AJFAND ONLINE
Volume 10 No. 5
May 2010
ISSN 1684 5374
AFRICAN JOURNAL OF FOOD AGRICULTURE NUTRITION AND DEVELOPMENT

THE EFFECT OF SOLVENTS ON RECOVERY OF POLYPHENOLS FROM THE PINK FUJI APPLE SKIN

Amzad HM*[1], Salehuddin SM[2], Mizanur RSM[3] and MJ Kabir[2]

Amzad Hossain

*Corresponding author e-mail: dramzadh@ums.edu.my and/or dramzadh@gmail.com

[1]Biotechnology Research Institute, University Sabah Malaysia, Locked Bag No. 2073, 88999 Kotakinabalu, Sabah, Malaysia
[2]Chemistry Division, Atomic Energy Centre, GPO Box 164, Ramna, Dhaka-1000, Bangladesh

[3]Department of Chemistry, University of Dhaka, Dhaka-1000, Bangladesh

AJFAND ONLINE
Volume 10 No. 5
May 2010
ISSN 1684 5374
AFRICAN JOURNAL OF FOOD AGRICULTURE NUTRITION AND DEVELOPMENT

ABSTRACT

Flavonoids constitute a group of polyphenols widely distributed in plants and are assumed to have beneficial effects on human health when present in food. The phenolic content of apple fruit skin and leaves was determined at the developmental stage of each organ. Phenolic levels decreased on a dry weight basis during the seasonal development of fruits and leaves with respect to their ontogenesis but the single compounds did not behave uniformly. A shift in flavanol pools from monomeric to oligomeric structures during fruit growth indicated the biosynthetic tendency towards the formation of procyanidins at the end of the growing period. A gas chromatography-mass spectrometry (GC-MS) method was developed for the separation and determination of three major flavones: sinensetin (SEN), rutin (RU) and 3′-hydroxy-5, 6, 7, 4′-tetramethoxyflavone (TMF) and rosmarinic acid (RA), a caffeic acid derivative in the pink skin of apple fruit. The GC-MS method was applied for the quantification of SEN, RU, TMF and RA in apple fruit collected from different local markets of Bangladesh during the period of November 2008. Apple fruit skin contains several bioactive phytochemicals including polyphenols such as flavones and phenolic acids. Dehydrated apple skin powder was used to evaluate the recovery of selected flavones and rosmarinic acid using water, methanol, acetone, chloroform, aqueous 50% methanol, and aqueous 70% acetone at 40^{o}C. The retrieved extracts were subjected to qualitative and quantitative GC-MS analysis. Highest amount of sinensetin (SEN) and rutin (RU) was found in the chloroform extract, which was obtained for 4 to 6 hours of extraction at 40^{o}C. Higher proportion of 3'-hydroxy-5, 6,7,4'-tetramethoxyflavone (TMF) was obtained in pure acetone and as well as 70% acetone where the extraction period 4 to 6 hours, respectively. Similar yield of rosmarinic acid (RA) was obtained in aqueous 70% acetone extracts when the periods of extraction were 2, 4, 6 and 8 hours, respectively.

Key words: Quantification, *Malus sylvestris*, Lipophillic flavones, GC-MS

INTRODUCTION

Phenolic acids and flavonoids are secondary metabolites found in most of the plants. These compounds protect plants against ultraviolet radiation, pathogens, and herbivores [1]. The daily intake of flavonoids by humans can range from 50 mg to 1 g, depending on the geographic regions and consumption of fruits and vegetables and their products [2]. Most of the protective effects of phenolics in biological systems are ascribed to their antioxidant abilities, capacity to transfer electrons, quenching of free radicals and chelating abilities [3], activate of [antioxidant enzymes, reduce alpha-tocopherol radicals and inhibit oxidases [4]]. There has been considerable interest in finding the naturally occurring antioxidants to replace synthetic food antioxidants such as butylated hydroxyl toluene. Several studies have been carried out to analyze the antioxidant potential of a variety of herbs [5, 6]. Among the different parts of plants studied, the leaves are reported to have highest antioxidant properties [7, 8, 9] and the most active principle among the phytochemicals is the phenolic fraction [10,11]. The phenolics have *in vivo* antioxidant activities and have been used as natural antioxidants in food [12,13].

Apple is one of the attractive and delicious fruits towards the consumers due to its appearance and attributes of firmness, taste, and health benefits. Flavonoids, contribute to the colour of the fruits and are widely believed to possess antioxidative, antimicrobial, antimutagenic and anticarcinogenic properties [14, 15, 16]. Epidemiological studies have shown an inverse relationship between the intake of fruits, vegetables and beverages rich in flavonoids and the incidence of coronary heart disease, but the relationship with cancer is not clear [17]. Apple is one of the main sources of flavonoid intake in the European diet, after onion and tea [15]. Flavonols such as quercetin 3-*O*-glycosides, monomeric and oligomeric flavan-3-ols such as catechin, epicatechin and procyanidins and anthocyanins such as cyanidin 3-*O* -glycosides are the major flavonoid presence in apple. These fruits also contain considerable amounts of hydroxycinnamic acid derivatives, which are mainly represented by chlorogenic acid [18,19]. Flavonoids and chlorogenic acid contribute to the quality aspects of apples and the red colour of the fruits is primarily due to the flavonoids cyanidin-3-*O* -galactoside located in the vacuoles of epidermal cells of peel [20,21], and the browning occurring in processed apple such as juices and ciders is mainly due to oxidation of chlorogenic acid by oxidative enzymes [15]. The biosynthesis of flavonoids in apple, as in other plant tissues, includes precursors from both the shikimate and the acetate-malonate pathways via several enzymatic steps [22, 23]. Flavonoids are generally present in plant tissues as glycosides. In apple, the predominant sugar involved in glycosylation is galactose. Other sugars involved are glucose, rhamnose, xylose, arabinose and the disaccharide rutinose. Unlike other flavonoids, flavan-3-ols are generally found in the free rather than in the glycosylated forms. The different flavonoid classes are predominantly located in the skin [24, 25].
Thus, it is concluded that culture and growing conditions have limited effects on the polyphenol profiles of the cortex and peel of apple fruits but did not discuss effects on actual concentrations [25]. There is considerable qualitative information on the development and environmental regulation of anthocyanin biosynthesis in apples [26,

27], but quantitative information on the amplitude of variation and hence the potential for control is almost lacking. For other flavonoids even the variation in content due to parietal and environmental factors is poorly studied.

The main objective of the present study was to observe the influence of different solvent extraction systems on three main polymethoxylated flavones in the skin of apple fruit, such as sinensetin (SEN), rutin (RU), 3´-hydroxy-5, 6, 7, 4´-tetramethoxyflavone (TMF) and the major phenolic acid, rosmarinic acid (RA).

MATERIALS AND METHODS

Chemicals and reagents

Standard samples of SEN, RU, TMF and RA were purchased from Indofine Chemical Co. (Hillsborough, NJ, USA). Solvents used for chromatographic separation were methanol, acetone, chloroform (GC grade) and water (GC grade) obtained from Merck (Darmstadt, Germany). Membrane filter (0.45-um pore size) from Millipore was used for filtration of the mobile phase and the samples. The other chemicals used have analytical or GC grade.

Sample collection

Different types of apple are available in Bangladesh markets all round the year. Fuji (*Malus domestica* Borkh) is one of the most popular apple cultivars grown in India. In this study, this variety was used. Apples were collected from different local markets of Bangladesh during the period of November 2008. The skin of this variety is initially yellowish green, but turns red when exposed to the sun's radiation. Ripe apples without visual symptoms of damage are usually harvested from both the outer and inner part of the tree canopy, resulting in apples with various levels of flavonoids in the peel. The colour of the apple ranged from pale green to dark red. After harvesting, the fruits were stored at 2^{o}C.

Extraction of Polyphenol

The fruits were cut out as a plug of skin with a cork borer (17mm diameter) followed by frozen in liquid nitrogen. Measured amount of skin (200 mg with the thickness of 1mm) was separated from the flesh under frozen condition, cut into six pieces with a scalpel and rapidly transferred to a tube containing 1mL of 0.01M HCl in methanol. The sample was sonicated for 30 min in an ultrasonic bath (CTU150, Coax Teknik, Lynge, Denmark) followed by 30 min shaking (1400 rpm) in a CM-9 Mixer (SARSTEDT, Numbrecht, Germany). The extract was transferred to another tube before re-extraction of the fruit materials with the same procedure as described above. The combined extract (2 mL) was filtered through a 0.45 µm Millex HA filter (Millipore, Molsheim, France) prior to GC-MS analysis. The entire procedure was carried out at 4^{o}C and shaded from incident light.

Preparation of samples for GC-MS analyses

One gram of the powder material of fruit peel was extracted successively with water, methanol, acetone, chloroform, 50% aqueous methanol and 70% aqueous acetone for

2, 4, 6, and 8 hours, respectively, at 40°C in a water bath with continuous shaking. The extracts were subjected to qualitative and quantitative GC-MS analysis. The extracts were completely evaporated by K-D evaporator. Each of the extracts was diluted with methanol followed by filtration through 0.45 µm membrane filters (Molsheim, France) prior to GC-MS analysis.

Identification and quantification of the samples by GC-MS

The GC-MS analysis of the crude methanol extract of the samples was performed using a Varian GC-MS (Model Varian CP 3800, USA) equipped with a VF-5 fused silica capillary column (30m x 0.25 i. d., film thickness 0.25 m, Varian, USA). An electron ionization system with the ionization energy of 70 eV was used for the detection of GC-MS. Helium was used as carrier gas with constant flow rate of 1 ml/min. Injector and mass transfer line temperature were set at 250 and 300°C, respectively, whereas the oven temperature was programmed from 50 to 200 at 8°C/min, followed by isothermal elution for 20 min and finally raised to 300°C at 10°C/min. Dilute samples (1/100, v/v, in methanol) of 0.2 µl were injected manually in the splitless mode. Identification of the crude methanol extract was based on GC retention time on VF-5 capillary column and computerized matching of mass spectra with standards (Mainlab, Replib and Tutorial data of GC-MS systems). The following reference compounds were used as markers: [Sinensetin (SEN), Rutin (RU), Tetramethoxyflavone (TMF) and Rosmarinic acid (RA)]. The markers were accurately weighed and dissolved in methanol to produce a series of concentrations. Standard calibration curves were established by plotting the peak areas against different concentrations of the reference compounds (varying from 5.0 to 1000 ng on column for SEN, RA and TMF and 10 to 1000 ng for RU). The external standard method was used for quantification of the markers in the apple extract.

The system suitability of the method was evaluated by the intra- and inter-day precision and accuracy of replicates. The accuracy was evaluated through recovery studies by adding known amounts of the standard solution to the extract. Controls from all samples were prepared and analyzed. The recovery experiment was performed at three different standard concentrations.

Statistical analyses

The experimental results were expressed as the means of triplicate measurements. Correlations were obtained by Pearson correlation coefficient in bivariate correlations. Means were compared by Tukey-HSD and LSD (least significant differences).

RESULTS

The fruits that were selected for this investigation were similar in sizes and all the results calculated based on dry weight basis.

Concentrations of the markers in methanol extracts of apple peel

The GC-MS method applied was a modification of what was reported [21] for the analysis of flavones present in apples. In the present study, a programmed method was

used for simultaneous assay of the authentic markers in which the chemical structures were shown in Figure 1. All standards were determined in a single GC-MS run. The standards were resolved and eluted at 5.35, 13.08, 17.52 and 25.35 min, with respect to RA, TMF, SEN and RU (Figure 2). The markers (5, 50, 500, 750 and 1000 ng on column for SEN, RA and TMF and 10, 100, 500, 750 and 1000 ng for RU) showed a good linearity in the range from 5.0 to 1000 ng in the calibration curves that were obtained by GC-MS analysis. All reference markers were present in the chromatographic profiles of the samples from various locations when the sample solution was analyzed by GC-MS (Figure 3). The peaks of RA, TMS, SEN and RU were confirmed by comparison of their retention times with reference standards (Indofine Chemical Co., Hillsborough, NJ USA)

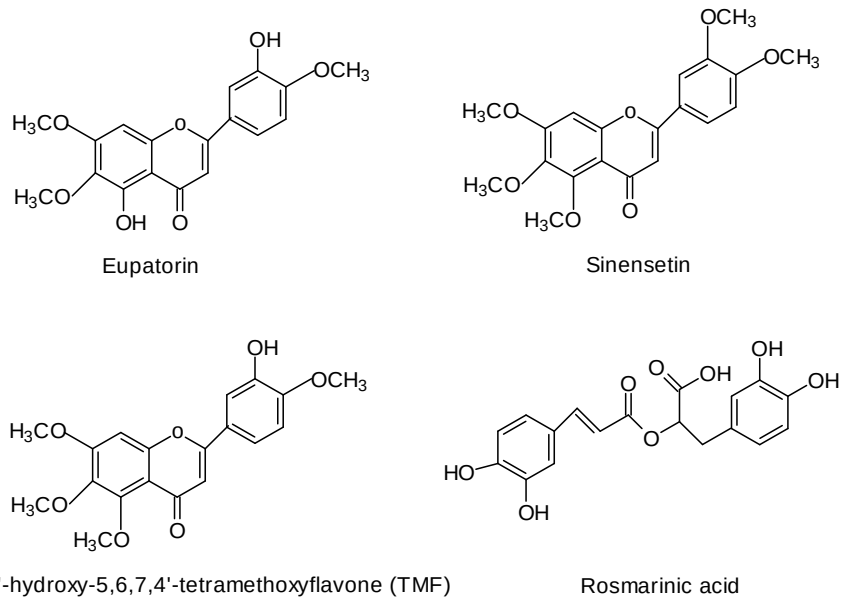

Figure 1: Chemical structures of markers

AJFAND ONLINE
Volume 10 No. 5
May 2010
ISSN 1684 5374
AFRICAN JOURNAL OF FOOD AGRICULTURE NUTRITION AND DEVELOPMENT

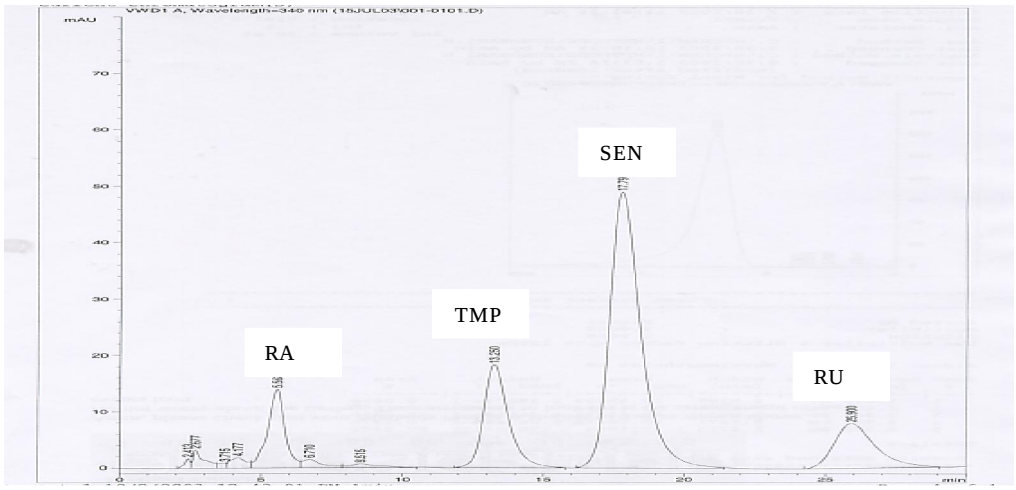

Figure 2: Chromatogram of the mixture of reference compounds, RA [A]; TMF [B]; SEN [C] and RU [D]

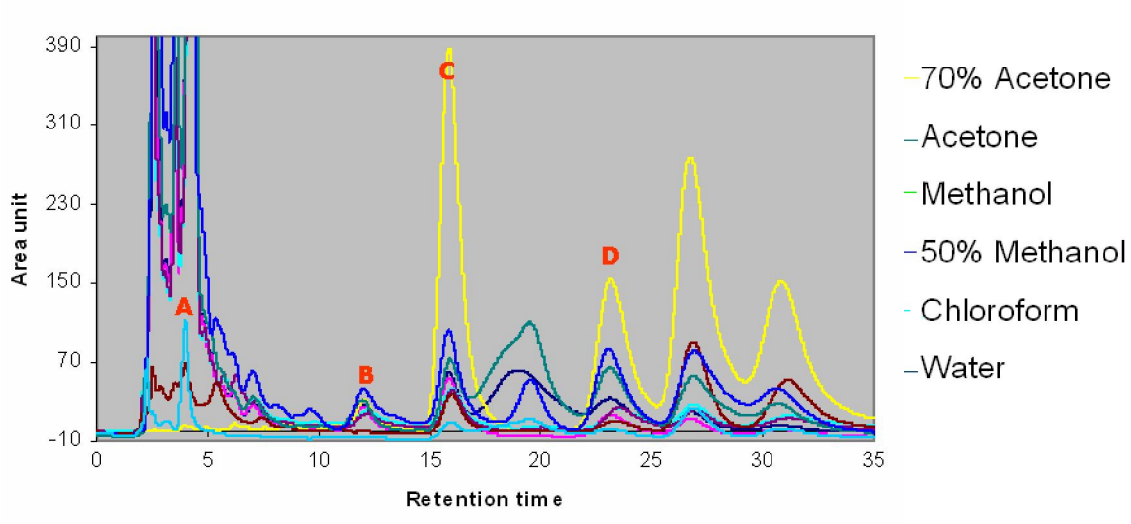

Figure 3: An overlaid of chromatogram of the apple fruit skin by different solvent

To assess the precision of these methods, standard solutions of RA, TMS, RU and SEN were determined six times on the same day and over a six-day period. The results showed a good precision, ranging from 5 to 100 µg/ml (Table 1). The accuracy of the method was evaluated through recovery studies. The recovery experiments were performed at three concentrations (5, 50 and 100 ng) of the standard added to sample solutions, in which the marker content had been determined, using a sample from Dhaka. The results for the recoveries of RA, RU, TMS and SEN were in the range of 96–103%. The limit of detection (LOD) of the GC-MS method, established at signals three times that of the noise for SEN, RU, TMF and RA, was 2.0, 2.5, 2.0 and 2.0 ng, respectively.

The GC-MS procedure was applied to determine the markers presence in the peel. Significant amounts of the markers (with a wide range) (Table 2) were present in the samples. The variation may be ascribed to environmental conditions and variation in sample sourcing. Although the fresh and similar size of fruits were collected for the present investigation, the chemical composition of the peel could be affected by the age and soil fertility levels [15, 26]. The values obtained for the markers appear to fall within the range reported for the markers in peel [29, 30, 31]. However, the overall levels of the marker concentration were considerably higher in samples. RA was the main component found in peel where the concentrations ranging from 0.072 to 1.653% of total dry skin weight. Concentrations of TMF, SEN and RU ranged from 0.002 to 0.29%, 0.433 to 0.029% and 0.010 to 0.146%, respectively (Table 2).

Flavonoids and phenolic acids have been implicated as natural antioxidants in plants, fruits and vegetables. Lipophilic flavones and caffeic acid derivatives were identified in the skin of apple and quantified by GC-MS. Caffeic acid derivatives, rosmarinic acid and lipophilic flavones were predominant phenolics in the methanol extract (Table 2) and their antioxidant properties have been well documented [6,20].

The GC-MS procedure was applied to identify as well as quantify the presence of the markers in apple peel that was extract with different solvents. The quantitative analysis was performed using external standard technique. As shown in Table 2, all the various solvent systems showed a wide range in the concentration of the markers. The amount of rosmarinic acid, the principal polyphenol of peel and the most polar compound among the markers was high in aqueous 70% acetone extract and these amount was significantly different form other solvent systems those were extracted for 2, 4 and 6 hours, respectively, (Table 2). Presence of RA, in different solvent systems were found in the increasing order of aqueous 70% acetone > aqueous 50% methanol > 100% methanol > water > 100% acetone > chloroform.

DISCUSSION

It was observed that the chloroform extracts contained the highest amount of RU and SEN when the peel was extracted 4 and 6 hours, respectively. There was a significant difference of the amount of SEN and RU in chloroform extracts compared to water, methanol, aqueous 50% methanol, aqueous 70% acetone and acetone used as

solvents. Higher concentration of TMF was observed 4 to 6 hours extraction with acetone as well as aqueous 70% acetone but the amount was not significantly different as compared to water and methanol extracts. Polymethoxylated flavones, the principal flavonoid aglycones, present in apple peel are exclusive with a methoxy group at C-5 a structural feature rare in flavonoids. The polarity of the basic ring structure of the polymethoxylated flavones used as markers is quite similar, although the addition of hydroxyl group(s) to ring B in TMF and rings A and B in RU may result in substantial increase in the polarity of the molecules (Fig. 1). The presence of the methoxy groups, five in SEN and four in TMF, renders them lipophillic hence more soluble in lipohillic solvents. Among the various solvents used in the present study for the extraction, chloroform has the lowest polarity, so the proportion of lipopholic markers should be high but the polar marker, RA, was not detected in the chloroform extracts by the study.

CONCLUSIONS

It was concluded that 4 to 6 hours of extraction using chloroform efflux of dehydrated apple skin provided complete recovery of SEN and RU. Similarly, 4 to 6 hours extraction with acetone and aqueous 70% acetone under reflux was optimum for recovery of TMF. The recovery of RA was high when apple skin was treated with water for 8 hours at 40°C. The optimum extraction conditions with different solvent followed by GC-MS method developed in the present study can be used to analyze the polymethoxylated flavones and phenolic acids in apple skin as well as other botanical resources.

ACKNOWLEDGEMENT

The authors are grateful to Sohela Akhtar, Head, Chemistry Division, Atomic Energy Centre, Dhaka, Bangladesh for her constant encouragement and helpful suggestions. They are also grateful to Mr. Zahidul Islam and Mr. Abbas Ali for their help to preparation the apple samples. The study was supported by a University Grant Commission, Dhaka, Bangladesh.

Table 1: Analytical characteristics of calibration curves for the marker phenolics

Constituent	Ret. Time (min)	Range (ng/ml)	Regression Equation	Detection Limit (µg)	R^2	Precision (%) (SD[a]) (n=5, 10ng/µl)	
						Inter-day	Intra-day
Sinensetin (SEN)	17.52	2 -1000	0.1917x-0.3250	2.0	0.9997	0.78	0.37
Rutin	25.35	2.5 -1000	0.0698x-0.0243	2.0	0.9994	0.83	0.42
3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF)	13.08	2 – 1000	0.1950x+0.3032	2.5	0.9998	1.52	1.21
Rosmarinic acid (RA)	5..35	2 - 1000	0.2619x+0.214	2.0	0.9995	0.91	0.83

[a] SD = standard deviation.

Table 2: Isolation of polyphenol with different solvent systems[a]

Percent concentrations of marker polyphenol in the pink skin apple fruit collected from different markets of Bangladesh between 1th November to 30th November 2008[a]

Marker	Time (hrs)	Water	Methanol	50% Methanol	Acetone	70% Acetone	Chloroform
RA	2	0.540 c	0.541 c	1.030 b	0.035 c	1.325 a	ND
	4	0.661 c	0.648 c	0.978 b	0.048 c	1.503 a	ND
	6	0.643 c	0.678 c	0.938 b	0.106 c	1.653 a	ND
	8	0.799 b	0.871 b	0.868 b	0.072 c	1.520 a	ND
TMF	2	0.006 ab	0.017 a	0.003 b	0.011 a	0.010 a	0.005 ab
	4	0.010 a	0.010 a	0.003 b	0.011 a	0.011 a	0.006 ab
	6	0.009 ab	0.009 a	0.003 b	0.011 a	0.012a	0.005 ab
	8	0.009 a	0.008a	0.002 b	0.011 a	0.010 a	0.004 b
SEN	2	0.030 c	0.067 b	0.029 c	0.098 b	0.087 b	0.365 a
	4	0.033 c	0.080 b	0.030 c	0.097 b	0.090 b	0.433 a
	6	0.037 c	0.074 b	0.031 c	0.093 b	0.097 b	0.407 a
	8	0.030 c	0.079 b	0.031 c	0.110 b	0.097 b	0.344 a
RU	2	0.027 b	0.099 b	0.030 c	0.088 b	0.098 b	0.124 a
	4	0.016 b	0.113 b	0.021 c	0.094 b	0.081 b	0.146 a
	6	0.018 a	0.086 b	0.015 c	0.126 a	0.068 b	0.140 a
	8	0.013 a	0.114 a	0.010 c	0.146 a	0.129 a	0.120 a

[a]Mean within column with different letters indicate significantly different values ($P < 0.05$). This experiments were performed in November 2008.

REFERENCES

1. **Harborne J B and CA Willam** Advances in flavonoid research since 1992. *Phytochemistry* 2000 ;**55** : 481–504.

2. **Lugasi A, Hovari J, Sagi KV and L Biro** The role of antioxidant phytonutrients in the prevention of diseases.*Acta Biol. Szeged* 2003;**47** : 119-125.

3. **Hirano I, Sasamoto W, Matsumoto A, Itakura H, Igrashi O and K Kondo** Antioxidant ability of various flavonoids against DPPH radicals and LDL oxidation.*Journal of Nutritional Science Vitaminol (Tokyo)* 2001;**47** : 357–362.

4. **Elliott A J, Scheiber S A, Thomas C and RS Pardini** Inhibition of gluthione reductase by flavonoids.*Biochemical Pharmacology* 1992;**44** : 1603–1608.

5. **Furuta S, Nishiba Y and I Suda** Fluorometric assay for screening antioxidative activity of vegetables.*Journal of Food Science* 1997;**62** : 526–528.

6. **Hirano I, Sasamoto W, Matsumoto A, Itakura H, Igrashi O and K Kondo** Antioxidant ability of various flavonoids against DPPH radicals and LDL oxidation.*Journal of Nutritional Science Vitaminol (Tokyo)* 2001;**47** : 375–390.

7. **Shahidi F, Janitha PK and PD Wanasundara** Phenolic antioxidants. *Critical Reviews of Food Science and Nutrition* 1992;**32** : 67–103.

8. **Chung H S, Chang L C, Lee S K, Shamon L A, Van Breemen R B, Mehta R G, Farnsworth N R, Pezzuto J M and KA Douglas** Flavonoids constituents of Chorizanthe diffusa with potential cancer chemopreventive activity.*Journal of Agricultural and Food Chemistry* 1999;**47** : 36–41.

9. **Venkatamuru K, Patel J D and S Rao** Fractionation of wood phenolics and their use in brandy.*Journal of Food Science and Technology* 1983;**20** : 16–18.

10. **Nakasugi T and K Komai** Antimutagens in the Brazilian folk medicinal plant Carqueja (Baccharis trimera Less).*Journal of Agricultural Food Chemistry* 1998;**46** : 2560–2564.

11. **Pietta P, Simonetti P and P Mauri** Antioxidant activity of selected medicinal plants.*Journal of Agricultural and Food Chemistry* 1998;**46** : 4487–4490.

12. **Zloch Z** Influence of quercetin and epicatechol on biochemical change in guinea pig during experimental C-hypovaminosis. *International Zeitschrift Vitaminforsch,* 1969;**39** : 269–280.

13. **Fuhrman B, Lavy A and M Aviram** Consumption of red wine meal reduces the susceptibility of human plasma and low density lipoprotein to lipid. *American Journal of Clinical Nutrition* 1995;**61** :349–554.

14. **Koes RE, Van Blokland R, Quattrocchio F, Van Tunen AJ and JNM Mol** Chalcone synthase promoters in petunia are active in pigmented and unpigmented cell types.*Plant Cell* 1990;**2** :379-392.

15. **Formica JV and W Regelson** Review of the biology of quercetin and related bioflavonoids.*Food and Chemical Toxicology* 1995;**33** :1061-1080.

16. **Robards K and M Antolovich** Analytical chemistry of fruit bioflavonoids. *Analyst,* 1997;**122** :11R-34R.

17. **Hollman PCH** Determinants of the absorption of the dietary flavonoids quercetin in man. PhD. thesis. Wageningen Agriculture University, Wageningen, The Netherlands.1997.

18. **Lancaster JE** Regulation of skin color in apples.*Critical Reviews in Plant Sciences* 1992;**10** :487-502.

19. **Nicolas JJ, Richard-Forget FC, Goupy PM, Amiot MJ and SY Aubert** Enzymatic browning reaction in apple and apple products.*Critical Reviews in Food Science and Nutrition* 1994;**34:** 109-157.

20. **Sun BH and FJ Francis** Apple anthocyanins: identification of cyanidin 7-arabinoside *Journal of Food Science* 1967;**32** :647-648.

21. **Lancaster JE, Grant JE, Lister CE and M Taylor** Skin color in apples-influence of copigmentation and plastid pigments on shade and darkness of red color in five genotypes. *Journal of the American Society for Horticultural Science* 1994;**119** :63-69.

22. **Stafford HA** Flavonoids Metabolism. CRC Press, Boca Raton, FL, pp. 1990; 101-132.

23. **Van der Meer IM, Stuitje AR and JNM Mol** Regulation of general phenylpropanoid and flavonoids gene expression. In: Verma, D.P.S., (Ed.), *Control of Gene Expression. CRC Press, Boca Raton, FL* ,1993; 125-155.

24. **McRae KB, Lidster PD, De Marco AC and AJ Dick** Comparison of the polyphenol profiles of the apple fruit cultivars by correspondence analysis. *Journal of the Science of Food and Agriculture* 1990;**50** :329-342.

25. **Guyot S, Thierry D, Soijet M B, Moutounet M and EJ Drilleau** Characterization of highly polymerized procyanidins in cider apple *(malus sylvestris var. kermerrien)* skin and pulp.*Phytochemistry* 1997;**44** :351-357.

26. **Guyot S, Marnet N, Laraba D, Sanoner P and JF Drilleau** Reversed-phase HPLC following thiolysis for quantitative estimation and characterization of the four main classes of phenolic compounds in different tissue zones of a French cider apple variety (Malus domestica Var. Kermerrien). *Journal of Agricultural and Food Chemistry* 1998;**46** : 1698-1705.

27. **Brand-Williams W, Curvelier M E and C Berset** Use of free radical method to evaluate antioxidant activity. *Lebensmittel- Wissenscaft Technologie* 1995; **28** : 25–30.

28. **Mohamed A, Awad, Anton de J, Lucie M and V Westing** Flavonoid and chlorogenic acid levels in apple fruit: characterisation of variation. *Scientia Horticulturae* 2000;**83** : 249-263.

29. **Stuart N, Ryan, William A, Laing and T Michael** A cysteine proteinase inhibitor purified from apple fruit. *Phytochemistry* 1998;**49(4):** 957-963.

30. **Beuerle E, Schreier P, Brunerle M, Bicchi L and L Schwab** Absolute configuration of octanol derivatives in apple fruits. *Phytochemistry* 1996; **43** : 145-149.

31. **Taylor O A, Fetuga B L and VA Oyenuga** Accumulation of mineral elements in five tropical leavy vegetables influenced by nitrogen fertilization and age. *Scientia Horticulturae* 1983;**18** : 313–322.