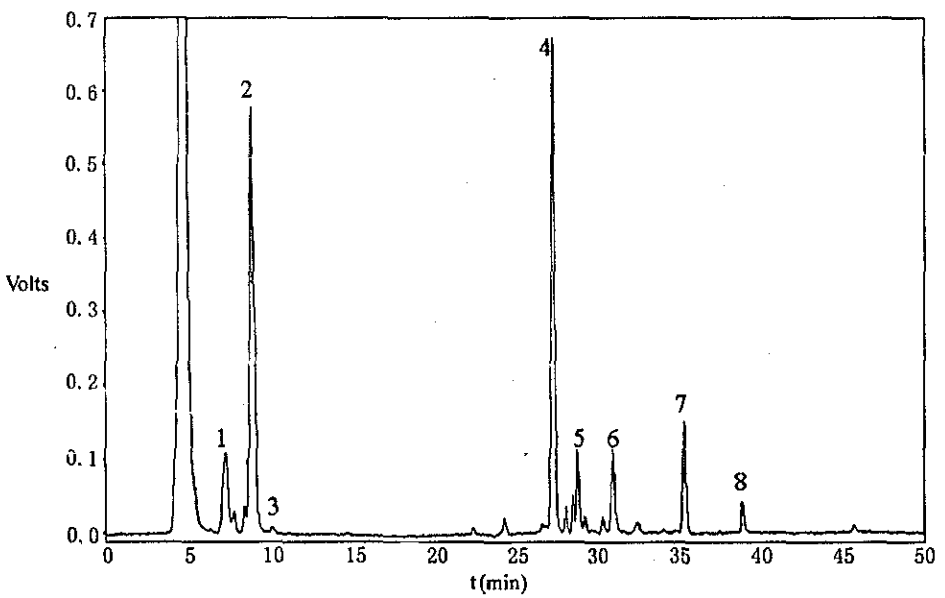

Fig. 2. LC–ELSD profile of *P. notoginseng* root extract. Compounds related to the peaks 1–8 were identified as notoginsenoside R1 and ginsenoside Rg1, Re, Rb1, Rc, Rb2, Rd and notoginsenoside K, respectively.

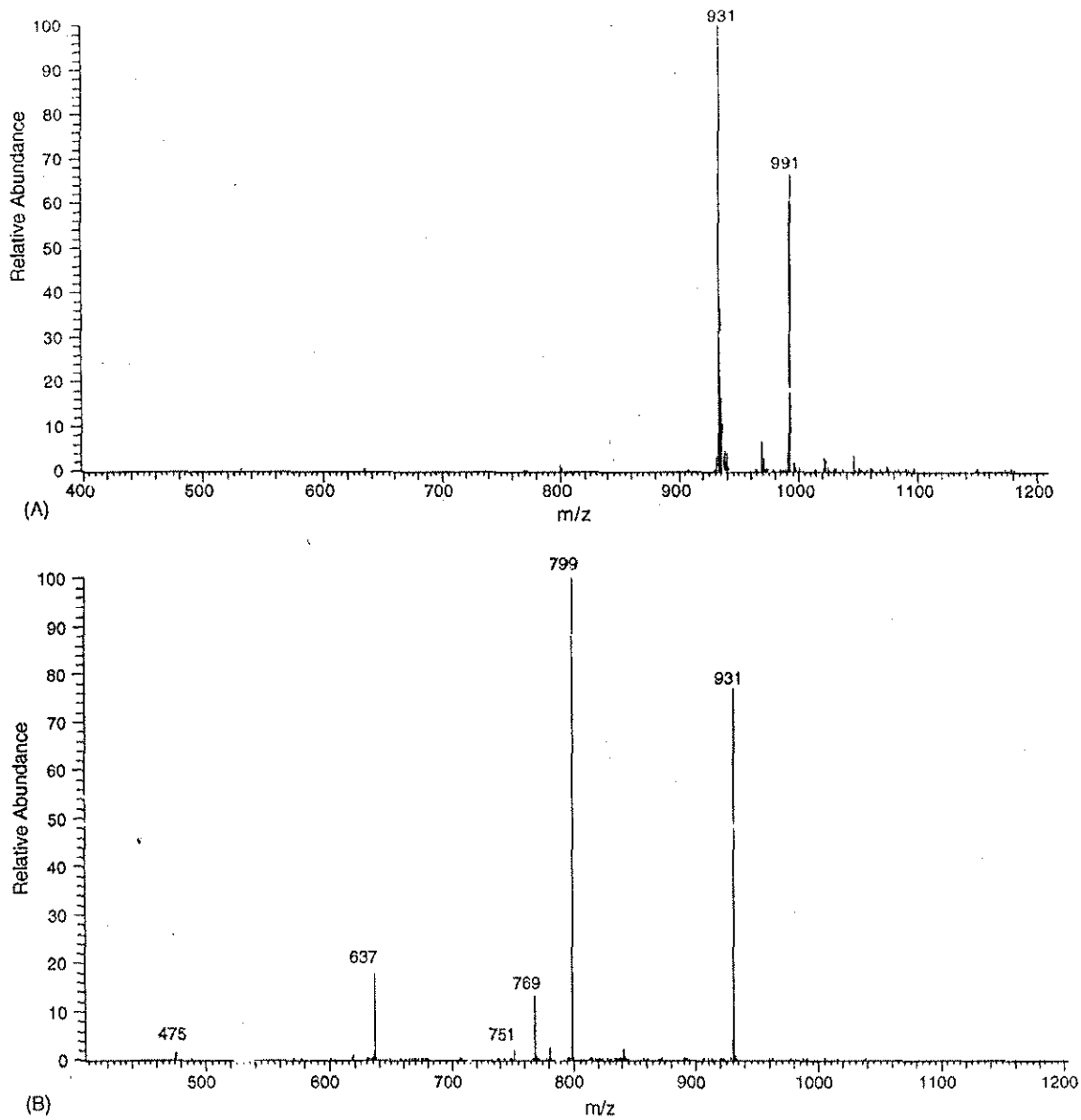

Fig. 3. (A) LC–ESI-MS spectrum of peak 1 obtained in the negative ion mode; (B) the CID spectrum of the parent ion *m/z* 931. The related compound was identified as notoginsenoside R1 by its retention time and CID pattern.

results by Fuzzati et al. [10] from the study on *P. ginseng*. The adduct ion $[M+AcO]^-$ was considered to be formed from acetic acid used in the mobile phase. The retention times, values of the mass-to-charge ratios (*m/z*) for the $[M-H]^-$ and $[M+AcO]^-$ ions and the CID fragments of the eight peaks shown in Fig. 2 are listed in Table 1. The molecular weights of peaks 1–8 were confirmed to be 932, 800, 946, 1108, 1078, 1078, 946 and 946 by LC–ESI-MS, respectively. Peaks 3, 7 and 8 had the same $[M-H]^-$ ion at *m/z* 945, which gave the molecular weight of 946 Da for all of these three compounds; similarly, peaks 5 and 6 had the same molecular weight of 1078 Da.

The LC–ESI-MS of peak 1 is shown in Fig. 3(A). Two ions with high abundance were found in the NI-MS, i.e. the deprotonated molecular ion $[M-H]^-$ at *m/z* 931 and its adduct ion $[M+AcO]^-$ at *m/z* 991. Fig. 3(B) shows the CID spectrum of the ion at *m/z* 931. All fragment ions in the CID spectrum were produced directly from the parent ion. The fragmentation pathway of the deprotonated molecular ion $[M-H]^-$ at *m/z* 931 is summarized in Fig. 4. The fragment ions of type Z and Y can be assigned to the characteristic cleavage of glycosidic bonds [16,17], and the pattern directly provides detailed structural information about the monosaccharide sequence. Five main fragment ions at *m/z* 799, 769,

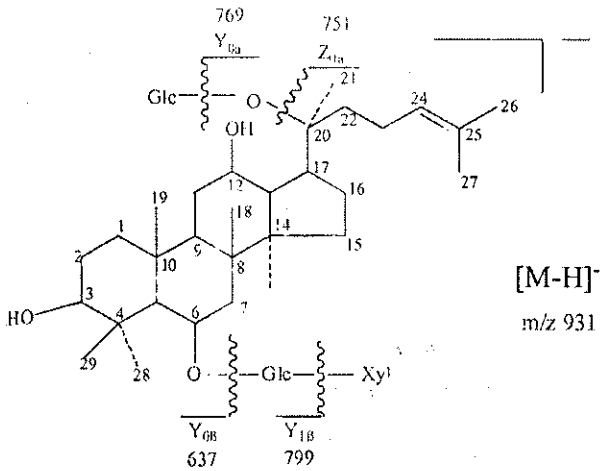

Fig. 4. Proposed fragmentation pathway for the deprotonated molecular ion $[M-H]^-$ of notoginsenoside R1.

751, 637, and 475 were observed in the CID of the parent ion, *m/z* 931 (Fig. 3(B)). The mass differences between the parent ion and the fragment ion *m/z* 799 and 769 were 132 and 162, respectively, corresponding to the loss of a pentose unit and a hexose unit. The simultaneous loss of the two sugar units indicates that there were two different terminal residues in the glycosidic moieties of the saponin structure, one residue was a pentose and the other was a hexose. The fragment ion at *m/z* 751 corresponded to the loss of a hexose unit and one molecule of water. The fragment ion at *m/z* 637 was directly produced from the parent ion of *m/z* 931, corresponding to the loss of a disaccharide consisting of a pentose and a hexose, indicating that the hexose in the disaccharide was the sugar that directly attached to the saponin aglycone. Similarly, the fragment ion at *m/z* 475 was also a daughter ion produced from *m/z* 931, corresponding to the loss of all sugar units. Based on the CID fragmentation pattern and the information obtained from LC–ELSD (retention time) and LC–ESI-MS (molecular weight), the compound corresponding to peak 1 was therefore identified as notoginsenoside R1.

The LC–MS of peak 2 gave *m/z* 799 as the deprotonated molecular ion $[M-H]^-$, which confirmed the molecular mass to be 800. Further experiments in the CID of the *m/z* 799 ($[M-H]^-$) produced two main fragment ions at *m/z* 637 and 475 (Table 1). The compound corresponding to peak 2 was therefore identified as ginsenoside Rg1 by comparing its retention time with that of the standard, and by congruent mass spectral data.

The LC–ESI-MS of peak 3 is shown in Fig. 5(A). The deprotonated molecular ion $[M-H]^-$ was at *m/z* 945, and the adduct ion $[M+AcO]^-$ was at *m/z* 1005, as a result, the molecular weight can be given as 946. The CID spectrum of $[M-H]^-$ is given in Fig. 5(B). Similar to peak 1, five main fragment ions at *m/z* 859, 799, 783, 637 and 475 were observed in the CID of peak 3. The fragment ions at *m/z* 799 and *m/z* 783 corresponded to the loss of a deoxyhexose unit and a hexose unit from the parent ion *m/z* 945. The results demonstrated that there were also two different terminal residues in the oligosaccharide chain, i.e. a deoxyhexose unit and a hexose unit. The fragment ion at *m/z* 859 was considered to be $[M-Rha+AcO]^-$ ion. The fragment ion at *m/z* 637 corresponded to the loss of a disaccharide consisting of a deoxyhexose and a hexose. The ion at *m/z* 475 was a result of losing all sugar units. The compound corresponding to peak 3 was similarly identified as ginsenoside Re by comparing the retention time with that of the standard, and by congruent mass spectral data, including the CID fragmentation patterns with literature reports [10–14]. Peaks 1–3 had the same CID fragment ion *m/z* 475, which corresponded to the common structure of the aglycone moiety, (20S)-protopanaxatriol.

The LC–MS of peak 7 also had two fragment ions, the deprotonated molecular ion $[M-H]^-$ at *m/z* 945 and the adduct ion $[M+AcO]^-$ at *m/z* 1005 (Fig. 6(A)). The CID spectrum of *m/z* 945 is given in Fig. 6(B), which had three main fragment ions at *m/z* 783, 621, 459. The fragment ion at *m/z* 783 was produced from the parent ion of *m/z* 945, corresponding to the loss of a hexose unit. The fragment ion at *m/z* 621 was also produced directly from the parent ion of *m/z* 945, but corresponded to the loss of a disaccharide consisting of two hexose units. The fragment ion at *m/z* 459 was a result of a subsequent loss of all attached hexose units. Based on the match of retention time and fragmentation pattern with the standard, the compound corresponding to peak 7 was therefore identified as ginsenoside Rd.

As shown in Figs. 3, 5 and 6 and Table 1, some saponins may share the same ESI-NI-MS pattern, and give the same two major ions, i.e. the deprotonated molecular ion and its adduction product. For instance, although the LC–ESI-NI-MS showed that peaks 3, 7 and 8 were eluted at different times, they had the same molecular weight ($[M-H]^-$, *m/z* 945). LC–ESI-NI-MS alone was therefore not enough information for positive identifications of these three isomers; however, by performing CID experiments for peaks 3 and 7, these two compounds showed different CID behaviors as discussed above, the difference in characteristic backbone and glycosidic moieties was evident, and therefore the assignment of the structure for these two isomers was straightforward. On the other hand, peak 8 showed the same CID pattern as peak 7, i.e. three fragmentation signals at *m/z* 783, 621 and 459 (Table 1) due to sequential losses of the attached hexose units (Δ162). All evidence showed that peaks 7 and 8 had the same aglycone i.e. (20S)-protopanaxatriol aglycone moiety and three hexose units, however, peak 8 was clearly separated from others, indicating that it was an independent compound. Ma et al. [5] reported that the FD mass spectrum of notoginsenoside K isolated from the root of *P. notoginseng* exhibited quasimolecular ions at *m/z* 985 $[M+K]^+$ and *m/z* 969 $[M+Na]^+$. Its FD–MS fragment ions included *m/z* 807 $[M-hexose+Na]^+$, *m/z* 748 $[M-hexose-2H_2O]^+$,

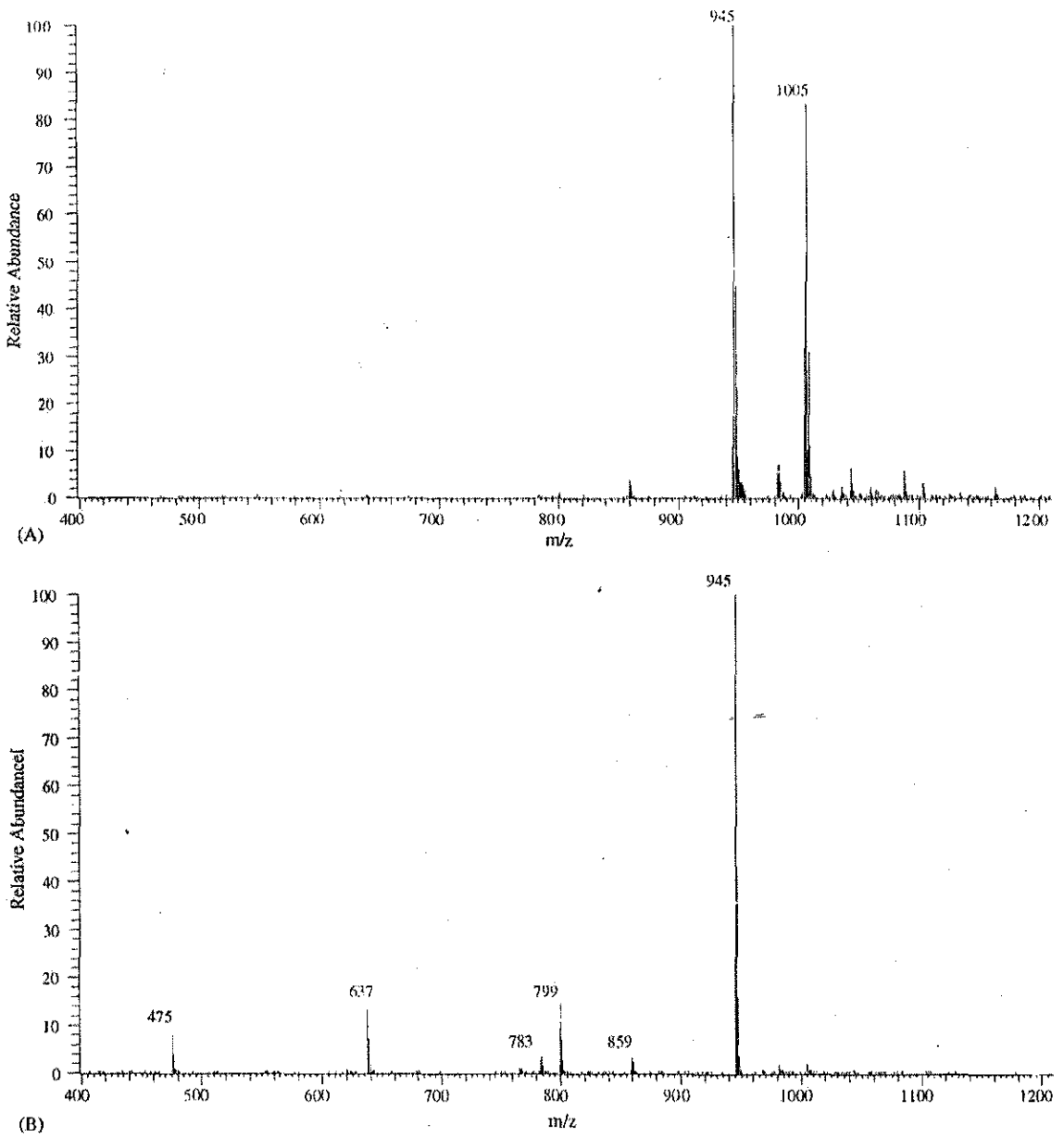

Fig. 5. (A) LC–ESI-MS of peak 3 obtained in the negative ion mode; (B) the CID spectrum of the parent ion *m/z* 945. The related compound was identified as ginsenoside Re by its retention time and CID pattern.

m/z 586 $[748\text{-hexose}]^+$ and *m/z* 424 $[586\text{-hexose}]^+$, indicating that there were three hexoses in the molecule. By comparing the retention times, and the CID fragment ions, such as *m/z* 783, 621 and 459 with those of the isomeric ginsenoside Re, Rd and notoginsenoside K, peak 8 was obviously a distinguished peak that was completely different from ginsenosides Re and Rd. We therefore tentatively identified peak 8 as notoginsenoside K by using the information as discussed above. However, the exact structure of this compound needs to be confirmed by NMR spectroscopy in next work.

The ion $[M-H]^-$ of peak 4 was at *m/z* 1107, and the CID experiment data showed that there are four fragment ions at *m/z* 945, 783, 621 and 459, so it can be elucidated that the compound corresponding to peak 4 is ginsenoside Rb1. Peaks 5 and 6 had the same ESI-MS ($[M-H]^-$ ion at *m/z* 1077 and CID pattern (fragment ions at *m/z* 945, 783, 621 and 459), and their retention times matched those of the standards, ginsenosides Rc and Rb2, respectively, thus identified as ginsenosides Rc and Rb2 accordingly. The ion at *m/z* 459 was a common deprotonated aglycone moiety, which corresponds to the (20S)-protopanaxadiol structure, it

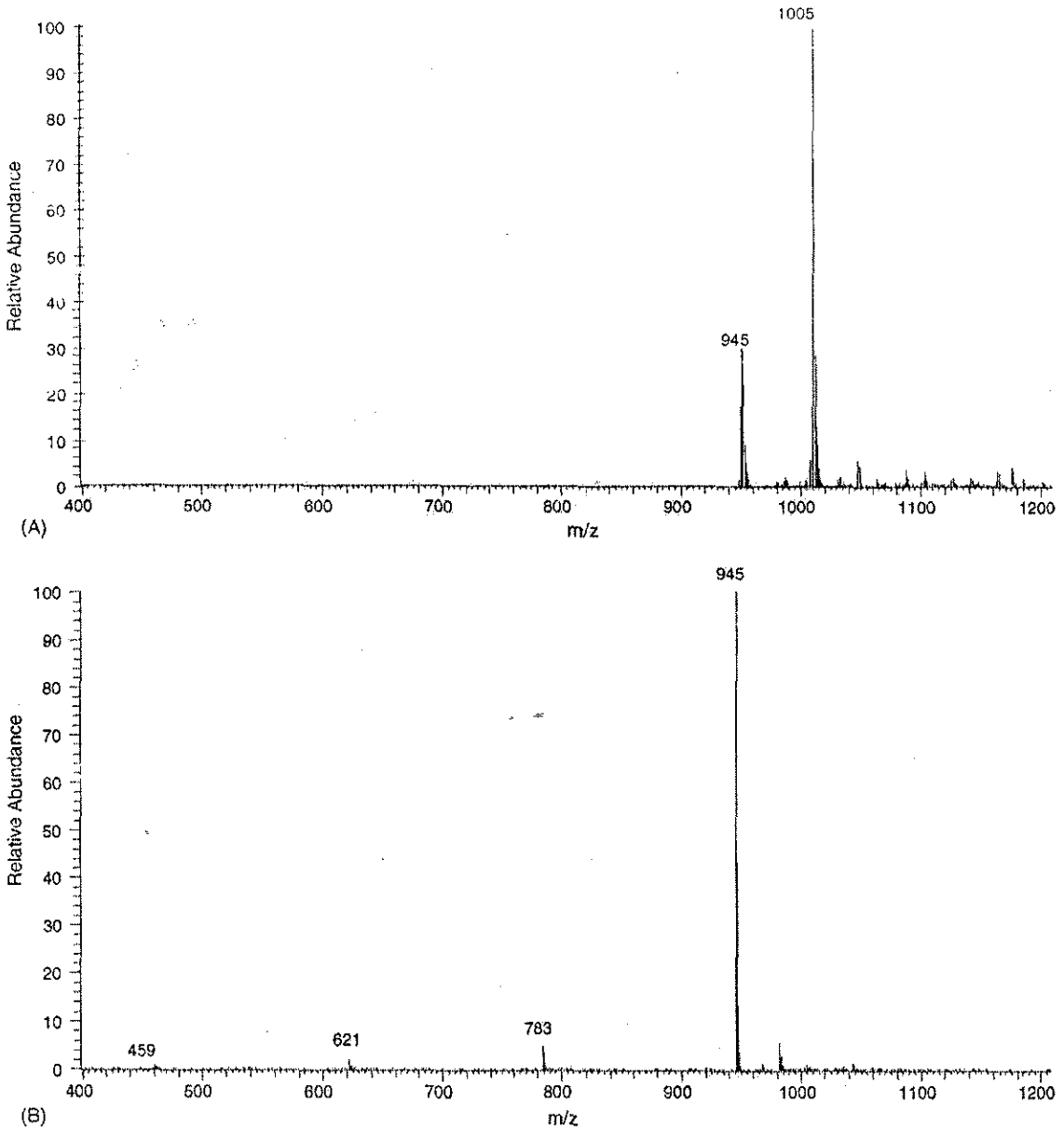

Fig. 6. (A) LC–ESI-MS spectrum of peak 7 in the negative ion mode; (B) the CID spectrum of the parent ion *m/z* 945. The related compound was identified as ginsenoside Rd by its retention time and CID pattern.

Table 1
The ESI-MS and corresponding CID data in the negative ion mode (*m/z* values) of peaks 1–8 in LC profile

Peak identification	t_R (min)[a]	$[M+AcO]^-$ (*m/z*)[b]	$[M-H]^-$ (*m/z*)[c]	CID (*m/z*)[d]
(1) Notoginsenoside R1	7.31	991	931	799[*M* – Xyl-H]$^-$, 769[*M* – Glc-H]$^-$, 751[*M* – H_2O-Glc-H]$^-$, 637[*M* – Xyl-Glc-H]$^-$, 475 [*M* – Xyl-2Glc-H]$^-$
(2) Ginsenoside Rg1	8.42	859	799	637[*M* – Glc-H]$^-$, 475 [*M* – 2Glc-H]$^-$
(3) Ginsenoside Re	9.20	1005	945	859[*M* – Rha + AcO-H]$^-$, 799[*M* – Rha-H]$^-$, 783[*M* – Glc-H]$^-$, 637[*M* – Glc-Rha-H]$^-$, 475[*M* – 2Glc-Rha-H]
(4) Ginsenoside Rb1	27.28	1167	1107	945[*M* – Glc-H]$^-$, 783[*M* – 2Glc-H]$^-$, 621[*M* – 3Glc-H]$^-$, 459 [*M* – 4Glc-H]$^-$
(5) Ginsenoside Rc	28.82	1137	1077	945[*M* – Ara(f)-H]$^-$, 783[*M* – Ara(f)-Glc-H]$^-$, 621[*M* – Ara(f)-2Glc-H]$^-$,459[*M* – Ara(f)-3Glc-H]$^-$

Table 1 (*Continued*)

Peak identification	t_R (min)[a]	$[M+AcO]^-$ (*m/z*)[b]	$[M-H]^-$ (*m/z*)[c]	CID (*m/z*)[d]
(6) Ginsenoside Rb2	31.02	1137	1077	945[*M* – Ara(p)-H]$^-$, 783[*M* – Ara(f)-Glc-H]$^-$, 621[*M* – Ara(f)-2Glc-H]$^-$,459[*M* – Ara(f)-3Glc-H]$^-$
(7) Ginsenoside Rd	35.35	1005	945	783[*M* – Glc-H]$^-$, 621 [*M* – 2Glc-H]$^-$, 459[*M* – 3Glc-H]$^-$
(8) Notoginsenoside K	38.52	1005	945	783[*M* – Glc-H]$^-$, 621 [*M* – 2Glc-H]$^-$, 459[*M* – 3Glc-H]

[a] Retention time.
[b] Molecular adduct ion.
[c] Deprotonated molecular ion.
[d] CID fragment ions.

can be seen that compounds of peaks 4–8 all had this ion in common, and the only difference among them was the number and type of glycosidic moieties.

4. Conclusions

In the present paper, the structure of the eight saponins in the crude extract of *P. notoginseng* have been identified by means of LC–ESI-MS methods. Although the method has been solely based on the match of retention time (LC–ELSD), molecular weight (LC–ESI-MS) and CID fragmentation patterns, the combinatorial use of these information did help to identify structures of the eight saponins in the crude extract of *P. notoginseng*. The present LC–MS method provided a reliable means to identify and distinguish saponins in the crude extract of *P. notoginseng*.

The CID technique was found particularly useful in the identification of some saponin isomers. The fragmentation patterns generated in CID experiment provided the characteristic of fragmentation of the saponin types. Our results also suggest that the LC–ESI-MS method could be applied to tentative identification of the type and molecular mass of known and unknown saponins of TCMs such as those in *P. notoginseng* crude extract even when there was no authentic standard available.

Acknowledgments

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Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions

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Abstract

Four different types of wheat bran were extracted and analyzed for phenolic acids using the Folin–Ciocalteu method and HPLC. The extracts and their hydrolysis products were also evaluated for their antioxidant activities. The total phenolic content of the red wheat bran was higher than that of the white wheat. We found that the majority of the phenolic acids existed in a bound form in wheat bran. These phenolic acids can be released by hydrolyzing the bran under alkaline or acidic conditions; however, the former was more efficient in the release of free phenolic acids than the latter. Ferulic, vanillic, and syringic acids were the major individual phenolic acids in the studied wheat bran. The main portion of the total ferulic acid was from alkaline hydrolysis. The alkaline hydrolysable fractions had greater antioxidant activities, while the acid hydrolysable fractions showed lower activities in both the red and white bran. The antioxidant activity of bran extract was stronger than that of free phenolic acids.

Keywords: Wheat bran; Phenolic acids; Antioxidant activity; Hydrolysis; Bond phenolics; Hydrolysable phenolics

1. Introduction

Phenolic acids are a group of natural products commonly found in many cereal grains. Higher concentrations of these compounds are found in the outer layers of the kernel which constitute the bran (Baublis, Clydesdale, & Decker, 2000; Baublis, Lu, Clydesdale, & Decker, 2002; Onyeneho & Hettiarachchy, 1992; Saadi, Lempereur, Sharonov, Autran, & Manfait, 1998). These phenolic acids may vary in structure due to difference in number and position of the hydroxyl groups on the aromatic ring. As a group, these naturally occurring compounds have been found to be strong antioxidants against free radicals and other reactive oxygen species (ROS), the major cause of many chronic human diseases such as cancer and cardiovascular diseases (Andreasen, Kroon, Williamson, & Garcia-Conesa, 2001a; Yu et al., 2002; Yu, Perret, Harris, Wilson, & Haley, 2003). The health benefits of cereal grains have significant implications for the improvement of food quality, particularly through applications in functional foods and nutraceuticals (Abdul-Hamid & Luan, 2000; Truswell, 2003). There are mainly two groups of phenolic acids in cereal bran: benzoic and cinnamic acid derivatives. Ferulic acid and other hydroxycinnamic acids (caffeic and *p*-coumaric acid derivatives) have been found to have good antioxidant activities (Andreasen, Kroon, Williamson, & Garcia-Conesa, 2001b; Emmons, Peterson, & Paul, 1999). The presence of the CH=CH–COOH group in the hydroxycinnamic acids is considered to be key for the significantly higher antioxidative efficiency than the COOH in the hydroxybenzoic acids (White & Xing, 1997).

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Wheat is one of the popular cereal grains, and its bran represents not only a good source of dietary fibres (Alabaster, Tang, & Shivapurkar, 1997; Møller, Dahl, & Bøckman, 1988), but also of phenolic acids, and the latter are known to contribute significantly to the total antioxidant activity of wheat (Baublis, Decker, & Clydesdale, 2000; Onyeneho & Hettiarachchy, 1992; Yu et al., 2003). Wheat bran extracts contain several phenolic acids, including vanillic, *p*-coumaric and, largely, ferulic acid (Kähkönen et al., 1999). These compounds, particularly ferulic acid, are not evenly distributed in the wheat; most are found in the bran (Baublis et al., 2002). Extract of wheat bran, having high concentration of phenolic acids, was shown to have stronger antioxidant activity than other fractions of wheat (Onyeneho & Hettiarachchy, 1992). In addition, wheat bran has been reported to be able to inhibit lipid oxidation catalyzed by either iron or peroxyl radicals (Baublis, Decker, et al., 2000). Most recently, Zhou, Laux, and Yu (2004) reported that wheat grain, bran and fractions had different antioxidant activities and total phenolic contents (TPC). Their study also showed that ferulic acid was a major contributor to the antioxidant activity. In a phosphatidylcholine liposome system, the percentage of liposome oxidation is reduced by increasing the concentration of isolated phenolic acids from whole-grain breakfast cereal (Baublis, Clydesdale, et al., 2000). Analytical procedures can significantly affect the antioxidant activity of phenolic acids because of the variable contents and types of phenolic acids through different sample preparations (Lehtinen & Laakso, 1997; Onyeneho & Hettiarachchy, 1992), extraction (Zieliński & Kozłowska, 2000) and hydrolysis procedures (Kader, Rovel, Girardin, & Metche, 1996; Nuutila, Kammiovirta, & Oksman-Caldentey, 2002). The hydrolysis method, in particular, can affect the yield and profile of phenolic acids in wheat bran because phenolic acids exist in esterified forms in the cell walls (Saadi et al., 1998). These bound phenolic acids can be hydrolyzed using an acid or an alkali to release so-called hydrolysable phenolic acids. Although the antioxidant activity of phenolic acids from other cereals has been intensively investigated, information on that in wheat bran is scarce (Baublis, Clydesdale, et al., 2000, Baublis, Decker, et al., 2000; Onyeneho & Hettiarachchy, 1992; Yu et al., 2003). Studies on the phenolic acid profile (both quantity and identity) in different varieties of wheat, and the effect of processing, e.g., hydrolysis and extraction methods, are particularly lacking.

The objectives of this study were, therefore, to qualify and quantify phenolic acids in wheat bran, to investigate the effect of hydrolysis and extraction conditions on the yield and profile of phenolic acids and to measure antioxidative activities of the extracts and the hydrolysis products.

2. Materials and methods

2.1. Materials

Hard red and soft white wheat bran samples were received from Hayhoe Mills Ltd. (ON, Canada). The same types of wheat bran were also obtained from AACC (American Association of Cereal Chemist) for comparison. Gallic, protocatechuic, *p*-hydroxybenzoic, gentisic, chlorogenic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, salicylic and *trans*-cinnamic acids were purchased from Sigma–Aldrich (Oakville, ON, Canada). Butylated hydroxytoluene (BHT), β-carotene (type I; synthetic 95%), linoleic acid and Tween 40 (polyoxyethylenesorbitan monopalmitate) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of analytical or HPLC-grade purity from Caledon Laboratories Ltd. (Georgetown, ON, Canada).

2.2. Extraction

Each of the four wheat bran samples was milled using a laboratory miller (A-10 S2 Kika-Labortechnik, Germany) and sieved through a 32-mesh screen. The fine flour (200 g) was transferred to an Erlenmeyer flask, defatted twice with hexane at a 4:1 ratio (v/w), and kept on a mechanical shaker for 1 h at room temperature. Each time, the mixture was filtered through a Whatman No. 1 filter paper, and the final defatted bran was dried in a hood at room temperature. The filtrate was evaporated to dryness and the residue was weighed as the total lipid content. The defatted bran was then extracted twice with 80% methanol at a 5:1 ratio (v/w) for 1 h at room temperature. The mixture was filtered through a Whatman No. 1 filter paper, and the combined supernatant was concentrated to dryness using a rotary evaporator at 40 °C. The methanolic extract was further freeze-dried and stored in a sealed container at 5 °C prior to being analyzed. Aliquots of the residue and the methanolic extract were also subjected to experiments related to hydrolysable phenolic acids.

2.3. Extractable phenolic acids

An aliquot of the methanolic extract, equivalent to 1 g bran, of each sample was re-dissolved in 4 ml of acidified water (pH 2 with HCl) and partitioned with 4 ml of ethyl ether, three times. The combined ether layer contained free phenolic acids (FPA). The water phase was neutralized to pH 7 with 2 M NaOH and dried using a vacuum evaporator. The residue was dissolved in 4 ml of 2 M NaOH and stirred for 4 h at room temperature. The solution was then acidified to pH 2, and extracted with ethyl ether as mentioned above. The resulting ether layer contained alkaline-hydrolysable phenolic acids (BHPA). The remaining water phase was treated with

3 ml of 6 M HCl and heated at 95 °C for 20 min. The solution was again partitioned with ethyl ether, which contains acid-hydrolysable phenolic acids (AHPA). All fractions were reconstituted in methanol, filtered through a 0.45 μm syringe filter (Acrodisc, Gelman Laboratory, Ann Arbor, MI) and subsequently subjected to total phenolic determination, HPLC analysis and antioxidant activity measurement.

2.4. Bound phenolic acids

The residue, after the methanol extraction, was hydrolyzed by two different protocols to determine the effect of hydrolysis conditions on the yield and profile of hydrolysable phenolic acids in the bran that were not extractable by aqueous methanol. Method A started with alkaline hydrolysis (1 g residue in 40 ml 2 M NaOH, 4 h, at room temperature) and was subsequently followed by acid hydrolysis of bran residue (6 M HCl, 1 h at 95 °C), resulting in two fractions A1 and A2, respectively. In method B, hydrolysis by acid was done first, and subsequently followed by alkaline hydrolysis, resulting in fractions B1 and B2. All fractions were extracted at pH 2 with ethyl ether (40 ml × 3). The same analyses were done for all these fractions.

2.5. Determination of total phenolic content

Each fraction (0.2 ml) was mixed with 1 ml of the Folin–Ciocalteu reagent and 0.8 ml of saturated sodium carbonate (Na_2CO_3) solution. The mixture was allowed to stand at room temperature for 30 min and then the absorbance was measured at 765 nm in a Varian Cary 3C spectrophotometer (Varian analytical instruments, Harbor City, CA). The total phenolic content was expressed as microgrammes of gallic acid equivalent (GAE) per millilitre of solution. The total phenolic contents in wheat bran were subsequently calculated from these data.

2.6. HPLC analysis

HPLC analysis was carried out using an Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and a diode-array detector. The analytical column was a Phenomenex Luna C18 (2) (250 × 4.6 μm; 5 μm) with a C18 guard column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 1.0 ml min^{-1} for a total run time of 70 min and the gradient programme was as follows: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min and 0% B to 100% B in 5 min. There was 10 min of post-run for reconditioning. The injection volume was 10 μl and peaks were monitored simultaneously at 280 and 320 nm for the benzoic acid and cinnamic acid derivatives, respectively. All samples were filtered through a 0.45 μm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectra and compared with those of the standards. Detection limits, for all compounds, were from 0.1 to 0.5 ppm, and the correlation coefficient R^2 was >0.9993 from 0.5 to 200 ppm. The detection limit was defined as the concentration at which the signal to noise ratio (S/N) was equal to or greater than three. All samples were prepared and analyzed in duplicate.

2.7. β-Carotene-linoleic acid model system (β-CLAMS)

The β-CLAMS method is based on the de-coloration of β-carotene by the peroxides generated during the oxidation of linoleic acid (a free radical chain reaction) at elevated temperature (Miller, 1971). In this study, the β-CLAMS was modified for the 96-well microplate reader. In brief, β-carotene (0.5 mg) was dissolved in ca. 2 ml of $CHCl_3$ in a 200 ml round-bottom flask, to which 25 μl of linoleic acid and 200 mg of Tween 40 were added. $CHCl_3$ was removed using a rotary evaporator. Oxygenated HPLC-grade water (100 ml) was added, and the flask was shaken vigorously until all material dissolved. The oxygenated water was obtained by bubbling water with compressed oxygen gas for at least 2 h at room temperature. This test mixture was prepared fresh and used immediately. To each well, 250 μl of the reagent mixture and 35 μl sample or standard solution or water (blank), were added. The plate was incubated at 45 °C. Readings were taken at 490 nm, immediately after and every 15 minutes, for 300 min, using a visible/UV microplate kinetics reader (EL 340, Bio-Tek Instruments, Inc., Winooski, VT). All phenolic acid standards were prepared at 200 ppm, and run in triplicate.

3. Results and discussion

3.1. Total phenolic contents

Eighty percent aqueous methanol gave the highest yield of extractables of the bran (8–9%). The total phenolic contents (TPC) of different fractions of the wheat bran are shown in Table 1. TPCs of the commercial wheat bran were similar to those of the AACC standards, although at individual fraction levels, they were significantly different. All fractions of the red wheat bran, except for the AHPA of the AACC red wheat bran, showed higher TPC than their corresponding white wheat bran. This result is consistent with what was reported by Maziya-dixon, Klopfenstein, and Leipold (1994). TPCs of wheat bran in our study ranged from 3.3 mg of gallic acid equivalents per gramme (GAE/g) of bran to 3.9 mg of GAE/g. The level of TPCs

Table 1
Total phenolic contents in different wheat bran fractions (μg GAE/g)[a]

Bran	Extractable phenolic acids (μg GAE/g[a])			Bound phenolic acids (μg GAE/g[a])		Total[e](μg/g)
	FPA[b]	BHPA[c]	AHPA[d]	BHPA[c]	AHPA[d]	
Red, AACC	336 ± 2.4	146 ± 11.4	151 ± 8.3	2312 ± 4.2	889 ± 14.6	3834 ± 6.8
White, AACC	281 ± 0.2	116 ± 2.5	165 ± 1.0	2145 ± 7.6	654 ± 13.3	3362 ± 24.3
Red, commercial	186 ± 1.4	148 ± 8.1	236 ± 6.1	2326 ± 4.1	1070 ± 6.2	3967 ± 5.8
White, commercial	185 ± 1.4	103 ± 1.4	170 ± 4.4	2266 ± 10.5	1060 ± 7.8	3784 ± 22.9

[a] Microgrammes gallic acid equivalents per gramme of bran.
[b] Free phenolic acid.
[c] Alkaline-hydrolysable phenolic acids.
[d] Acid-hydrolysable phenolic acids.
[e] Sum of all fractions of bran.

was close to, but slightly higher than, what was reported by Zhou and Yu (2004). The bound phenolic contents were significantly higher than the extractable phenolic contents in all varieties, indicating that the major phenolic acids in wheat bran were not extractable by aqueous methanol but released upon alkaline or acid hydrolysis. Adom, Sorrells, and Liu (2003) also found that phenolic content of wheat occurred mostly in the bound form and

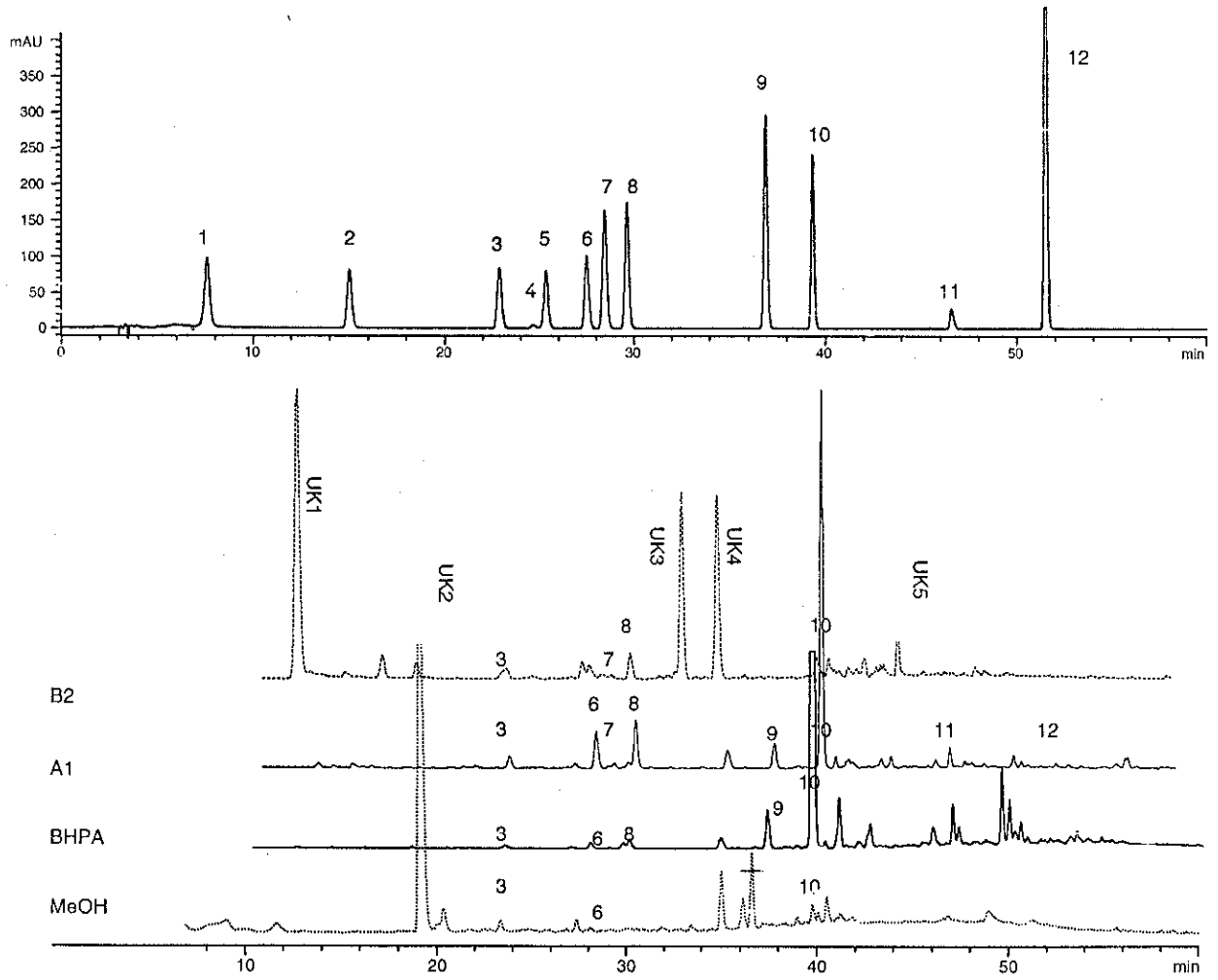

Fig. 1. HPLC chromatograms of a standard mixture of phenolic acids (top) and profiles of typical fractions of wheat bran extracts. Peak 1, gallic acid; 2, protocatechuic acid; 3, *p*-hydroxybenzoic acid; 4, gentistic acid; 5, chlorogenic acid; 6, vanillic acid; 7, caffeic acid; 8 syringic acid; 9, *p*-coumaric acid; 10, ferulic acid; 11, salicylic acid; 12, *trans*-cinnamic acid. A1, bound phenolic acids hydrolyzed by alkaline in method A; B2, bound phenolic acids hydrolyzed by alkaline in method B; MeOH, aqueous methanol (80%)-extractable phenolic acids; BHPA, alkaline-hydrolysable phenolic acids in the methanol-extractable fraction (see Section 2). All chromatograms were from the commercial red wheat bran.

the bound phenolic content was 2.5–5.4-fold higher than the free phenolic content in wheat grains. Our data confirmed their finding (Table 1).

3.2. Individual phenolic acids

The 12 standard phenolic acids shown in Fig. 1 have been reported in wheat (Onyeneho & Hettiarachchy, 1992; Sosulski, Krygier, & Hogge, 1982; Wu, Haig, Pratley, Lemerle, & An, 1999); however, only ferulic, vanillic, caffeic, syringic, *p*-hydroxybenzoic, *p*-coumaric, salicylic and *trans*-cinnamic acids were detected in the various fractions of bran analyzed in this study. Different fractions contained different phenolic acid profiles (Fig. 1). The crude methanolic extract of the commercial red wheat bran contained few FPAs, but it was the unknown compound (UK2) at near 19 min that dominated the HPLC profile. Concentrations of individual phenolic acids in different fractions of wheat bran are shown in Table 2 and 3. Ferulic acid, *p*-hydroxybenzoic acid

Table 2
Phenolic acids in different fractions of AACC wheat bran

Phenolics	Extractable phenolic acids (μg/g)			Bound phenolic acids (μg/g)		Total (μg/g)[d]
	FPA[a]	BHPA[b]	AHPA[c]	A1[b]	A2[c]	
Red wheat bran						
3. *p*-Hydroxybenzoic acid	3.41 ± 0.06	2.11 ± 0.11		12.9 ± 1.37	20.8 ± 0.29	39.2 ± 0.62
6. Vanillic acid	7.92 ± 0.46	4.78 ± 0.04	10.7 ± 0.02	19.8 ± 0.30	39.4 ± 0.31	82.6 ± 0.19
7. Caffeic acid	0.80 ± 0.00					0.80 ± 0.00
8. Syringic acid	10.5 ± 0.28	20.53 ± 0.84	14.2 ± 0.04	61.7 ± 0.00	17.9 ± 0.14	125 ± 0.34
9. *p*-Coumaric acid	0.84 ± 0.02	1.01 ± 0.00		33.1 ± 0.06		35.0 ± 0.03
10. Ferulic acid	7.66 ± 0.28	5.71 ± 0.17		1905 ± 2.97		1918 ± 1.59
11. Salicylic acid				89.0 ± 0.00		89.0 ± 0.00
White wheat bran						
3. *p*-Hydroxybenzoic acid		1.40 ± 0.00	2.55 ± 0.02	8.14 ± 0.08	12.5 ± 0.08	24.6 ± 0.04
6. Vanillic acid	5.62 ± 0.10	3.20 ± 0.01		20.3 ± 1.15	16.5 ± 0.23	45.5 ± 0.53
8. Syringic acid	4.16 ± 0.00	10.5 ± 0.02	9.82 ± 0.21	40.0 ± 0.40	7.14 ± 0.20	71.5 ± 0.16
9. *p*-Coumaric acid		1.30 ± 0.01		36.8 ± 0.08		38.1 ± 0.05
10. Ferulic acid	4.19 ± 0.45	10.4 ± 0.04	2.44 ± 0.06	1359 ± 3.18		1376 ± 4.00
12. *trans*-Cinnamic acid				2.70 ± 0.04		2.70 ± 0.04

Data are means ± standard deviation ($n = 3$) determined by HPLC analysis.
[a] Free phenolic acid.
[b] Alkaline hydrolysables.
[c] Acid hydrolysables.
[d] Sum of all fractions.

Table 3
Phenolic acids in different fractions of commercial wheat bran

Phenolics	Extractable phenolic acids (μg/g)			Bound phenolic acids (μg/g)		Total (μg/g)[d]
	FPA[a]	BHPA[b]	AHPA[c]	A1[b]	A2[c]	
Red wheat bran						
3. *p*-Hydroxybenzoic acid		2.68 ± 0.12	4.55 ± 0.06	7.46 ± 1.98	13.6 ± 0.00	28.3 ± 0.96
6. Vanillic acid	1.41 ± 0.01	8.18 ± 0.20		13.4 ± 0.56	55.0 ± 0.79	78.0 ± 0.35
7. Caffeic acid				1.78 ± 0.00		1.78 ± 0.00
8. Syringic acid		23.8 ± 0.60	24.1 ± 0.42	26.6 ± 0.82	108 ± 0.94	182 ± 0.23
9. *p*-Coumaric acid		1.60 ± 0.03	0.25 ± 0.00	41.7 ± 0.47	2.98 ± 0.06	46.6 ± 0.22
10. Ferulic acid	2.46 ± 0.08	37.5 ± 0.52	4.76 ± 0.05	1934 ± 2.31	43.1 ± 0.33	2020 ± 0.94
11. Salicylic acid				1.88 ± 0.00		1.88 ± 0.00
White wheat bran						
3. *p*-Hydroxybenzoic acid		2.54 ± 0.03	4.32 ± 0.18	10.4 ± 1.30	28.3 ± 0.96	45.5 ± 0.61
6. Vanillic acid	2.21 ± 0.00	1.90 ± 0.00	5.88 ± 0.04	18.1 ± 0.00	52.7 ± 3.31	80.8 ± 1.48
8. Syringic acid		8.04 ± 0.15	8.16 ± 0.25	42.5 ± 0.00	86.4 ± 0.37	145 ± 0.16
9. *p*-Coumaric acid		1.38 ± 0.00	0.39 ± 0.01	34.9 ± 0.00	2.08 ± 0.06	38.8 ± 0.03
10. Ferulic acid	4.13 ± 0.00	40.0 ± 0.13	2.86 ± 0.00	1904 ± 13.08	40.8 ± 0.18	1992 ± 5.81

Data are means ± standard deviation ($n = 3$) determined by HPLC analysis.
[a] Free phenolic acid.
[b] Alkaline hydrolysables.
[c] Acid hydrolysables.
[d] Sum of all fractions.

and vanillic acid were the main FPAs extracted into the crude methanol extract; however, only ferulic acid was released in significantly higher amount upon alkaline hydrolysis of the residue (Table 3, A1). The concentrations of individual phenolic acids of the commercial wheat bran were higher than those of the AACC samples. Also, the concentrations of individual phenolic acids in red bran were higher than those in white bran (Tables 2 and 3). These results were consistent with the TPC data.

3.3. Hydrolysis of wheat bran

Most phenolic acids in cereals primarily occur in the bound form as conjugates with sugars, fatty acids, or proteins (White & Xing, 1997); therefore, it is important that a hydrolysis process is adopted in order to obtain maximum yield of the phenolic acids of cereal grains such as wheat and wheat bran.

HPLC profiles of bound phenolic acids obtained by two hydrolysis methods are shown in Table 4. Using method A, vanillic, caffeic, syringic, *p*-hydroxybenzoic, *p*-coumaric, salicylic, *trans*-cinnamic acids and ferulic acid were released from the bound form and detected by HPLC. Ferulic acid was the predominant phenolic acid in fraction A1, whereas an unknown peak (UK 1), at 11 min, dominated the profile of fraction A2. This peak was also observed in fractions B1 and B2, obtained using method B (Fig. 1). The HPLC profile of B1 was similar to A2; however, in B2, in addition to the aforementioned unknown peak, three other unknown products (UK 3–5) were observed (Fig. 1, Table 4). The different hydrolysis protocols clearly affected the concentrations of phenolic acids. In general, method A was more efficient in releasing the phenolic acids, particularly ferulic acid (Fig. 1, Table 4). However, salicylic acid was an exception; it was mainly released by method B after acid hydrolysis. Acidic and alkaline hydrolyses are often used to cleave the ester bond in separation and characterization of specific phenolic compounds (Nuutila et al., 2002) but, as our results show (Table 4), the hydrolysis conditions, acid or alkaline only, or in different sequence, can significantly affect the total yield and profile of phenolic acids. Cinnamic acid derivatives, *p*-coumaric, caffeic and ferulic acids, were found to degrade under hot acidic conditions (Gao & Mazza, 1994; Robbins, 2003). This may partially explain why acid hydrolysis gave a low yield of hydrolysable phenolic acids.

3.4. Antioxidant activities of wheat bran extracts

Percent original absorbance was used as an endpoint for the evaluation of the antioxidant activity measured using the β-CLAMS method. The higher the percentage, the stronger was the antioxidant activity at a certain time interval. Although different methods have been used to interpret the data (Tsao & Yang, 2003), we have chosen to use the data at 180 min in this discussion. As shown in Fig. 2A, the alkaline-extractable fractions had greater antioxidant activities, while the acid-extractable fractions, in general, showed the lowest in both of red and white bran. Methanol-extractable fractions of wheat bran also showed strong antioxidant activities (approximately 67% on average). During the first 60 min, the antioxidant activity of the methanol-extractable fraction of the commercial white bran inhibited the peroxidation slightly better than did BHT.

The antioxidant activity of wheat bran extracts only indicates the total antioxidant capacity of the mixture. The role of individual phenolic acids and their contribution to the total antioxidant activity cannot be deciphered from the mixtures. To find the antioxidant capacities of the phenolic acids identified in the bran, individual compounds were subjected to the same evaluation using the β-CLAMS test. As shown in Fig. 2B, although the concentrations were the same, the antioxidant activities of these compounds were significantly different. Caffeic, syringic, and ferulic acids were the strongest antioxidants, whereas *trans*-cinnamic and vanillic acids were the weakest. This may explain why extracts with high contents of ferulic acid, such as those

Table 4
Effect of different hydrolysis conditions on the individual phenolic acids (μg/g defatted bran)[a]

Phenolic acids	Method A[b]			Method B[b]		
	A1	A2	Total	B1	B2	Total
p-Hydroxybenzoic acid	8.92	11.04	20.0	17.5	5.24	22.8
Vanillic acid	13.0	60.8	73.8	30.0	14.5	44.4
Syringic acid	27.5	109	137	81.7	35.7	117
trans-Cinnamic acid	0.88		0.88			
Caffeic acid	2.28		2.28			
p-Coumaric acid	41.4	2.9	44.3	1.66		1.66
Ferulic acid	1932		1932	34.4	10.3	44.7
Salicylic acid	1.88		1.88	158		158

Data are the average of duplicates determined by HPLC analysis. Data were from the commercial red wheat bran.

[a] All concentrations were on dry matter basis.

[b] See Fig. 1 caption.

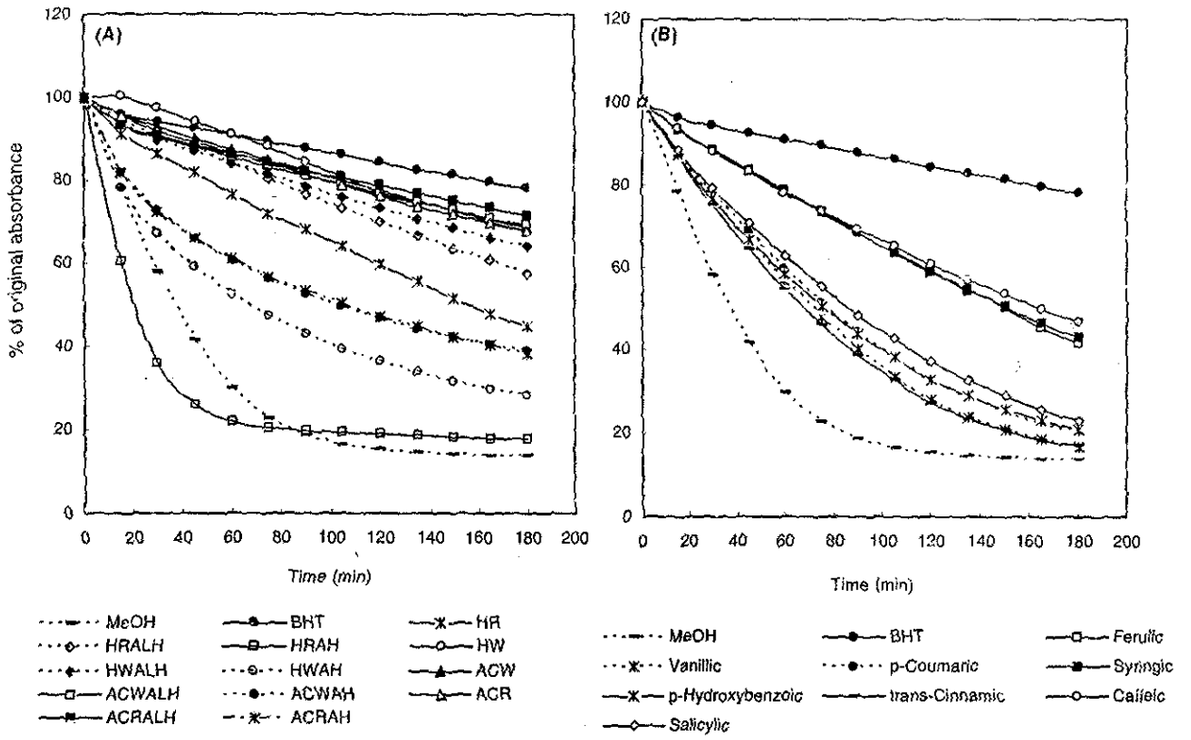

Fig. 2. Antioxidant activities of wheat bran extracts (A) and typical wheat phenolic acids (B). HR, aqueous methanol-extractable fraction from the commercial red wheat bran; HW, aqueous methanol-extractable fraction from the commercial white wheat bran; ACR, aqueous methanol-extractable fraction from the AACC red wheat bran; ACW, aqueous methanol-extractable fraction from the AACC white wheat bran; HRALH, alkaline-hydrolysable bound phenolic acids in the commercial red wheat bran; HRAH, acid-hydrolysable bound phenolic acids in the commercial red wheat bran; HWALH, alkaline-hydrolysable bound phenolic acids in the commercial white wheat bran; HWAH, acid-hydrolysable bound phenolic acids in the commercial white wheat bran; ACRALH, alkaline-hydrolysable bound phenolic acids in the AACC red wheat bran; ACRAH, acid-hydrolysable bound phenolic acids in AACC red wheat bran; ACWALH, alkaline-hydrolysable bound phenolic acids in the AACC white wheat bran; ACWAH, acid-hydrolysable bound phenolic acids in AACC white wheat bran.

by alkaline hydrolysis had higher antioxidant activity. We also found that the methanolic extracts had strong antioxidant activity despite having a low concentration of phenolic acids. Other antioxidant phytochemicals may have contributed to the activity. The unknown peak in the extract (Fig. 1) is considered one such compound producing strong antioxidant activity. Its identity is currently being investigated.

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Isolation and purification of acteoside and isoacteoside from *Plantago psyllium* L. by high-speed counter-current chromatography

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Abstract

Two isomeric phenylethanoid glycosides, acteoside and isoacteoside were isolated and purified from the seeds of *Plantago psyllium* L. for the first time by high-speed counter-current chromatography (HSCCC) using a solvent system consisting of ethyl acetate–water (1:1, v:v). By injecting 200 mg of the *n*-butanol extract of *P. psyllium* for five consecutive times, the two-step HSCCC procedure yielded a total of 165 mg of acteoside and 17.5 mg of isoacteoside from 978 mg extract. The recovery rates for acteoside and isoacteoside were 90 and 84%, respectively, and the purities were 98 and 94%, respectively. The HSCCC fractions were analyzed by HPLC and the structures were identified by UV, LC–APCI–MS in negative ion mode, and confirmed by NMR experiments.

Keywords: *Plantago psyllium*; Acteoside; Isoacteoside; Phenylethanoid glycoside; HSCCC; LC–APCI–MS; NMR

1. Introduction

Plantago psyllium L. belongs to the *Plantago* family. Its seeds have been used as demulcents and in the treatment of chronic constipation [1]. The major bioactive constitute in the seeds of *P. psyllium* are phenolic compounds, including acteoside and isoacteoside (Fig. 1). Acteoside and related phenylethanoid glycosides are widely distributed in the plant kingdom [2–7] and have been found to have various biological activities, including anti-hepatotoxic [8], anti-inflammatory, anti-nociceptive [9] and antioxidant [10,11] activities. The preparative separation and purification of acteoside and related phenylethanoid glycosides from many plants by conventional methods are tedious and usually require repeated chromatographic steps on silica gel and Sephadex LH-20 column [3–7]. The overall yields of these methods were poor, because the hydroxyl groups in the phenylethanoid glycosides make these compounds strongly adsorbed onto the solid support during separation [12].

High-speed counter-current chromatography (HSCCC) is a support-free all liquid chromatographic technique that has been successfully applied to separation and isolation of many natural products [12–18]. The HSCCC method is considered as a suitable alternative for the separation of phenolic compounds [19–21]. Lei et al. [12] successfully separated acteoside and 2′-acetyl acteoside from *Cistanches salsa* (C.A. Mey) G. Beck by using HSCCC, however, no report has been published on the use of HSCCC for the separation and purification of acteoside and isoacteoside from the seeds of *P. psyllium*. The identification of acteoside and other phenolic compounds by mass spectrometry also has been limited to electrospray ionization (ESI)–MS, fast-atom bombardment (FAB)–MS and field desorption (FD)–MS [6,22,23]. The objective of this paper was to develop a simple and efficient method for the preparation of acteoside and isoacteoside from

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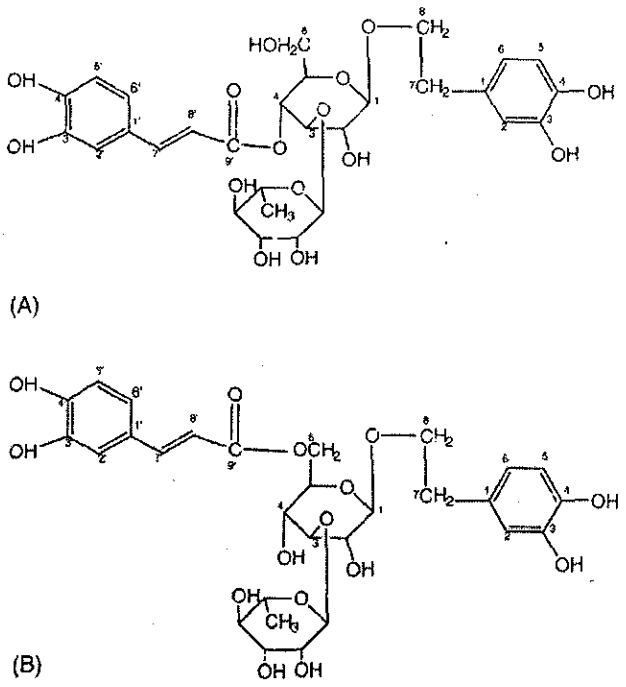

Fig. 1. Structures of acteoside (A) and isoacteoside (B).

P. psyllium seeds by high-speed counter-current chromatography. In our experiment, LC coupled with on-line APCI mass spectrometry and NMR experiments were used for the analysis and identification of these two isomeric phenylethanoid glycosides.

2. Experimental

2.1. Chemicals and reagents

Acteoside, caffeic acid and hydroxytyrosol were purchased from Sigma–Aldrich (Oakville, ON). *P. psyllium* seeds were provided by Prof. Zeyuan Deng (NanChang University, China). All solvents were of HPLC grade and purchased from Caledon Laboratories Ltd. (Georgetown, Canada).

2.2. Sample preparation

P. psyllium seeds (25 g) were milled to powder (ca. 50 mesh) by using a Retsch (MM 2000) machine, soaked in 500 mL of 80% aqueous methanol for 12 h for three times at room temperature. Each time, the extraction mixture was filtered through a Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, UK). All three filtrates were combined and concentrated to 300 mL in vacuo at <40 °C. The resulting aqueous solution was defatted twice, each with 300 mL of hexane and then extracted successively for three times, each with 300 mL *n*-butanol. The *n*-butanol layers were combined and concentrated to dryness in vacuo at <40 °C, which yielded 978 mg of crude extract. The extract was stored at −4 °C before HSCCC separation.

2.3. HSCCC separation procedure

The preparative HSCCC was carried out using a Model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, MD, USA). This apparatus had three preparative coils, connected in series (total volume, 325 mL). The revolution speed of the apparatus could be regulated between 0 and 2000 rpm. The HSCCC system was equipped with an HPLC pump (Pharma-Tech Research, Baltimore, Maryland, USA), a Model 450 UV detector (Alltech, USA), a Model L 120 E flat-bed recorder (Linseis Inc., Princeton Jct, USA), a fraction collector (Advantec MFS Inc., USA) and a sample injection valve with a 10 mL sample loop.

A mixture of ethyl acetate–water (1:1, v:v) was shaken vigorously in a separatory funnel and let stand at room temperature until there were two clearly separated phases. The two phases were then used in the HSCCC after they reached equilibrium.

The entire coiled column was first filled with the upper layer which serves as the stationary phase. Then, the pump was set at 1045 rpm. The lower layer (mobile phase) was pumped into head end of the column at a flow-rate of 1.5 mL/min. A sample (200 mg) dissolved in 8 mL of the mixture of ethyl acetate–water (1:1, v:v) was loaded into the injection valve after the system reached hydrodynamic equilibrium. The effluent from the outlet of the column was continuously monitored by a UV detector at 254 nm and collected into test tubes with a fraction collector set at 4 min for each tube. Five consecutive injections, each containing ca. 200 mg of the *n*-butanol extract in 8 mL of the two-phase solvent system, were made. Fractions from the HSCCC that had only pure compound as determined by HPLC were combined, and freeze-dried. Those containing a mixture of the compounds were pooled, concentrated and re-injected to the system for further purification.

2.4. LC conditions

An Agilent Technology 1100 Series HPLC system equipped with a quaternary pump, a degasser, a thermostatic auto-sampler and a photodiode array detector (DAD), was used for the analysis of phenylethanoid glycosides in the *n*-butanol extract and fractions collected from the HSCCC separation. The analysis was carried out with a Phenomenex ODS-C_{18} column (150 mm × 4.6 mm, 5 μm) and a C_{18} guard column. The binary mobile phase consisted of acetonitrile (solvent A) and water containing 2% acetic acid (solvent B). All solvents were filtered through a 0.45 μm filter prior to use. The flow-rate was kept constant at 1.0 mL/min for a total run time of 30 min. The system was run with a gradient program: 100% B to 75% B in 20 min, 75% B to 0% B in 5 min, and

0% B to 100% B in 5 min. The sample injection volume was 10 μL. Peaks of interest were monitored at 320 nm by a DAD detector.

2.5. LC–APCI–MS for identification

LC–MS experiments were carried out using an LC coupled to a Finnigan LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) which was equipped with an atmospheric pressure chemical ionization (APCI) source. The mass spectrometer conditions were optimized for acteoside prior to sample analysis in order to achieve maximum sensitivity. As a result, the shear gas and auxiliary flow rates were set at 54 and 12 (arbitrary units), respectively. The capillary voltage was fixed at −28 kV and its temperature was controlled at 200 °C. The vaporizer temperature was set at 450 °C and the current of discharge needle was at 4.50 μA. The entrance lens voltage was fixed at 60 V. The tube lens offset was −21 V, the multipole 1 offset was 5 V and the multipole 2 offset was 12.5 V. The lens voltage was 38 V. The electron multiplier voltage was set at 400 V for ion detection. Full scan of ions was performed in the negative ion mode with a molecular mass range set from 50 to 2000. The same separation conditions were used as in the LC experiment.

2.6. Acid hydrolysis and monosaccharide analysis

One milligram of compound 1 or 2 was hydrolyzed with 1 ml of 1 M H_2SO_4 which was heated at 90 °C in a water bath for 1.5 h. The hydrolyzed product was diluted with water and filtered through a 0.45 μm syringe filter before being analyzed by HPLC using the same conditions. Monosaccharides were analyzed in a Dionex (Sunnydale, CA, USA) DX-500 ion chromatograph using a Dionex CarboPac PA1 column (4 mm × 250 mm) and a Dionex PA guard column (4 mm × 25 mm). A pulsed amperometric detector (PAD) was used for detection. A gold electrode was used as the working electrode and silver/silver chloride as the reference electrode. The mobile phase consisted of 100 mM NaOH (solvent A), 30 mM NaOH (solvent B) and water (solvent C). Prior to sample injection, the instrument was run with 100% B for 15 min, and then a combination of 8% A and 92% C for 10 min. After injection, 8% A and 92% C were run for 7 min and then 100% C for 18 min. The column was held at 35 °C with a flow rate of 1 mL/min. The injection volume was 50 μL for both samples.

2.7. NMR for identification

Proton NMR spectra were recorded on a Bruker Avance-600 spectrometer (Bruker BioSpin Ltd., Canada). Compounds 1 and 2 were dissolved in CD_3OD, separately. TMS was an internal standard. In addition, H–H COSY and C–H correlation were also recorded.

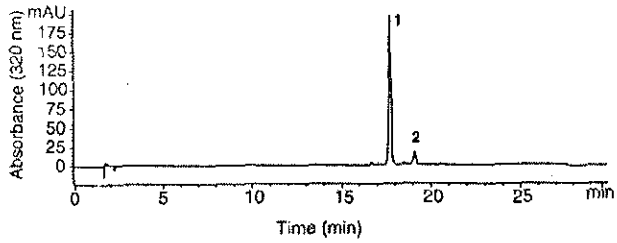

Fig. 2. HPLC profile of the *n*-butanol extract of *P. psyllium* seed. Peak 1, compound 1; peak 2, compound 2. Experimental conditions: column, Phenomenex C_{18}-ODS column (150 mm × 4.6 mm, 5 μm) and a C_{18} guard column; mobile phase: acetonitrile (A) and 2% acetic acid (B), the gradient program: 100% B to 75% B in 20 min, 75% B to 0% B in 5 min, and 0% B to 100% B in 5 min; sample injection volume: 10 μL; flow rate: 1.0 mL/min; detection: 320 nm.

3. Results and discussion

3.1. HSCCC separation

The LC chromatogram of *n*-butanol extract from *P. psyllium* is given in Fig. 2. Two major peaks were separated and detected with retention times at 17.6 and 19.2 min. Compound 1 was identified as acteoside by congruence in its retention time and UV spectra with that of authentic acteoside.

In a HSCCC experiment, selection of the two-phase solvent system is the first and critical step; a good solvent system can provide an ideal partition coefficient (K) for the target compounds. The key of solvent optimization is first to find a solvent combination in which the sample is freely soluble, then to adjust this solvent combination to ensure that the K value of the target compounds is close to 1 [24–26]. The K value of a two-phase solvent system is critical for efficient separation. If it is much smaller than 1, the solutes will be eluted close to each other near the solvent front, which may result in loss of peak resolution; if the K valve is much greater than 1, the solutes will be eluted in excessively broad peaks, and may lead to extended elution time [13].

In our experiment, we selected five series of solvent systems according to the solubility of the target compounds. LC was used to measure the sample concentration in each phase, from which the K values of the target compounds were calculated. The K values of compounds 1 and 2 in these systems are given in Table 1. A solvent system containing ethyl acetate–*n*-butanol–ethanol–water (4:0.6:0.6:5, v:v:v:v) has been used in HSCCC to separate acteoside and 2′-acetyl acteoside from *Cistanches salsa* (C.A. Mey) G. Beck [12]. However, we found that this system was not suitable for the separation of the two phenylethanoid glycosides from *P. psyllium*; the K values of compounds 1 and 2 were greater than 1.5, and the settling time of the two-phase solvent system was rather long. A modified version of this system with solvent ratios at 4:0.6:0.6:4, v:v:v:v was also tried. Although the two phases separated quickly, the K value was too high for both com-

Table 1
The K (partition coefficient) values of compound 1 and 2 in different solvent systems[a]

Solvent system	Compound 1	Compound 2
Ethyl acetate–*n*-butanol–ethanol–water (4:0.6:0.6:5)	1.58	1.94
Ethyl acetate–*n*-butanol–ethanol–water (4:0.6:0.6:4)	2.34	2.76
Ethyl acetate–*n*-butanol–water (0.75:0.25:1)	1.63	1.74
Hexane–ethyl acetate–methanol–water (0.5:0.5:0.25:0.75)	0.68	0.89
Ethyl acetate–water (1:1)	0.82	0.92

[a] Experimental procedure: approximately 1 mg of each sample was weighed in a 10 mL test tube into which 1 mL of each phase of the pre-equilibrated two-phase solvent system was added. The test tube was capped and shaken vigorously for 1 min, and allowed to stand until it separated completely. An aliquot of 100 μL of each layer was taken out and evaporated separately to dryness in vacuo at <40 °C. The residue was dissolved in 100 μL methanol and analyzed by LC for determining the partition coefficient (K) of compound 1 and 2. The K value was expressed as the peak area of target compound in the upper phase divided by that in the lower phase.

pounds 1 and 2 (Table 1). The K values were reduced but still greater than 1 when ethanol was removed in a system containing ethyl acetate–*n*-butanol–water (0.75:0.25:1, v:v:v). A system containing hexane–ethyl acetate–methanol–water (0.5:0.5:0.25:0.75, v:v:v:v) was found to have a short settling time, but poor separation due to the low K values (Table 1). The solvent ratios of the these systems produced K values very close to the targeted value of 1, and the ratios could still be optimized further, however, due to the number of solvents involved, large number of combinations will have to be tested. To avoid this, we examined several two-solvent systems. Ethyl acetate–water (1:1, v:v) gave the best results. This system is simple, and the two phases reached equilibrium very quickly. The K values for compounds 1 and 2 in this system were 0.82 and 0.92, respectively, which produced a good separation (Table 1, Fig. 3). This system was then used in preparative separation and purification of the two main components in the *n*-butanol extract of *P. psyllium*.

Fig. 3A shows the HSCCC separation of one of the five injections containing ca. 200 mg of the *n*-butanol extract of *P. psyllium* using this solvent system. The stationary phase of this system, ethyl acetate–water (1:1, v:v), once filled into the coils, was found to give satisfactory resolution for five consecutive runs of separation. Fractions that were confirmed by HPLC to contain only compound 1 or 2 were combined separately, and those containing both compounds were pooled, freeze-dried and re-subjected to the HSCCC for further separation (Fig. 3B). The two-step HSCCC separation described above yielded a total of 165 mg of compound 1 and 17.5 mg of compound 2 from 978 mg *n*-butanol extract. The recovery rate of compounds 1 and 2 were 89.8 and 84.1%, respectively. The chromatographic purity of the freeze-dried compounds 1 and 2 were 98.4 and 94.2%, respectively, and these samples were directly used for LC–APCI–MS and NMR analyses.

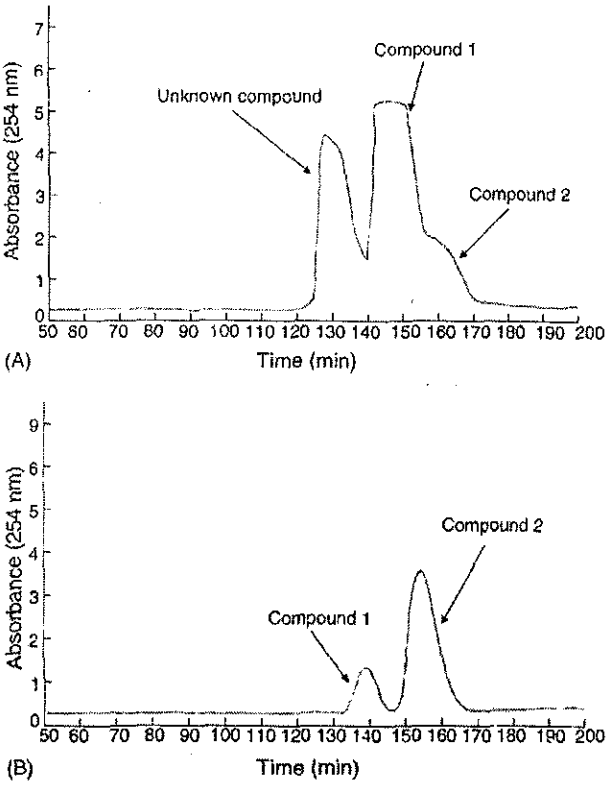

Fig. 3. HSCCC chromatograms of the *n*-butanol extract of *P. psyllium* seed. (A) Crude extract; (B) combined fractions containing both compounds 1 and 2. Experimental conditions: coil volume, 325 mL; rotation speed, 1045 rpm; flow rate, 1.5 mL/min; sample injection volume, 10 mL; sample injected, 200 mg extract dissolved in 8 mL of the mixture of acetate–water (1:1, v:v).

3.2. Identification by LC–APCI–MS and NMR experiments

Tentative identification of the compounds was first achieved by congruent retention times and UV spectra with that of the authentic acteoside standard (Fig. 4). Compound 1 was thus identified as acteoside; compound 2 was an unknown

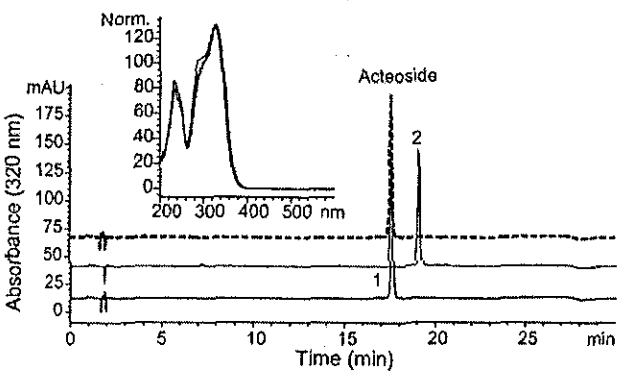

Fig. 4. HPLC chromatograms and UV spectra of standard acteoside, purified compounds 1 and 2. Separation conditions were the same as described in Fig. 2.

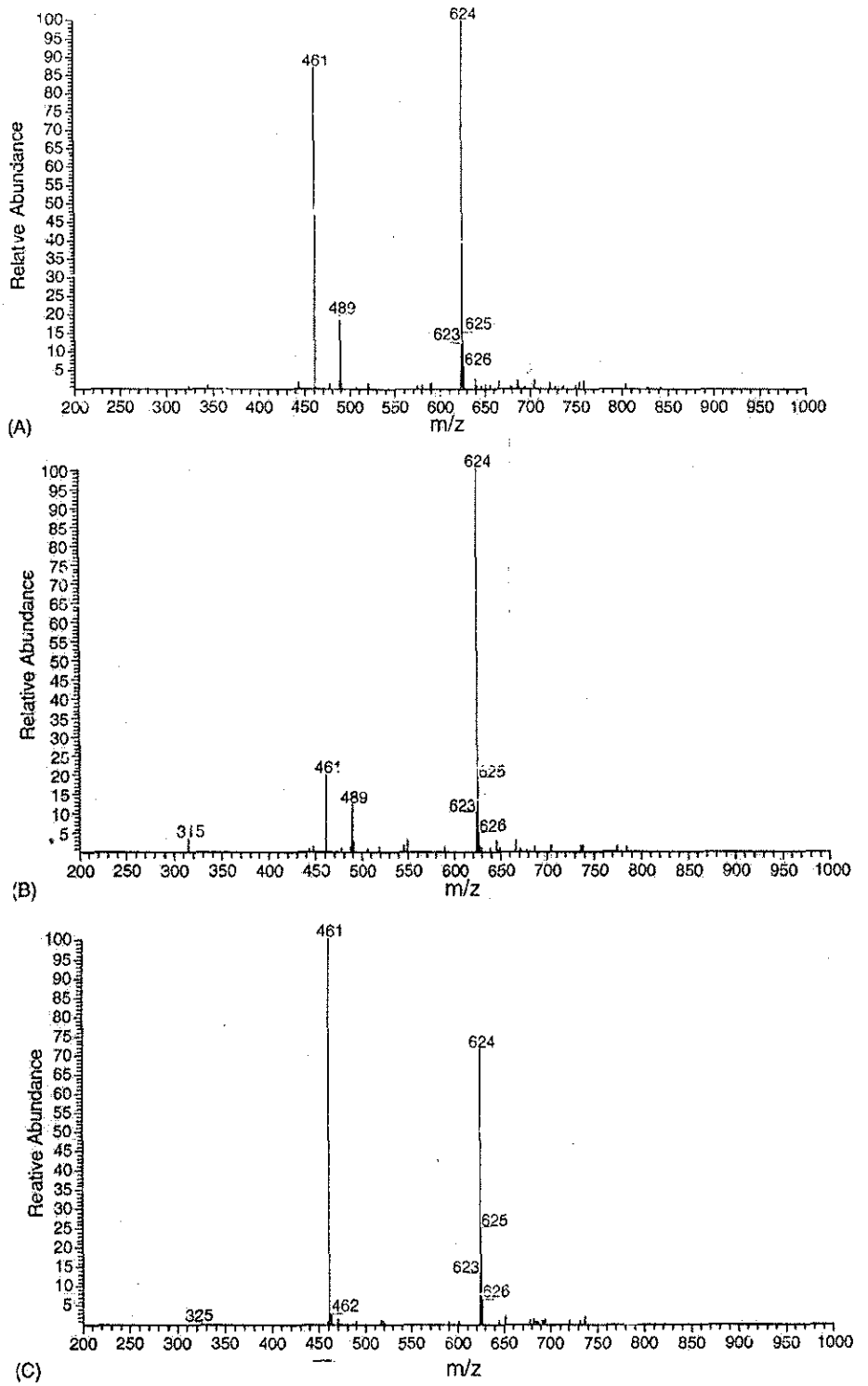

Fig. 5. (A) LC–APCI–MS/MS spectrum of *m/z* 623 of compound 1; (B) LC–MS^3 spectrum of the parent ion *m/z* 461 of compound 1; (C) LC–MS^3 spectrum of the parent ion *m/z* 489 of compound 1; (D) LC–APCI–MS/MS spectrum of *m/z* 623 of compound 2; (E) LC–MS^3 spectrum of the parent ion *m/z* 461 of compound 2; LC–MS^3 spectrum of the parent ion *m/z* 489 of compound 2; (F) LC–MS^3 spectrum of the parent ion *m/z* 489 of compound 2.

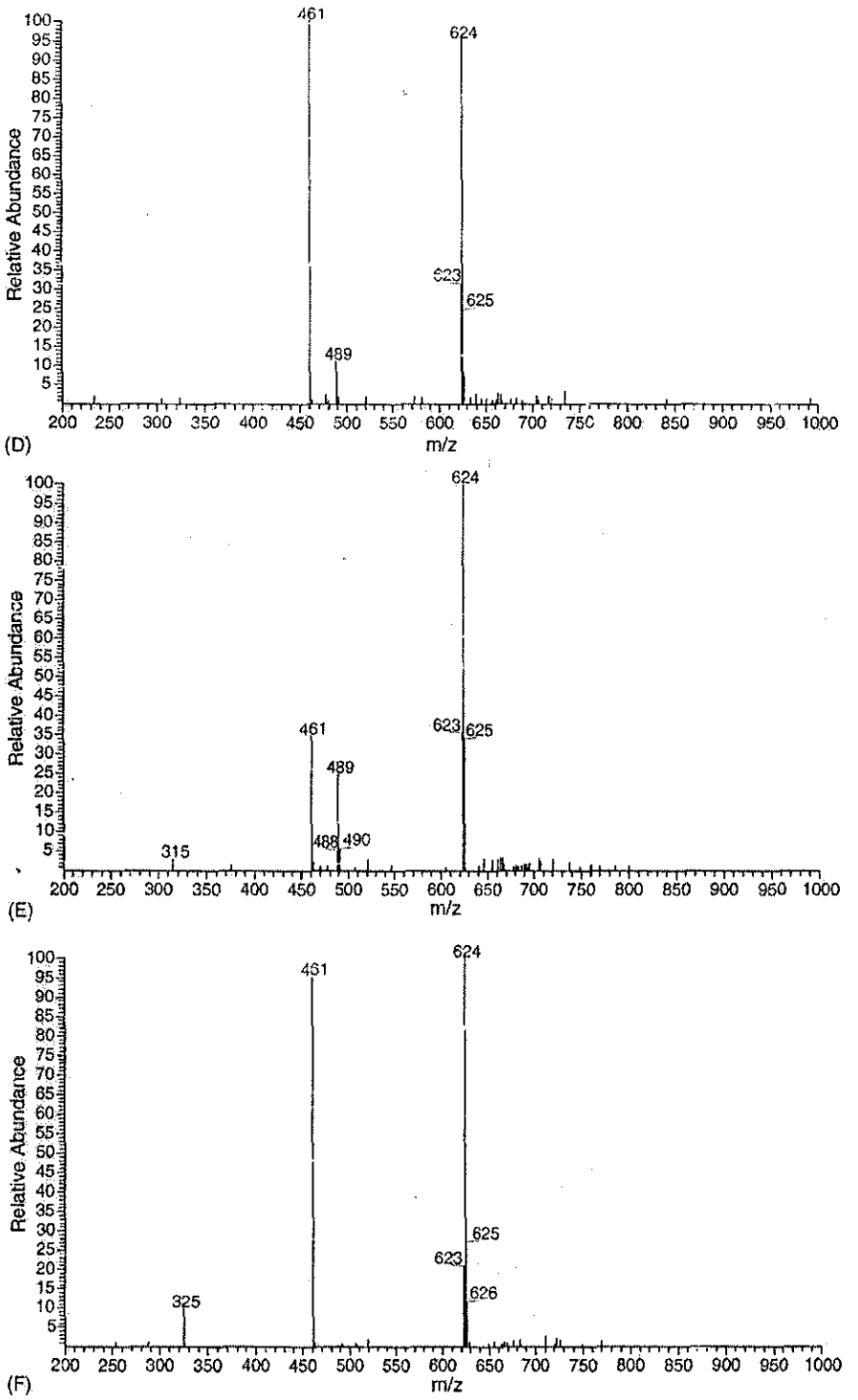

Fig. 5. (*Continued*).

compound. The UV spectrum of compound 1 showed the characteristic of phenylethanoid glycosides with absorption peaks at 232, 246, 289, 332 nm. The UV spectrum of compound 2 was highly similar (232, 246, 286, 328 nm).

Further identification of the structures of these two compounds by LC–APCI–MS, MS^2 and MS^3 are shown in Fig. 5. The LC–APCI–MS of compound 1 in the negative mode gave *m/z* 623 as the deprotonated molecular ion $[M–H]^-$, which confirmed the molecular mass as 624, the same as that for acteoside. Further experiments in MS^2 of the *m/z* 623 ion ($[M–H]^-$) produced two main fragment ions at *m/z* 489 and 461 (Fig. 5A). The ion at *m/z* 461 is considered to be from the loss of the caffeoyl moiety $[M–H–162]^-$ from the parent ion *m/z* 623, and 489 is produced from the cleavage of the bond between C8′ and C9′$[M–H–134]^-$ (Figs. 5A and 6). The MS^3 spectrum of the ion at *m/z* 461 yielded one very weak ion at *m/z* 315 by losing a rhamnose unit (Figs. 5B and 6). The MS^3 spectrum of the ion at *m/z* 489 yielded one very weak daughter ion at *m/z* 325 by losing a 3,4-dihydroxyphenethyl moiety and a carbonyl group (C9′) of the caffeic acid (Figs. 5C and 6). All MS, MS^2 and MS^3 data for compound 1 were the same as that for the authentic acteoside. Possible fragmentation pathways of acteoside are illustrated in Fig. 6.

For compound 2, the LC–APCI–MS data provided *m/z* 623 as the deprotonated molecular ion $[M–H]^-$, which indicates that it has the same molecular mass of 624 as acteoside. The MS^2 spectrum of the ion at *m/z* 623 and MS^3 spectra of the ion at *m/z* 461 and 489 (Fig. 5D–F) were all the same as those of compound 1 (acteoside) (Fig. 5A–C), suggesting that these two compounds are isomers with similar structures.

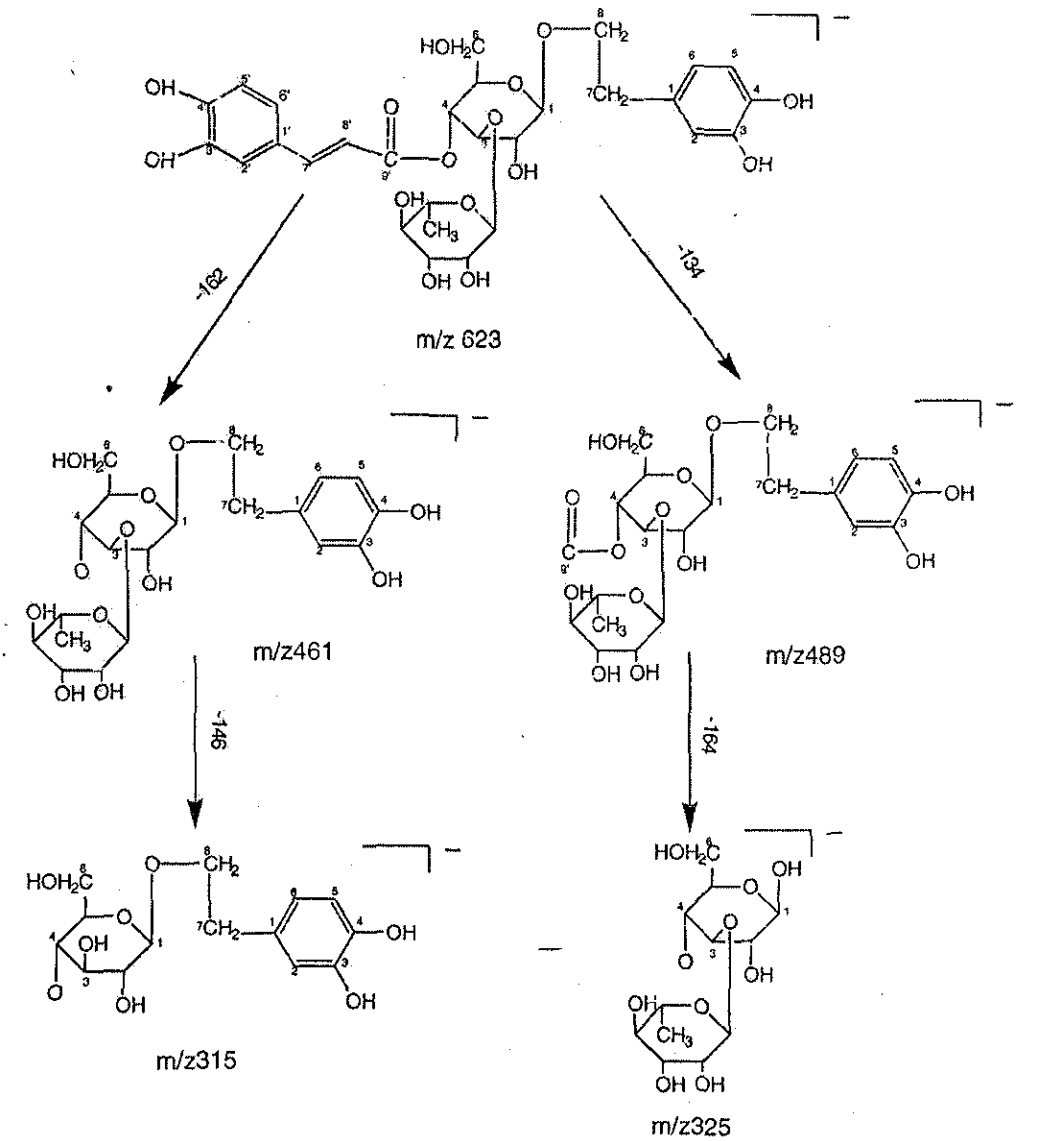

Fig. 6. Proposed fragmentation pathway for acteoside.

Table 2
Proton NMR data of acteoside and isoacteoside[a]

Moiety	C position	δ_H (J)	
		Acteoside	Isoacteoside
Hydroxytyrosol	1		
	2	6.702 (d, 1.8)	6.713 (d, 1.8)
	3		
	4		
	5	6.715 (d, 7.8)	6.674 (d, 7.8)
	6	6.607 (dd, 7.8, 1.8)	6.575 (dd, 7.8, 1.8)
	7	3.760 (dd, 12.6, 7.2), 4.044 (dd, 12.6, 7.2)	3.750 (dd, 9.6, 6.6) 4.025 (dd, 9.6, 6.6)
	8	2.890 (m)	2.821 (m)
Caffeic acid	1		
	2	7.095 (d, 1.8)	7.076 (d, 1.8)
	3		
	4		
	5	6.819 (d, 8.4)	6.809 (d, 7.8)
	6	6.997 (dd, 8.4, 1.8)	6.926 (d, 7.2)
	7	7.634 (d, 15.6)	7.603 (d, 16.2)
	8	6.314 (d, 15.6)	6.328 (d, 15.6)
	9		
Glucose	1	4.417 (d, 8.4)	4.327 (d, 7.8)
	2	3.432 (dd, 9.0, 8.4)	3.350 (dd, 9.0, 6.0)
	3	3.856 (t, 9.0)	3.571 (t, 9.0)
	4	**4.952 (d, 9.6)**	**3.432 (dd, 9.6)**
	5	3.575 (m)	3.598 (m)
	6	**3.664 (dd, 11.7), 3.570 (dd, 11.7)**	**4.536 (dd, 12.0), 4.399 (dd, 12.0)**
Rhamnose	1	5.229 (d, 1.2)	5.220 (s)
	2	3.906 (dd, 3.0, 1.8)	3.986 (d, 3.0)
	3	3.606 (dd, 8.4, 3.2)	3.750 (dd, 9.6, 3.0)
	4	3.354 (dd, 8.4)	3.454 (dd, 9.0)
	5	3.607 (q, 3.6)	4.025 (q, 3.6)
	6	1.133 (d, 6.0)	1.288 (d, 5.4)

[a] δ_H: chemical shift in ppm; splitting patterns and the coupling constant J values (Hz) are in parentheses. The main differences between these two compounds are in bold.

Data collected from our LC–APCI–MS, MS^2 and MS^3 experiment provided much detailed structural information about acteoside and isoacteoside than the MS methods reported in the literature [6,22,23].

Acid hydrolysis of compounds 1 and 2 was performed to determine if the two isomers were only different in sugar content. However, all hydrolysis products were proven to be the same for the two compounds. Caffeic acid and hydroxytyrosol were confirmed by LC–MS, and two sugar moieties were confirmed to be glucose and rhamnose at a molecular ratio of 1:1 by a Dionex (Sunnyvale, CA) DX-500 ion chromatograph.

Further studies in ^{1}H-NMR and 2D-NMR experiments (long-range COSY, ROESY, and CH correlation) of these two compounds, showed that all NMR data of compound 1 matched with the reported NMR data for acteoside, and all NMR data for compound 2 matched with those of isoacteoside [23,27]. ^{1}H-NMR chemical shifts and the coupling constants of the two compounds are shown in Table 2. The major differences in ^{1}H-NMR between compounds 1 and 2 are the protons on C4 and C6 of the glucose moiety. Based on all available data, we therefore conclude that compounds 1 and 2 in *P. psyllium* are acteoside and isoacteoside, respectively.

4. Conclusions

Our study demonstrated that HSCCC could be a highly useful technique for isolating and purifying acteoside and isoacteoside from the seeds of *P. psyllium*. A simple two-phase solvent system containing ethyl acetate:water (1:1, v/v) provided fast and efficient separation, and good purity and recovery rates for the two major phenylethanoid glycosides in *P. psyllium* extract.

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