ANTIOXIDANT PROPERTIES OF COCOA POWDER

M.J. ABBE MALEYKI and A. ISMAIL[1]

Department of Nutrition and Dietetics
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
43400, UPM Serdang
Selangor Darul Ehsan, Malaysia

ABSTRACT

This study was aimed to determine antioxidant properties of cocoa powder. Crude cocoa extracts were fractionated using prepacked column (25 cm × 2.0 cm) with Sephadex LH 20 and an increase in water–acetone (85:15, 70:30, and 40:60, v/v) as elution. The resulting fractions 1 (F1), 2 (F2) and 3 (F3) were tested for phenolic contents, antioxidant capacity, identification of bioactive compounds liquid chromatography-mass spectrometry (LC-MS) and stability test. Theobromine and caffeine were major compounds detected in F1 and F2. Monomer, dimer and trimer were identified in F3 as m/z 289, 578 and 867, respectively. Addition of F1 and F2 could reduce antioxidant capacity of F3. Catechin and epicatechin in F3 was stable when stored at 4 and −20C for 5 months. High antioxidant capacity in F3 was likely due to the monomeric, dimeric and trimeric phenolic compounds. The presence of methylxanthines could reduce antioxidant capacity of flavonoids in cocoa powder.

PRACTICAL APPLICATIONS

Numerous studies have been reported on the health benefits of cocoa powder. Polyphenol compounds present in cocoa powder could significantly contribute to their health-promoting activities. The study of bioactive compounds (polyphenols and methylxanthines) toward antioxidant capacity could reveal their actual antioxidant properties. Stability of individual polyphenol compounds under different storage condition and time could help in preserving their quality when producing products from cocoa powder.

---

[1] Corresponding author. TEL: 6089472435; FAX: 6089426769; EMAIL: amin@medic.upm.edu.my

INTRODUCTION

The studies of cocoa and their related products have become an area of interest owing to their health-promoting properties. In recent years, cocoa and cocoa products, namely cocoa powder, dark chocolate and cocoa liquor, have been shown to suppress the development of atherosclerotic lesions (Kurosawa *et al.* 2005), decreased platelet function (Murphy *et al.* 2003), increased dermal blood flow (Neukam *et al.* 2007), and inhibit the proliferation of human breast cancer cells (Ramljak *et al.* 2005) and exerted hypoglycemic properties (Tomaru *et al.* 2007).

Aforementioned health-promoting properties of cocoa were attributed to their phenolic compounds, mainly flavonoids. Generally, cocoa contains significant amount of procyanidin monomers, namely catechin, epicatechin and dimer to tetradecamer (Adamson *et al.* 1999; Rios *et al.* 2003; Kelm *et al.* 2006; Tomas-Barberan *et al.* 2007). These procyanidins also showed potent antioxidant capacity *in vitro* and *in vivo* (Adamson *et al.* 1999; Vinson *et al.* 1999). However, different types of cocoa, different countries of origin, degree of fermentation and roasting might have different composition of polyphenol content (Vinson *et al.* 1999; Wollgast and Anklam 2000; Azizah *et al.* 2007; Tomas-Barberan *et al.* 2007).

Our previous studies showed that cocoa beans, cocoa powder and cocoa liquor extracts reduced the severity of hepatocarcinogenesis and possessed antihyperglycemic properties in streptozotocin-induced rats (Amin *et al.* 2004a,b; Ruzaidi *et al.* 2005). In addition, recent studies by Kurosawa *et al.* (2005) and Vinson *et al.* (2006) demonstrated that diet supplemented with cocoa powder had beneficial effects on the development of atherosclerotic lesions in animal models. Their findings suggested that significant effects of cocoa powder are mainly due to antioxidant activity of polyphenol compounds.

Apart from polyphenol content, cocoa was also rich in methylxanthine, namely caffeine and theobromine (Greer *et al.* 2001; Rios *et al.* 2003). A study reported that caffeine supplementation be able to decrease insulin-mediated glucose uptake and glucose disposal (Lee *et al.* 2005). In addition, administration of theobromine purified from cocoa reduced the lipid profiles in hypercholesterolemic animal (Eteng and Ettarh 2000). Therefore, the health effects of cocoa and cocoa products could also be due to other compounds than polyphenols. This present study was initiated to fractionate and identify the main bioactive compounds in cocoa extract in order to understand their contribution toward antioxidant capacity.

As cocoa contains mixtures of bioactive compounds, it is important to preserve their phenolic quality both under physiological and laboratory conditions. As the preparation of extract was time-consuming and needed to be

stored before use, the study of their stability under typical storage is warranted. To a greater extent, recent studies reported the degradation of catechin, epicatechin and their dimers under simulated physiologic pH (Zhu *et al.* 2003; Klimczak *et al.* 2007). Under typical storage conditions, a decrease of polyphenols in beverages was reflected by their low antioxidant capacity when stored at different temperatures and prolonged time period (Sang *et al.* 2005). A study indicated that auto-oxidation and epimerization were two major reactions involved in the instability of phenolic compounds under typical experimental conditions (Zhu *et al.* 2002a). To the best of our knowledge, no study has been done on the stability of phenolic compounds under typical storage condition. Hence, our present study is also initiated to investigate the stability of phenolic compounds under the aforementioned condition.

MATERIALS AND METHODS

Chemicals

Catechin, epicatechin and potassium peroxodisulfate ($K_2S_2O_8$) were purchased from Sigma Aldrich (St. Louis, MO). Folin–Ciocalteu reagent, ferric chloride ($FeCl_3$), ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) and sodium carbonate (Na_2CO_3) were purchased from Merck (Darmstadt, Germany). 2,4,6-Tripyridyl-s-triazine (TPTZ) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) were obtained from Fluka Chemie GmbH (Steinheim, Germany). Sodium nitrite ($NaNO_2$) and aluminum chloride ($AlCl_3$) were purchased from BDH Chemicals (Poole, England) and Sephadex LH-20 from Amersham Biosciences (Uppsala, Sweden). Other common reagents used were of high-performance liquid chromatography (HPLC) grade.

Preparation of Crude Cocoa Extract

Crude cocoa extract was prepared according to a study described by Ruzaidi *et al.* (2005). Commercially available Malaysian cocoa powder was purchased from KL-Kepong Cocoa Products Sdn. Bhd., Port Klang, Selangor, Malaysia. The cocoa powder was defatted by means of 10-folds of petroleum ether using Soxhlet apparatus. Crude extract was prepared by extracting defatted cocoa powder (40 g) with 80% (v/v) ethanol for 2 h in the ratio of 1:10. The ethanol was removed from the extract using a rotary evaporator (Buchi Rotavor R-200, Flawil, Switzerland) for 40 min at 55C. The resulting aqueous cocoa extract (40 mL) was used for fractionation.

Fractionation of Cocoa Extract

Fractionation of cocoa extract was done using prepacked column (25 cm × 2.0 cm) with Sephadex LH 20 (Amersham) at room temperature

according to the method as described by Osakabe *et al.* (1998). The modification was done on sample preparation, which was prepared as described before. Stepwise increased in water–acetone (85:15, 70:30 and 40:60) as elution medium was used. Five milliliters of aqueous extract was carefully loaded onto the column. Flow rate of 2.0 mL/min was maintained along the procedure. One hundred milliliters of the resulting fractions, namely Fraction 1 (F1), Fraction 2 (F2) and Fraction 3 (F3), were collected and evaporated to dryness at 50C for 1 h (Buchi Rotavor R-200) to remove water and acetone. The resulting fractions were weighed and diluted with known amount of respective elution medium for total phenolic content, antioxidant capacity, identification of bioactive compounds and stability test.

Total Polyphenol Content

Total polyphenol content was determined using a Folin–Ciocalteu assay, which measure the formation of blue-green complexes between phenolic compounds and Folin–Ciocalteu reagent (Velioglu *et al.* 1998). First, 100 μL of appropriately diluted samples were added to 0.75 mL of diluted Folin–Ciocalteu reagent (Folin–Ciocalteu reagent: distilled water, 1:10). Then, the mixture was kept at room temperature for 5 min. Subsequently, 750 μl of 6% (w/v) Na_2CO_3 was added to it. The solution was allowed to stand at room temperature for 90 min. Absorbance was read at 725 nm using an ultraviolet (UV)-Vis spectrophotometer (SECOMAM, Anthelie Advanced 5, Domont, France). Catechin in the range of 100–1,000 μmol was used as the standard. Total polyphenols were expressed as mol catechin equivalent (CE)/g fraction.

Total Flavonoid Content

Total flavonoid content was determined based on the spectrophotometric method (Jia *et al.* 1999). One milliliter of appropriately diluted (1:50) samples was added to deionized water. Then, 0.3 mL of 5% (w/v) $NaNO_2$ was added to the mixture. After 5 min, 0.3 mL of 10% (w/v) $AlCl_3$ was added to the mixture and kept for another 6 min. Finally, the mixture was added with 2 mL NaOH (1 M) and made up to 10 mL with deionized water. Absorbance was read at 510 nm using a UV-Vis spectrophotometer (SECOMAM, Anthelie Advanced 5). Catechin in the range of 100–1,000 μmol was used as the standard. Total flavonoids were expressed as mol CE/g fraction.

Antioxidant Capacity

Ferric Reducing/Antioxidant Power Assay. This assay followed the methods described previously (Benzie and Strain 1996; Katalinic *et al.* 2005). In principle, ferric reducing/antioxidant power (FRAP) assay measures the

change in absorbance at 593 nm due to the formation of blue-colored complex between ferrous ion (Fe^{2+}) and TPTZ. Prior to this, colorless ferric ion (Fe^{3+}) was oxidized to ferrous ion (Fe^{2+}) by the action of electron-donating antioxidants. Freshly prepared FRAP reagent was warmed at 37C in a water bath which gives the initial reading ($A_{initial}$; $t = 0$ min). This reagent was prepared by mixing 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3$ and 0.3 M acetate buffer (pH 3.6) in the ratio of 1:1:10. For sample, 100 µL of each fraction was added to 100 µL of deionized water and 1.8 mL of FRAP reagent. The mixture was incubated at 37C for 4 min. Absorbance was read at 593 nm using a UV-Vis spectrophotometer (SECOMAM, Anthelie Advanced 5). FRAP value was calculated based on the following equation.

FRAP value = $A_{final} - A_{initial}$

A_{final} Final absorbance at 593 nm (4 min)

$A_{initial}$ Initial absorbance at 593 nm (0 min)

A reducing ability in FRAP assay was calculated with reference to the reaction given by $FeSO_4.7H_2O$ at concentrations ranging from 100 to 1,000 µmol. The values were expressed as mol Fe^{2+} equivalents/g fraction.

CE Antioxidant Capacity Assay. Scavenging activity of the fractions was determined based on the inhibition of cation radicals of ABTS with slight modifications on the sample volume and the use of reference calibration curve (Katalinic *et al.* 2005).

ABTS radical cation (ABTS$^{\bullet+}$) solution was prepared by adding 0.04 g of ABTS in 10 mL of distilled water. The ABTS$^{\bullet+}$ was produced by dissolving initially prepared ABTS solution in 2.45 mM potassium peroxodisulfate, and the mixture was allowed to stand at room temperature for 12 h. The resulting ABTS$^{\bullet+}$ solution was further diluted with distilled water to an absorbance of 0.70 ± 0.02 at 734 nm before use.

Twenty milliliters of diluted ABTS$^{\bullet+}$ solution was added to 100 µL of fraction or catechin solution as the standard, and the reaction mixture was incubated at 30C for 6 min. Subsequently, the decrease in absorbance at 734 nm was determined using a UV-Vis spectrophotometer (SECOMAM, Anthelie Advanced 5). Percentage inhibition of the ABTS$^{\bullet+}$ was calculated based on the following equation.

Inhibition $(\%) = [(A - B)/A] \times 100$

A Absorbance of control ($t = 0$ min)

$$B \qquad \text{Absorbance of sample/standard } (t = 6 \text{ min})$$

The decrease in absorbance due to the inhibition of radical cation was determined using the standard curve plotted using a catechin solution at concentrations ranging from 100 to 1,000 µmol. The values were expressed as mol CE/g fraction.

Identification of Bioactive Compounds

Bioactive compounds in each fraction were determined using liquid chromatography-mass spectrometry (LC-MS) (Finnigan LCQ Deca, San Jose, CA) with slight modifications on LC-MS conditions (Natsume *et al.* 2000). Liquid chromatography system (Agilent 1100, Palo Alto, CA) equipped with quaternary pump, auto-injector, degasser and Diode Array Detector (DAD). Separation of phenolic compounds was done using a reversed-phase C_{18} column (Alltech, Licosphere, Deerfield, IL) (250 mM × 4 mm, 5 µm I.D.) and gradient elution of (A) water–trifluoroacetic acid (99.9:0.1, v/v) and (B) acetonitrile–trifluoroacetic acid (99.9:0.1, v/v). Specifically, linear gradient elutions of 0–10% (A) for 5 min, 10–25% (A) for 25 min and 25–100% (A) for 5 min with a flow rate of 0.8 mL/min was used for the analysis. The spectrum was monitored at 280 nm. Mass spectrometer (MS) conditions used were protonated molecular ion $[M + H]^+$ MS equipped with an electron spray ionization (ESI) source, capillary temperature at 275C and capillary voltage at 31.00 V. Full scan mass covered the range of m/z 200–2000. Data acquisition was performed using Finnigan XCalibur version 1.4.

Stability Test

Stability test of polyphenols was evaluated according to previous method (Su *et al.* 1999). F3 was selected for this test as it contains most polyphenol compounds. For stability evaluation, an aliquot of fraction was stored at different storage conditions (−20C, 4–6C and 26–28C for 0, 24, 48 h and 5 months). Following the described conditions, samples were chromatographed using a HPLC (Natsume *et al.* 2000), as mention before. The amount of catechin and epicatechin (mg/g fraction) was quantified based on external standards (100–1,000 µg/mL).

Statistical Analysis

Data were expressed as mean ± standard deviation of three replications. One-way analysis of variance (SPSS version 12.0, Chicago, IL) and Tukey's post hoc test were used to determine the mean differences for total phenolic and flavonoid contents, antioxidant capacity and stability of polyphenol

compounds at a significance level of $P < 0.05$. Pearson's correlation (r value) was used to determine correlation between antioxidant capacity and polyphenol contents.

RESULTS AND DISCUSSION

Total Phenolic and Flavonoid Contents

The resulting F1, F2 and F3 yielded 62.4 ± 0.002 (6.24%), 1.8 ± 0.00 (0.18%) and 9.6 ± 0.002 mg (1.0%) dry matter per g cocoa extract, respectively. The yields were significantly different ($P < 0.05$) when compared with each other. Total phenolic and flavonoid contents of each fraction are depicted in Fig. 1. The contents of total phenolic and flavonoids are in the range of 0.16–0.60 mmole and 0.04–0.99 µmole CE/g fraction, respectively. There were significant differences ($P < 0.05$) in total phenolic content between each fraction. Flavonoid content was significantly different ($P < 0.05$) except between F1 and F2 and F1 and crude extract. Total phenolic content was in the order of F3 > crude extract > F2 > F1. Total flavonoid content was in the order of F3 > crude extract > F1 > F2. Osakabe *et al.* (1998) reported that different fractions of cocoa extract prepared from cocoa liquor showed potent antioxidant activity, but the study had not determined the total phenolic and flavonoid
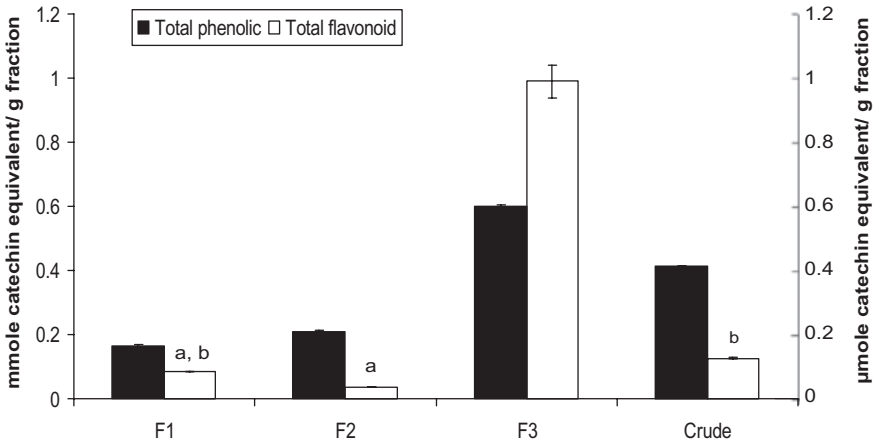

FIG. 1. TOTAL PHENOLIC AND FLAVONOID CONTENTS OF DIFFERENT COCOA POWDER FRACTIONS

Results are expressed as mean ± standard deviation of triplicate values. Total phenolic and flavonoid are expressed as mmole and µmole catechin equivalent/g fraction, respectively. All values are significantly different at the level of $P < 0.05$ except those with identical letters.

contents of the fractions. Of note, both quantity (phenolic and flavonoid content) and quality (antioxidant activity) are important part in phenol anti-oxidant of cocoa and cocoa products (Vinson *et al.* 1999). Hence, to extend the knowledge on quality of both phenolic and flavonoid contents, determination of antioxidant capacity was carried out.

Antioxidant Capacity

Antioxidant capacity based on FRAP and CE antioxidant capacity assays was shown in Fig. 2. F3 showed significant ($P < 0.05$) highest antioxidant capacity compared with the other fractions. Antioxidant capacity of fractions were in the order of F3 > crude powder extract > F2 > F1. Combination of F1 in F3 and F2 in F3 (1:9) was done to determine the synergism effect of fractions. The addition of F1 and F2 resulted in decreased antioxidant capacity of F3 (Fig. 2). Previous study demonstrated that methylxanthines as well as phenolic compounds are present in cocoa powder (Tomas-Barberan *et al.* 2007). Hence, we postulated that methylxanthines could present in F1 and F2
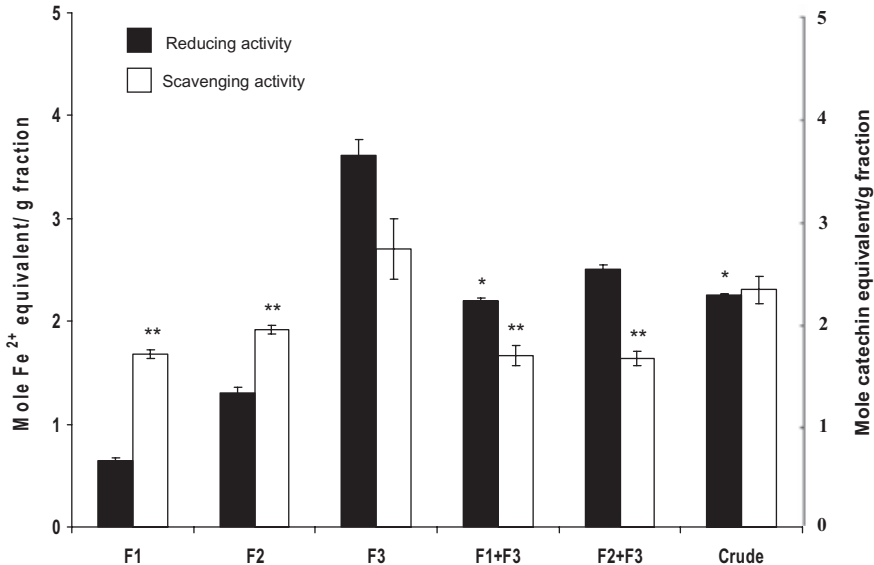

FIG. 2. REDUCING POWER AND SCAVENGING ACTIVITY OF DIFFERENT COCOA POWDER FRACTIONS

Results are expressed as mean ± standard deviation of triplicate values. Single (*) and double asterisks (**) indicate that values are not significantly different at $P > 0.05$ within reducing and scavenging activity, respectively. F1, fraction 1; F2, fraction 2; F3, fraction 3; Crude, crude cocoa extract.

owing to their low antioxidant capacity while phenolic compounds in F3 as indicated by high antioxidant capacity. Pearson's correlation was used to give credence to the contribution of phenolic and flavonoid toward antioxidant capacity. The results indicated that phenolic content significantly ($P < 0.05$) contributed toward reducing power and scavenging activity with $r = 0.99$ and 0.94, respectively (Fig. 3a,b). Similarly, flavonoid content significantly ($P < 0.05$) contributed toward reducing power and scavenging activity with $r = 0.82$ and 0.73, respectively (Fig. 3c,d). Lee *et al.* (2003) reported that antioxidant capacity highly contributed toward phenolic and flavonoid contents. It was reported that phenolic in cocoa extract ranging from monomer to decamers possessed strong antioxidant capacity (Zhu *et al.* 2002b). A high antioxidant capacity in F3 was likely attributed to their phenolic and flavonoid contents. The findings indicated that the compounds present in F1 and F2 lowered the antioxidant capacity of F3.

Previous study indicated that a fraction derived from cocoa liquor extract had highest antioxidant capacity compared with the other fractions. The candidate compounds detected were catechin and epicatechin in the cocoa liquor extract (Osakabe *et al.* 1998). However, no further study was reported on the bioactive compounds present in the other fractions. Hence, identification of individual bioactive compounds in each fraction was carried out to further understand and confirm their relative contribution toward antioxidant capacity and phenolic content of cocoa powder.

Identification of Bioactive Compounds

Bioactive compounds in each fraction was identified by means of ESI LC-MS. Protonated molecular ionization $[M + H]^+$ of each fraction was depicted in Fig. 4. The present results indicated that the bioactive compounds were well ionized in positive ionization mode. Figure 4a shows theobromine and caffeine as main compounds in F1. Theobromine and caffeine contents were 7.63 ± 0.93 and 12.22 ± 0.13 mg/g fraction, respectively. Theobromine and caffeine were eluted earlier as it was more polar compared with phenolic compounds (Adamson *et al.* 1999). Similar to F1, F2 contains theobromine, caffeine and an additional compound, theophylline (Fig. 4b). However, both theobromine and caffeine were significantly lower ($P < 0.05$) compared with F1 with 0.99 ± 0.03 and 1.61 ± 0.31 mg/g fraction, respectively. Theophylline shares the same molecular weight to that of theobromine, but it was ionized at different time. These findings showed that methylxanthines were well separated in cocoa powder extract, and are predominant compounds in F1 and F2. Hence, it showed that low phenolic content and low antioxidant capacity of F1 and F2 were due to methylxanthines.

Phenolic monomers up to trimer were identified in F3 (Fig. 4c). Flavonoid monomers in F3 were identified as catechin and epicatechin at same
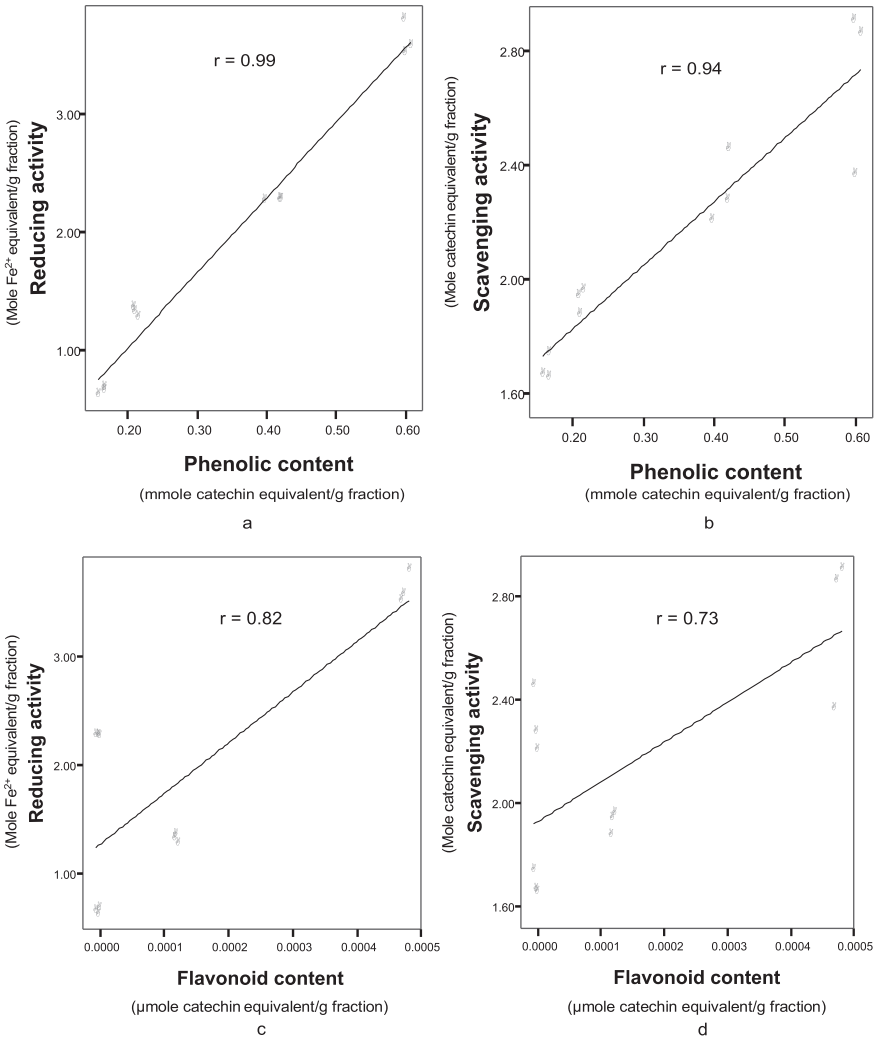
FIG. 3. CORRELATION BETWEEN (a) REDUCING ACTIVITY AND PHENOLIC CONTENT;
(b) SCAVENGING ACTIVITY AND PHENOLIC CONTENT; (c) REDUCING ACTIVITY AND
FLAVONOID CONTENT; (d) SCAVENGING ACTIVITY AND FLAVONOID CONTENT
The correlations were significantly different at level of $P < 0.05$.

m/z 291. Based on HPLC-DAD analysis, epicatechin content was higher than that of catechin with 56.24 ± 3.0 and 39.81 ± 3.9 mg/g fraction, respectively. This result was in agreement with Osakabe *et al.* (2002), which indicated that epicatechin and catechin were major compounds in cocoa powder. Along with
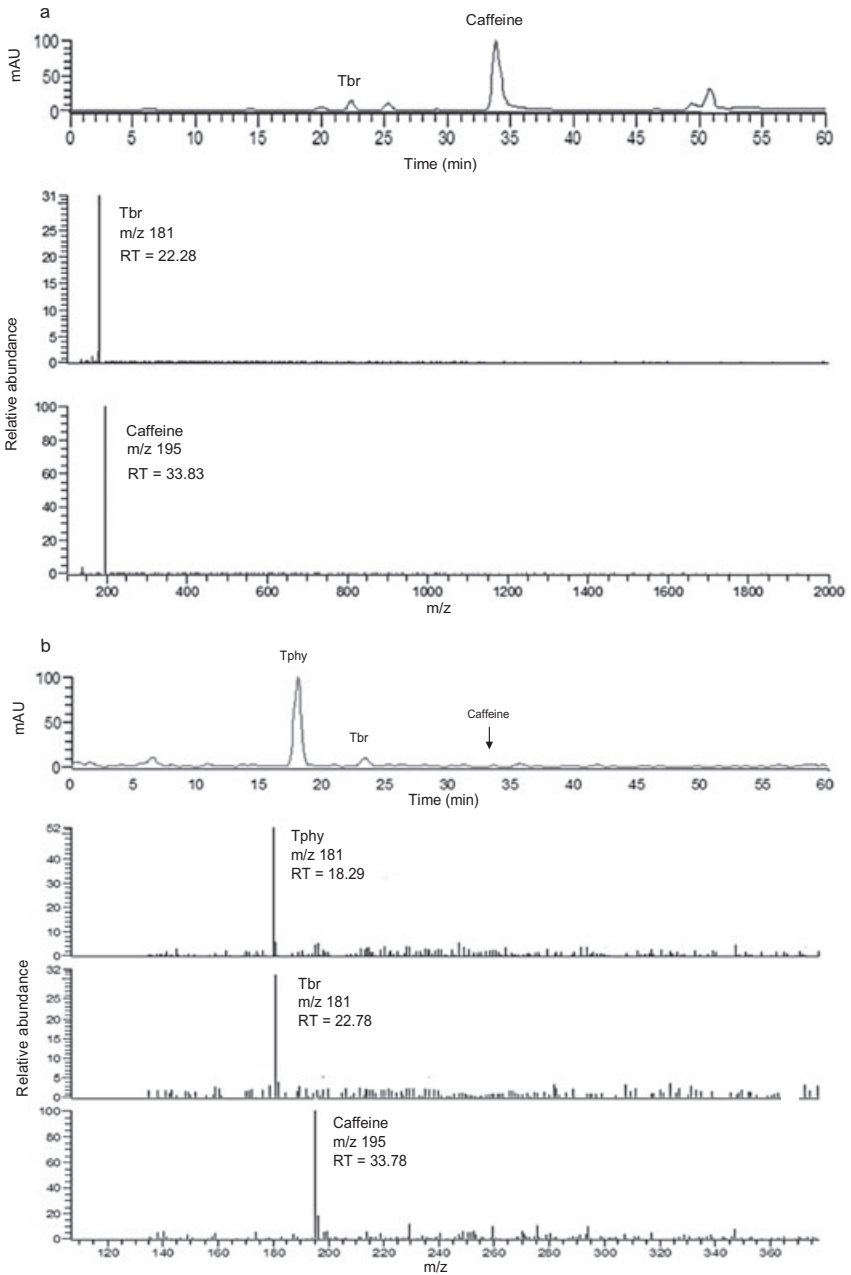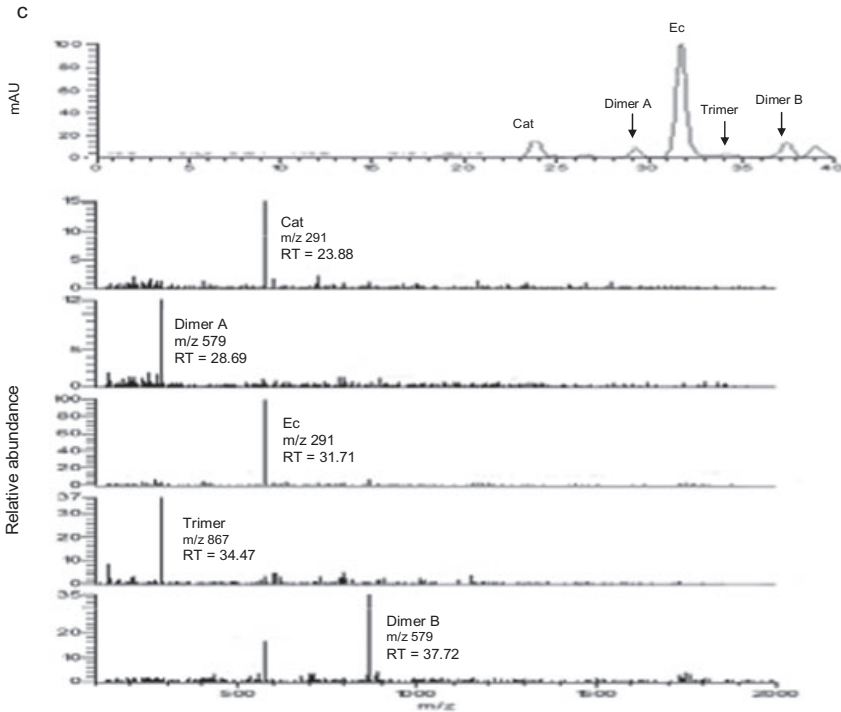
FIG. 4. EXTRACTED ION CHROMATOGRAM AND PROTONATED MOLECULAR ION
[M + H]+ OF (a) FRACTION 1; (b) FRACTION 2; (c) FRACTION 3
Cat, catechin; Ec, epicatechin; Tbr, theobromine; Tphy, theophylline.

FIG. 4. *Continued*

monomeric compounds, two dimers were identified in F3 as Dimer A and B based on their m/z 579. In this study, Dimer A and B were quantified based on CE as there are limited commercially available standards than monomeric compounds. Dimer A and B were significantly lower ($P < 0.05$) with 4.53 ± 0.08 and 2.03 ± 0.04 mg CE/g fraction, respectively, than monomers catechin and epicatechin. One trimer was identified based on their m/z 867 with 3.51 ± 0.5 mg CE/g fraction. Tomas-Barberan *et al.* (2007) demonstrated that dimers and trimers were quantified in unfermented and unroasted cocoa powder. Similarly, dimers were quantified in conventional cocoa powder produced from fermented and dried cocoa beans. Trimer could only be identified in conventional cocoa powder.

There are factors to be considered in explaining the observed findings. First, the reversed-phase LC-MS used in this study might limit the detection of polymeric compounds in F3. Previous study showed that only monomeric epicatechin and catechin, dimer, trimer and tetramer could be detected by Reverse Phase (RP) LC-MS (Natsume *et al.* 2000). Second, as cocoa powder

derived from fermented, dried and roasted cocoa beans during primary and secondary processing, the loss of phenolic compounds was higher than that of cocoa liquor (De Brito *et al.* 2000). Several phenolic compounds were lost in cocoa powder produced from fermented, dried and roasted beans compared with cocoa powder produced from unfermented beans (Tomas-Barberan *et al.* 2007).

Although the loss of oligomeric phenolics occurred, the above results indicated that individual flavonoids in F3, namely monomer, dimers and trimer flavonoids, could strongly contribute toward phenolic and flavonoid contents and antioxidant capacity. This finding was in agreement with previous study which demonstrated that cocoa flavonoids correlated to its antioxidant capacity (Adamson *et al.* 1999). The present study indicates that the addition of F1 and F2 decreased the antioxidant capacity of F3 (Fig. 2). Methylxanthines in F1 and F2 could possess pro-oxidant properties and suppressed the antioxidant capacity of F3. It was noted that both caffeine and theobromine exhibit pro-oxidant properties (Lee 2000). Furthermore, caffeine itself did not contribute toward antioxidant capacity (Moreira *et al.* 2005; Kofink *et al.* 2007).

Stability of Phenolic Compounds

Figure 5 shows the stability of cocoa powder catechin and epicatechin at different storage conditions and time, respectively. Generally, the stability of catechin and epicatechin mirrored the same trend at different storage conditions when stored up to 5 months. Both compounds were relatively stable when stored at −20C for 5 months. The compounds significantly increased at 5 months compared with the other storage time and conditions. Storage at 4C showed slight increase ($P > 0.05$) of the compounds over 5 months compared with the initial state. The compounds were significantly ($P < 0.05$) reduced when stored at 26–28C for 5 months compared with the same time points at 4C and −20C. However, investigation of exact mechanism involved in the changes of phenolic compounds was not done. Determination of the degree of epimerization and the presence of enantiomers could partly explain these findings. Recent study showed that roasted cocoa beans and cocoa products contained additional compound, flavan-3-ol(-)-catechin along with typical compounds, (+)-catechin and (−)-epicatechin. The new compound was formed during manufacturing process through epimerization of (-)-epicatechin to its epimer(-)-catechin (Kofink *et al.* 2007). Of note, our typical RP-HPLC analysis was limited in separating enantiomers of catechin and epicatechin, namely (−)-catechin, (+)-catechin, (−)-epicatechin and (+)-epicatechin.

Besides epimerization and the presence of enantiomers, the study of phenolic stability under typical storage conditions are of great importance as it
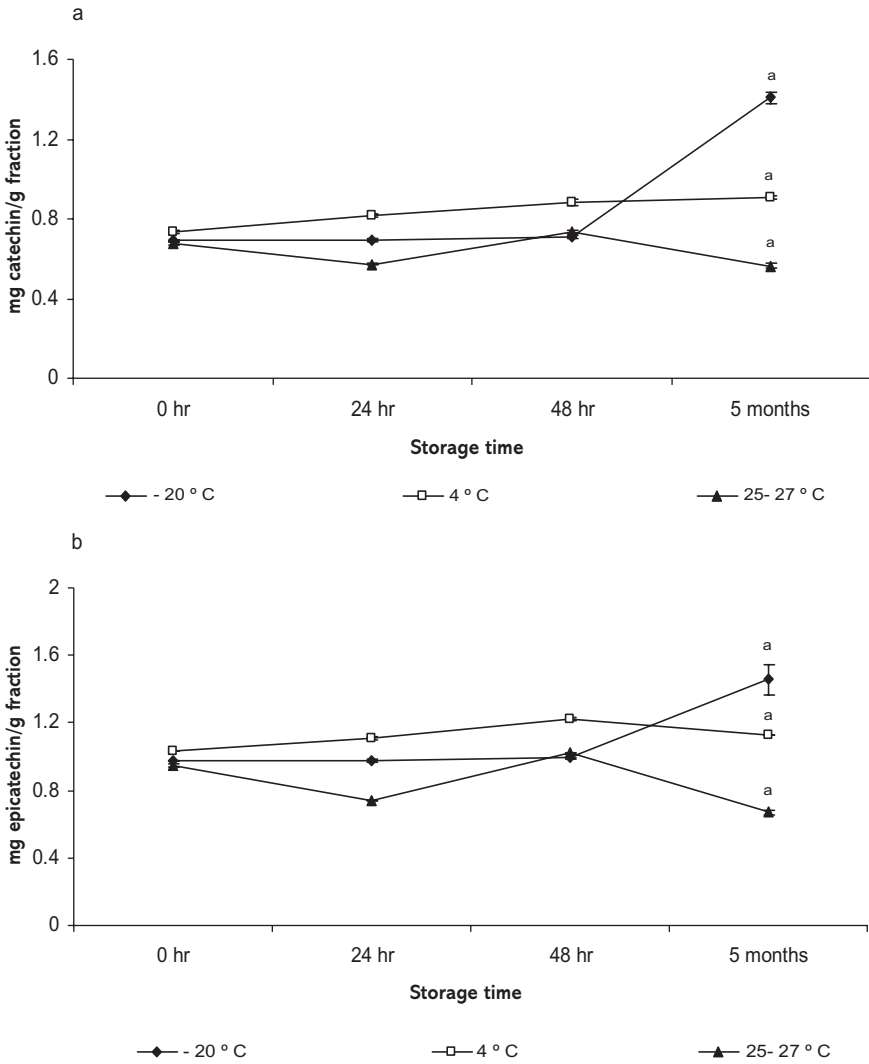
FIG. 5. STABILITY OF (a) CATECHIN AT DIFFERENT STORAGE CONDITION AND TIME;
(b) EPICATECHIN AT DIFFERENT STORAGE CONDITION AND TIME
Results were expressed as mean ± standard deviation of three replicate values. Identical
superscript indicated that values are significantly different at the level of $P < 0.05$ within
the same time point.

could reflect their antioxidant capacity. A study showed that decreasing of antioxidant capacity in beverages stored at different temperature and time was due to loss of phenolic content (Sang *et al.* 2005). Shin *et al.* (2007) reported that antioxidant capacity of fruits was higher when kept at 10C than that kept at 20C for 3 days.

CONCLUSIONS

The present study indicated that antioxidant capacity of cocoa powder could be contributed by the presence of phenolic compounds especially flavonoids. The flavonoids were identified as catechin, epicatechin, dimers and trimer. Methylxanthines (theobromine, caffeine and theophylline) were well separated from cocoa powder and showed low antioxidant capacity compared with other fractions. The presence of methylxanthines could reduce the antioxidant capacity of flavonoids. The individual polyphenolic compounds were relatively stable when stored at 4C and increased significantly when stored at −20C for 5 months.

REFERENCES

ADAMSON, G.E., LAZARUS, S.A., MITCHELL, A.E., PRIOR, R.L., CAO, G., JACOBS, P.H., KREMERS, B.G., HAMMERSTONE, J.F., RUCKER, R.B., RITTER, K.A. *ET AL.* 1999. HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. J. Agric. Food Chem. *47*, 4184–4188.

AMIN, I., FAIZUL, H.A. and AZLI, R. 2004a. Effect of cocoa powder extract on plasma glucose levels in hyperglycaemic rats. Nutr. Food Sci. *34*, 116–121.

AMIN, I., KOH, B.K. and ASMAH, R. 2004b. Effect of cacao liquor extract on tumor marker enzymes during chemical hepatocarcinogenesis in rats. J. Med. Food *7*, 7–12.

AZIZAH, O., AMIN, I., NAWALYAH, A.G. and ILHAM, A. 2007. Antioxidant capacity and phenolic content of cocoa beans. Food Chem. *100*, 1523–1530.

BENZIE, I.F.F. and STRAIN, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. Anal. Biochem. *239*, 70–76.

DE BRITO, E.S., GARCIA, N.H.P., GALLAO, M.I., CORTELAZZO, A.L., FEVEREIRO, P.S. and BRAGA, M.R. 2000. Structural and chemical

changes in cocoa (*Theobroma cacao*) during fermentation, drying and roasting. J. Sci. Food Agric. *81*, 281–288.

ETENG, C.M.U. and ETTARH, R.R. 2000. Comparative effects of theobromine and cocoa extract on lipid profile in rats. Nutr. Res. *20*(10), 1513–1517.

GREER, F., HUDSON, R., ROSS, R. and GRAHAM, T. 2001. Caffeine ingestion decreases glucose disposal during a hyperinsulinemic-euglycemic clamp in sedentary humans. Diabetes *50*, 2349–2354.

JIA, Z., MENGCHENG, T. and JIANMING, W. 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. *64*, 555–559.

KATALINIC, V., MODUN, D., MUSIC, I. and BOBAN, M. 2005. Gender differences in antioxidant capacity of rat tissue determined by 2, 2′ – azinobis (3-ethylbenzothiiazoline 6-sulfonate; ABTS) and ferric reducing antioxidant power (FRAP) assays. Comp. Biochem. Physiol. *140*, 47–52.

KELM, M.A., JOHNSON, J.C., ROBBINS, R.J., HAMMERSTONE, J.F. and SCHMITZ, H.H. 2006. High-performance liquid chromatography separation and purification of cacao (*Theobroma cacao* L.) procyanidins according to degree of polymerization using a diol stationary phase. J. Agric. Food Chem. *54*, 1571–1576.

KLIMCZAK, I., MALECKA, M., SZLACHTA, M. and GLISZCZYNSKA-SWIGLO, A. 2007. Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juices. J. Food Compost. Anal. *20*, 313–322.

KOFINK, M., PAPAGIANNOPOULOS, M. and GALENSA, R. 2007. Catechin in cocoa and chocolate: Occurence and analysis of an atypical flavan-3-ol enantiomer. Molecules *12*(7), 1274–1288.

KUROSAWA, T., ITOH, F., NOZAKI, A., NAKANO, Y., KATSUDA, S.-I., OSAKABE, N., TUBONE, H., KONDO, K. and ITAKURA, H. 2005. Suppressive effect of cocoa powder on atherosclerosis in Kurosawa and Kusanagi-hypercholesterolemic rabbits. J. Atheroscler. Thromb. *12*, 20–28.

LEE, C. 2000. Antioxidant ability of caffeine and its metabolites based on the study of oxygen radical absorbing capacity and inhibition of LDL peroxidation. Clin. Chim. Acta. *295*, 141–154.

LEE, K.W., KIM, Y.J., LEE, H.J. and LEE, C.Y. 2003. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. J. Agric. Food Chem. *51*(25), 7292–7295.

LEE, S., HUDSON, R., KILPATRICK, K., GRAHAM, T.E. and ROSS, R. 2005. Caffeine ingestion is associated with reductions in glucose uptake independent of obesity and type 2 diabetes before and after exercise training. Diab. Care *28*, 566–572.

MOREIRA, D.P., MONTEIRO, M.C., RIBEIRO-ALVES, M., DONAGELO, C.M. and TRUGO, L.C. 2005. Contribution of chlorogenic acids to the iron-reducing activity of coffee beverages. J. Agric. Food Chem. *53*, 1399–1402.

MURPHY, K.J., CHRONOPOULOS, A.K., SINGH, I., FRANCIS, M.A., MORIARTY, H., PIKE, M.J., TURNER, A.H., MANN, N.J. and SIN-CLAIR, A.J. 2003. Dietary flavanols and procyanidin oligomers from cocoa (*Theobroma cacao*) inhibit platelet function. Am. J. Clin. Nutr. *77*, 1466–1473.

NATSUME, M., OSAKABE, N., YAMAGISHI, M., TAKIZAWA, T., NAKA-MURA, T., MIYATAKE, H., HATANO, T. and YOSHIDA, T. 2000. Analyses of polyphenols in cacao liquor, cocoa, and chocolate by normal-phase and reverse-phase HPLC. Biosci. Biotechnol. Biochem. *64*(12), 2581–2587.

NEUKAM, K., STAHL, W., TRONNIER, H., SIES, H. and HEINRIC, U. 2007. Consumption of flavanol-rich cocoa acutely increases microcirculation in human skin. Eur. J. Nutr. *46*, 53–56.

OSAKABE, N., YAMAGISHI, M., SANBOGI, C., NATSUME, M., TAK-IZAWA, T. and OSAWA, T. 1998. The antioxidative substances in cacao liquor. J. Nutr. Sci. Vitaminol. *44*, 313–321.

OSAKABE, N., YASUDA, A., NATSUME, M., TAKIZAWA, T., TERAO, J. and KONDO, K. 2002. Catechins and their oligomers linked by C4 → C8 bonds are major cacao polyphenols and protect low-density lipoprotein from oxidation in vitro. Exp. Biol. Med. *227*(1), 51–56.

RAMLJAK, D., ROMANCYZK, L.J., METHNEY-BARLOW, L.J., THOMP-SON, N., KNEZEVIC, V., GALPERIN, M., RAMESH, A. and DICKSON, R.B. 2005. Pentameric procyanidin from *Theobroma cacao* selectively inhibits growth of human breast cancer cells. Mol. Cancer Ther. *4*(4), 537–546.

RIOS, L.Y., GONTHIER, M.-P., REMESY, C., MILA, I., LAPIERRE, C., LAZARUS, S.A., WILLIAMSON, G. and SCALBERT, A. 2003. Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects. Am. J. Clin. Nutr. *77*, 912–918.

RUZAIDI, A., AMIN, I., NAWALYAH, A.G., HAMID, M. and FAIZUL, H.A. 2005. The effect of Malaysian cocoa extract on glucose levels and lipid profiles in diabetic rats. J. Ethnopharmacol. *98*, 55–60.

SANG, S., LEE, M.-J., HOU, Z., HO, C.T. and YANG, C.S. 2005. Stability of tea polyphenol (-)-epigallocatechin-3-gallate and formation of dimers and epimers under common experimental conditions. J. Agric. Food Chem. *53*, 9478–9484.

SHIN, Y., LIU, R.H., NOCK, J.F., HOLLIDAY, D. and WATKINS, C.B. 2007. Temperature and relative humidity effects on quality, total ascorbic acid,

phenolics, flavonoids concentrations, and antioxidant activity of straw-berry. Postharvest. Biol. Technol. *45*, 349–357.

SU, Q., ROWLEY, K.G. and O'DEA, K. 1999. Stability of individual caro-tenoids, retinol and tocopherols in human plasma during exposure to light after extraction. J. Chromatogr. B *729*, 191–198.

TOMARU, M., TAKANO, H., OSAKABE, N., YASUDA, A., INOUSE, K.-I., YANGISAWA, R., OHWATARI, T. and UEMATSU, H. 2007. Dietary supplementation with cacao liquor proanthocyanidins prevents elevation of blood glucose levels in diabetic obese mice. Nutrition *23*, 351–355.

TOMAS-BARBERAN, F.A., CIENFUEGOS-JOVELLANOS, E., MARIN, A., MUGUERZA, B., GIL-IZQUIERDO, A., CERDAA, B., ZAF-RILLA, P., MORILLAS, J., MULERO, J., IBARRA, A. *ET AL.* 2007. A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. J. Agric. Food Chem. *55*, 3926–3935.

VELIOGLU, Y.S., MAZZA, G., GAO, L. and OOMA, B.D. 1998. Antioxi-dant activity and total phenolics in selected fruits, vegetables, and grain products. J. Agric. Food Chem. *46*, 4113–4117.

VINSON, J.A., PROCH, J. and ZUBIK, L. 1999. Phenol antioxidant quantity and quality in foods: Cocoa, dark chocolate, and milk chocolate. J. Agric. Food Chem. *47*(12), 4821–4824.

VINSON, J.A., PROCH, J., BOSE, P., MUCHLER, S., TAFFERA, P., SHUTA, D., SAMMAN, D.N. and AGBOR, G.A. 2006. Chocolate is a powerful ex vivo and in vivo antioxidant, an antiatherosclerotic agent in an animal model, and a significant contributor to antioxidants in the European and American diets. J. Agric. Food Chem. *54*, 8071–8076.

WOLLGAST, J. and ANKLAM, A. 2000. Review on polyphenols in *Theo-broma cacao*: Changes in composition during the manufacture of choco-late and methodology for identification and quantification. Food Res. Int. *33*, 423–447.

ZHU, Q.Y., HOLT, R.R., LAZARUS, S.A., ENSUNSA, J.L., HAMMER-STONE, J.F., SCHMITZ, H.H. and KEEN, C.L. 2002a. Stability of the flavan-3-ols epicatechin and catechin and related dimeric procyanidins derived from cocoa. J. Agric. Food Chem. *50*, 1700–1705.

ZHU, Q.Y., HOLT, R.R., LAZARUS, S.A., OROZCO, T.J. and KEEN, C.L. 2002b. Inhibitory effects of cocoa flavanols and procyanidin oligomers on free radical-induced erythrocyte hemolysis. Exp. Biol. Med. *227*(5), 321–329.

ZHU, Y.Z., HAMMERSTONE, J.F., LAZARUS, S.A., SCHMITZ, H.H. and KEEN, C.L. 2003. Stabilizing effect of ascorbic acid on flavan-3-ols and dimeric procyanidins from cocoa. J. Agric. Food Chem. *51*, 828–833.