Assessing the Variability of Extractable and Non-Extractable Phenolic Compounds in a Diverse Apple Breeding Population: Implications for Nutritional Quality and Health Benefits"

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ABSTRACT

Polyphenols, particularly the extractable fraction, have been extensively studied, whereas literature on non-extractable polyphenols is limited or non-existent, despite both types exhibiting health-related activities. Most bioavailability and intervention studies attribute these activities solely to extractable polyphenols. In this study, both extractable and non-extractable polyphenols were analyzed in 15 apple varieties sourced from US apple farmers. The apples were harvested, peeled, frozen, and subsequently analyzed using high-performance liquid chromatography (HPLC). Six compounds, including procyanidin B1, epicatechin, 4-O-caffeoylquinic acid, chlorogenic acid, and ellagic acid, were identified and quantified in both extractable and non-extractable samples. The concentrations of non-extractable polyphenols (NEPP), such as ellagic acid B1 (1.12 ± 0.78 mg/ml), procyanidin B1 (1.29 ± 0.69 mg/ml), and catechin (1.100 ± 1.27 mg/ml), were significantly higher ($P < 0.05$) than those found in extractable polyphenols (EPP): 0.44 ± 0.20 mg/ml, 0.65 ± 0.03 mg/ml, and 0.56 ± 0.02 mg/ml, respectively. Cultivars 'APP6' and 'APP15' exhibited significantly higher concentrations of chlorogenic acid in NEPP, with mean values of 0.96 ± 0.12 mg/ml and 0.86 ± 0.51 mg/ml, respectively, compared to concentrations of 0.24 ± 0.34 mg/ml and 0.19 ± 0.42 mg/ml in EPP in the same cultivar. Generally, the concentrations of NEPP observed in this study were generally higher than those of EPP. Therefore, non-extractable polyphenols play a significant role in contributing to the health-related benefits typically associated only with extractable polyphenols. This underscores the importance of considering NEPP in both intervention and observational studies, where they are often overlooked.

INTRODUCTION

Plant secondary metabolites, especially polyphenols (both extractable and non-extractable polyphenols), are renowned for their biological and pharmaceutical activities, including anticarcinogenic, antiallergic, antibiotic, antihypertensive, and hypoglycaemic properties (Arts and Hollman, 2005). Despite growing scientific evidence linking a polyphenol-rich diet to disease prevention, current research on polyphenols often overlooks a crucial fraction: non-extractable polyphenols (NEPPs). Non-extractable polyphenols are those not detectable in aqueous organic solvent extracts and typically remain unreleased from the food matrix after ingestion and digestion, resistant to the acidic pH of the stomach or gastric enzymes (Perez-Jimenez et al., 2013; Ogah et al., 2014). These polyphenols enter the colon intact and require extensive modification by colonic microflora for release (Boyer and Liu, 2004). NEPPs, undetectable by conventional extractable polyphenol (EPP) methods, are thus often excluded from food and dietary intake analyses in bioavailability, intervention, and observational studies (Perez-Jimenez et al., 2013). EPP represents only those polyphenols extractable by organic solvents, typically released from the food matrix upon consumption via gastric enzymes. This category includes pyruvic acid, procyanidins, flavonols, dihydrochalcones, and anthocyanins (Boyer and Liu, 2004). Chemically, NEPP contains some polyphenols also found in EPPs, such as proanthocyanidins, phenolic acids, and hydrolysable tannins, but these evade detection by standard EPP extraction procedures. Consequently, NEPP has received scant attention compared to the vast literature on EPP, which exceeds 30,000 references

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(Perez-Jimenez et al., 2013). In polyphenol research, residues post-organic solvent extraction (EPP extraction) are typically disregarded and assumed free of polyphenols. However, these residues harbor another fraction of polyphenols with distinct biological activities. The significance of NEPP cannot be overstated; it is considered a component of dietary fiber and EPP, thereby contributing to all health-related properties commonly associated with dietary fiber and EPP. NEPP exhibits diverse biological activities, including anti-inflammatory effects, reduction of oxidative stress, inhibition of protein glycation, antiproliferative effects, and flow-mediated vasodilator effects (Perez-Jimenez et al., 2013). Apples, a staple food consumed by billions worldwide, with annual production exceeding 62 million tonnes and valued at over $180 billion, are notably rich in polyphenols. Given that apples (Malus × domestica) are consumed whole (i.e., with both EPP and NEPP), a substantial amount of NEPP is ingested daily, contributing to the polyphenol health effects in apples. Thus, this study aims to identify, quantify, and compare the concentrations of individual extractable and non-extractable polyphenols in selected apple varieties to elucidate the potential impacts of NEPP in bioavailability, intervention, or observational studies involving EPP.

MATERIALS AND METHODS

Sample collection: The apple fruits used in this study comprised 15 different varieties harvested at maturity in the USA. The samples, identified as APP1, APP2, APP2, APP4, APP5, APP6, APP7, APP8, APP9, APP10, APP11, APP12, APP13, APP14, and APP15, were harvested according to the Cornell starch iodine index levels 3-5 (Blanpied and Silsby, 1992). Fruits were selected from well-exposed parts of the tree, excluding the upper and lower canopy regions, and stored in a cold room at 2 °C.

Sample Preparation:Using an apple peeler set to a depth of approximately 5-6 mm, 10 fruits per variety were peeled. The composite collection was placed in 50 ml disposable Falcon centrifuge tubes, immediately chilled with dry ice, and subsequently transferred within 8 hours to a freezer set at -80 °C for storage until analysis.

Extraction of the Extractable Polyphenol Fraction: Twenty grams of apple peel samples were homogenized using an Ultra-Turrax in acidic methanol (50:50 v/v, pH 2.0), allowed to stand for 1 hour at room temperature with continuous shaking, and then centrifuged at 2,100 g for 10 minutes at 4 °C. The acidic methanolic extract was collected from the supernatant. The precipitate was then extracted with a 70:30 v/v acetone/water solution (1:5 ratio v/v) by shaking for 60 minutes, followed by centrifugation at 2,100 g for 10 minutes at 4 °C. The combined supernatants were analyzed for extractable polyphenols using HPLC.

Extraction of Non-extractable Polyphenols: The residues from the extractable polyphenol extraction were used for non-extractable polyphenol extraction. Ten grams of the residue obtained after successive acidic methanol and acetone extractions (as described above) were hydrolyzed with 50 ml methanol/sulfuric acid (1:1 v/v) at 80 °C for 12 hours. The sample was then centrifuged at 15,000 g for 3 minutes, and the supernatant was collected for analysis by HPLC.

Identification and Quantification of Polyphenols by HPLC: The polyphenol composition of apple peel was analyzed using HPLC following the method described by Tsao et al. (2003). Approximately 300 µl of the phenolic extract was injected into an HPLC system equipped with a 5 µm C-18 kinetic biophenyl column, a guard column (250 x 4.6 mm), a quaternary pump, a degasser, a thermostatic autosampler, and a diode array detector (DAD) set at wavelengths of 280-525 nm. The mobile phase consisted of nano-pure water supplemented with 0.5% (v/v) acetic acid and 5 mM ammonium acetate (solvent A), and acetonitrile supplemented with 0.5% (v/v) acetic acid and 5 mM ammonium acetate (solvent B). The gradient program involved a flow rate of 1.0 ml/min over 60 minutes: 0-5 min, 100% to 90% A; 5-20 min, 90% to 85% A; 20-40 min, 85% to 70% A; 40-45 min, 70% to 0% A; 45-50 min, 0% A; 50-51 min, 100% A; and 51-60 min, 100% A. The detector wavelength was set to 280 nm for quantitative data collection of phenols. Compound identification was achieved by comparing HPLC-DAD peak profiles with those of pure standards under identical elution conditions. All samples were prepared and analyzed in duplicate, and results were expressed as mg/ml of fresh weight.

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue VI June 2024

Data Analysis: All phenotypic data (concentrations of extractable and non-extractable polyphenols) were subjected to one-way analysis of variance (ANOVA) using SPSS. Statistically significant differences were determined at $p < 0.05$, and post hoc analysis was performed using the Least Significant Difference (LSD) method to separate means.

Results and Discussion : The two families of polyphenols, hydrocyanic acid and proanthocyanidin, were chosen for comparison based on studies by Perez-Jimenez et al. (2013), which identified them as the most significant polyphenols among non-extractable polyphenols. Figure 1 displays chromatograms of each standard compound. The analysis revealed multiple peaks for both extractable and non-extractable phenolic compounds, with 6 peaks clearly identified and characterized in each sample using hydrocyanic acid and proanthocyanidin standards. Among the EPP, Epicatechin and 4-o-caffeoylquinic acid had the highest concentrations, while ellagic acid and chlorogenic acid had the lowest concentrations (Figures 2 and 3). In the non-extractable samples, ellagic acid B1 (1.12 + 0.78 mg/ml), procyanidin B1 (1.29 + 0.69 mg/ml), and catechin (1.100 + 1.27 mg/ml) exhibited higher concentrations. These findings are consistent with Perez-Jimenez et al. (2013), who reported higher non-extractable polyphenol content in most Spanish fruits compared to extractable polyphenols. Additionally, Khanizade et al. (2007) similarly found that apples contained the highest levels of proanthocyanidin followed by hydrocyanic acid. The variation in extractable and non-extractable polyphenol content in the sample indicates that residues typically discarded after extraction contain significant amounts of important polyphenols. These compounds are known to possess various health benefits such as anticarcinogenic, antiallergic, antibiotic, antihypertensive, and hypoglycemic properties, attributes usually associated only with extractable polyphenols (Arts and Hollman, 2005). Perez-Jimenez et al. (2013) also reviewed the health-related properties of non-extractable polyphenols, highlighting their antioxidant and antiproliferative roles in gastrointestinal health. Animal studies have shown their potential to reduce tumor formation in the intestine and modulate gene expression.

Of the fifteen (15) cultivars studied, cultivars APP6 and APP15 exhibited relatively higher concentrations of chlorogenic acid, with values of 4.61 ± 0.00 mg/ml and 2.01 ± 0.10 mg/ml, respectively, compared to 0.24 ± 0.34 mg/ml and 0.19 ± 0.42 mg/ml found in extractable polyphenols (EPP) in the same cultivars (Figures 12 and 13). Cultivars ‘APP2’ and ‘APP9’ showed higher concentrations of catechin, with mean values of 4.61 + 0.00 and 2.01 ± 0.10 mg/ml. This study represents the first comparison of extractable and non-extractable phenolics in these cultivars. Generally, non-extractable phenolics were found to have higher concentrations than extractable phenolic compounds. Variations in compound concentrations among cultivars may be attributed to genotypic differences, suggesting potential flavor variations influenced by these compounds, which are known to contribute to fruit flavor. Hydroxycinnamic acid has been identified as the predominant phenolic acid in apples (Watanabe et al., 2006). Chlorogenic acid, due to its water solubility, is considered the most important phenolic acid in apples regardless of the polyphenol profile (Treutter, 2000; Gutiérrez-Escobar et al.,2021). According to Montagnana et al. (2012), chlorogenic acid offers health benefits such as lowering blood pressure, anti-inflammatory effects, managing type 2 diabetes, and inhibiting platelet aggregation. For instance, studies by Watanabe et al. (2006) indicate that daily intake of 140 mg of chlorogenic acid from cocoa can reduce blood pressure in individuals with mild hypertension. Chlorogenic acid also influences glucose metabolism; Ong et al. (2012) reported improved glucose tolerance and potential enhancement of glucose transport in skeletal muscle. Van Dijk et al. (2009) demonstrated that 1 g of chlorogenic acid significantly reduces glucose and insulin levels within 15 minutes of ingestion. The mechanism involves delaying α-glucosidase action and potentially affecting glucose transporters in the gut. Chlorogenic acid may also inhibit glucose-6-phosphate in the liver, thereby lowering plasma glucose levels.

Although non-extractable polyphenol (NEPP) content in apples has not been extensively analyzed compared to extractable polyphenols (EPP), existing data underscore the dietary importance of this polyphenol fraction. Procyanidins B1 in apples exhibit cardioprotective, anti-inflammatory, anticarcinogenic, antiviral, and antibacterial properties, attributed to their antioxidant role in human physiology (Arranz et al., 2009). Despite generally low concentrations of phenolic acids such as ellagic acid, 4-O-caffeoylquinic acid, and chlorogenic acid, apples are valued for their antioxidant properties ((Teissedre et al. 1996).

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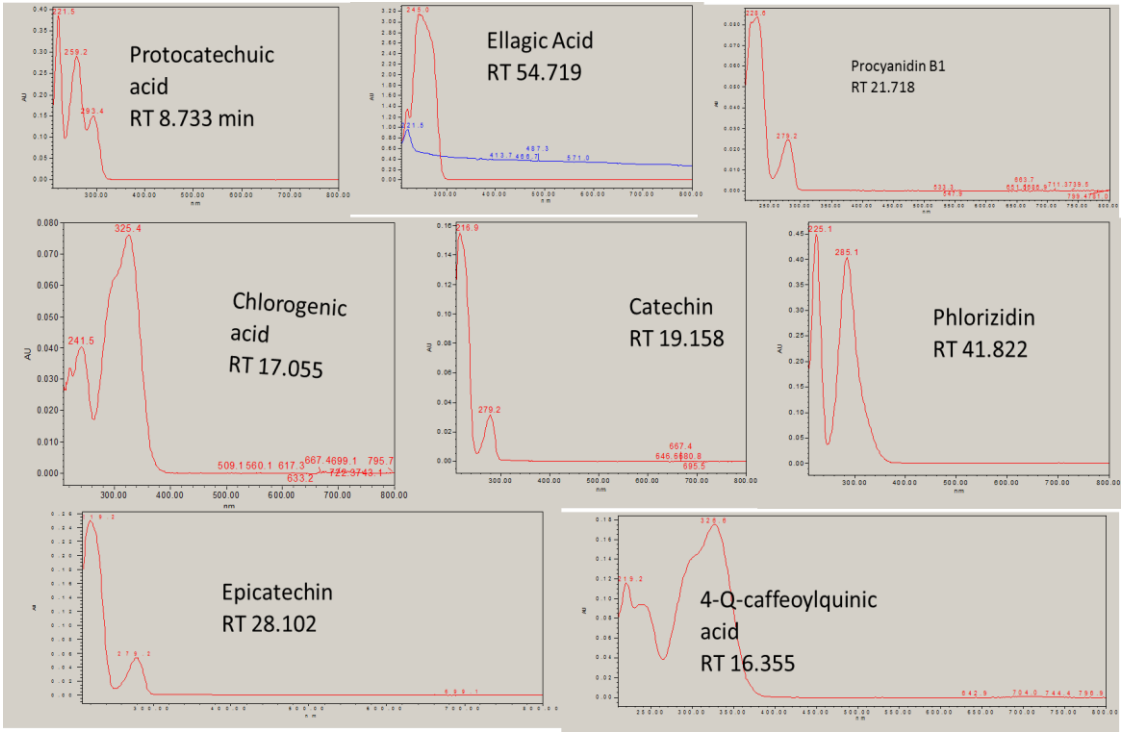

Figure 1. Chromatograms of some standard phenolic compounds

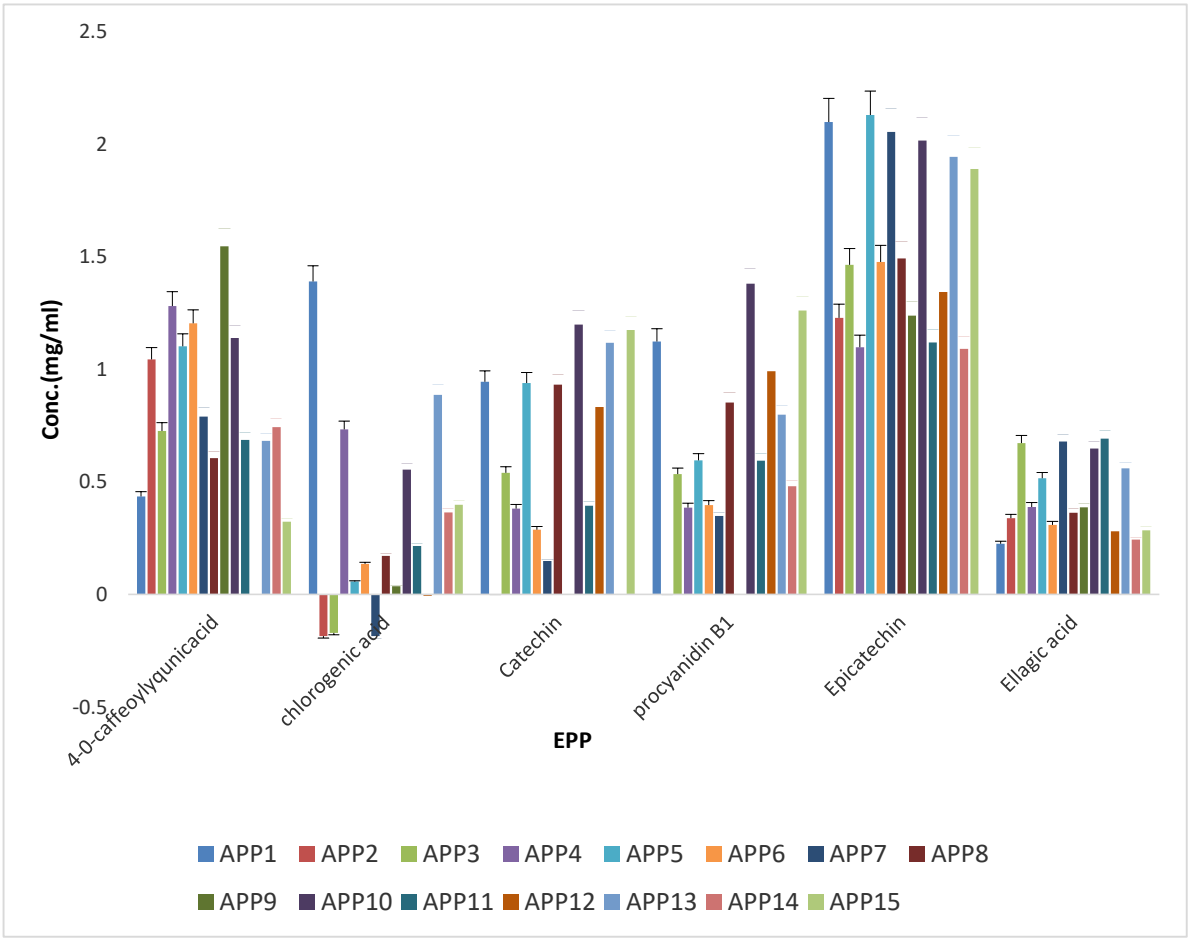

Figure 2 The concentrations of EPP compounds in apple accessions

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Among the EPP, Epicatechin and 4-o caffeoylquinic acid had the highest concentration while ellagic acid and chlorogenic acids were the lowest in concentrations Figures 2 and 3 while in NPP, epicatechin and procyanidinB1 had the highest concentration followed while chlorogenic acid was the least figures 4 and 5.

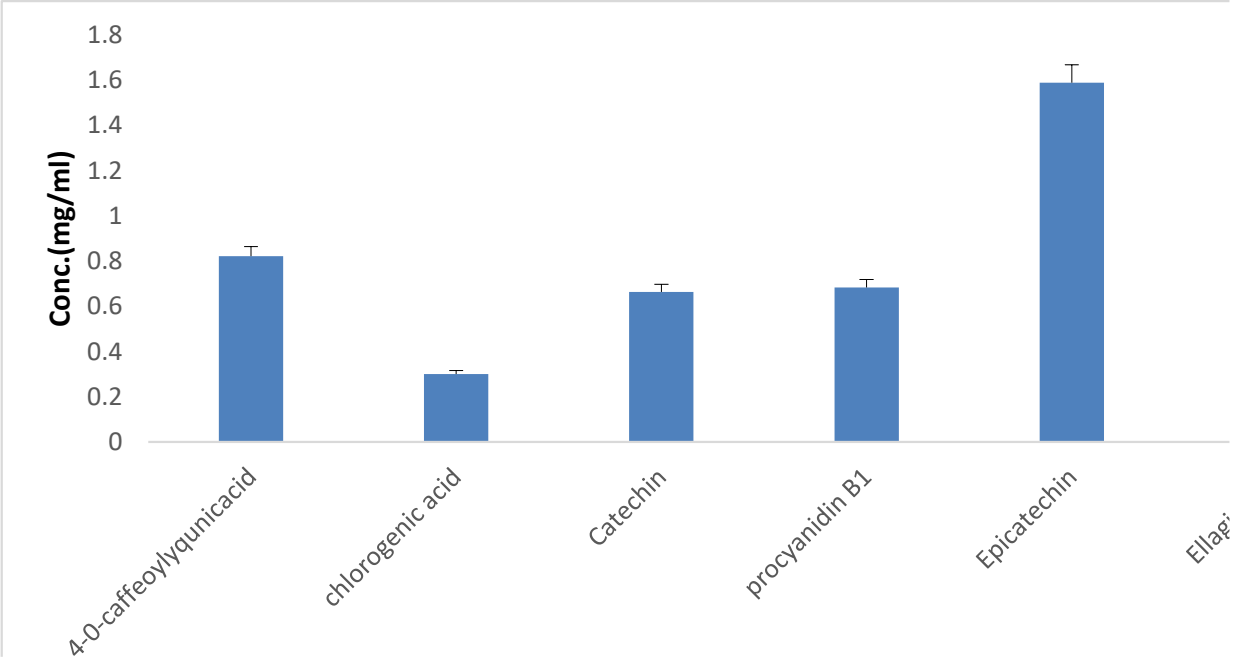

Figure 3: The concentrations of different EPP in apple.

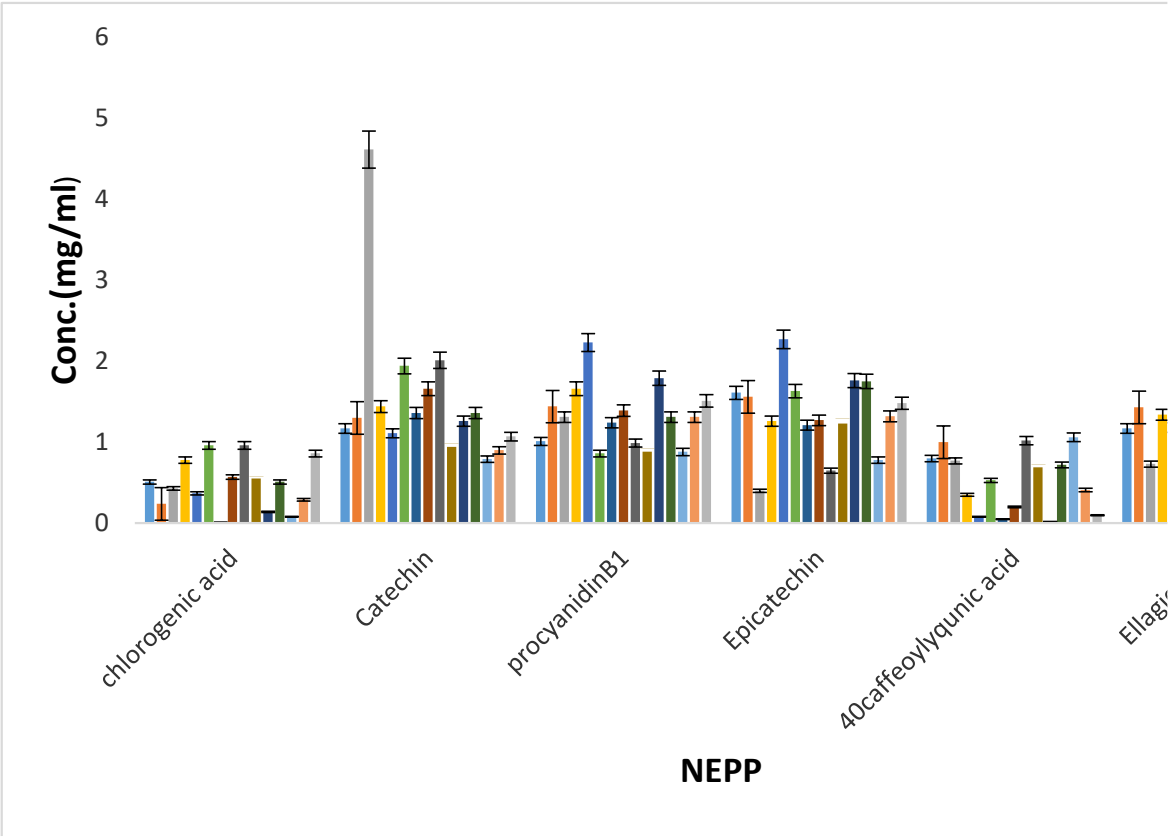

Figure 4: The concentrations of NEPP in Apple parent Cultivars

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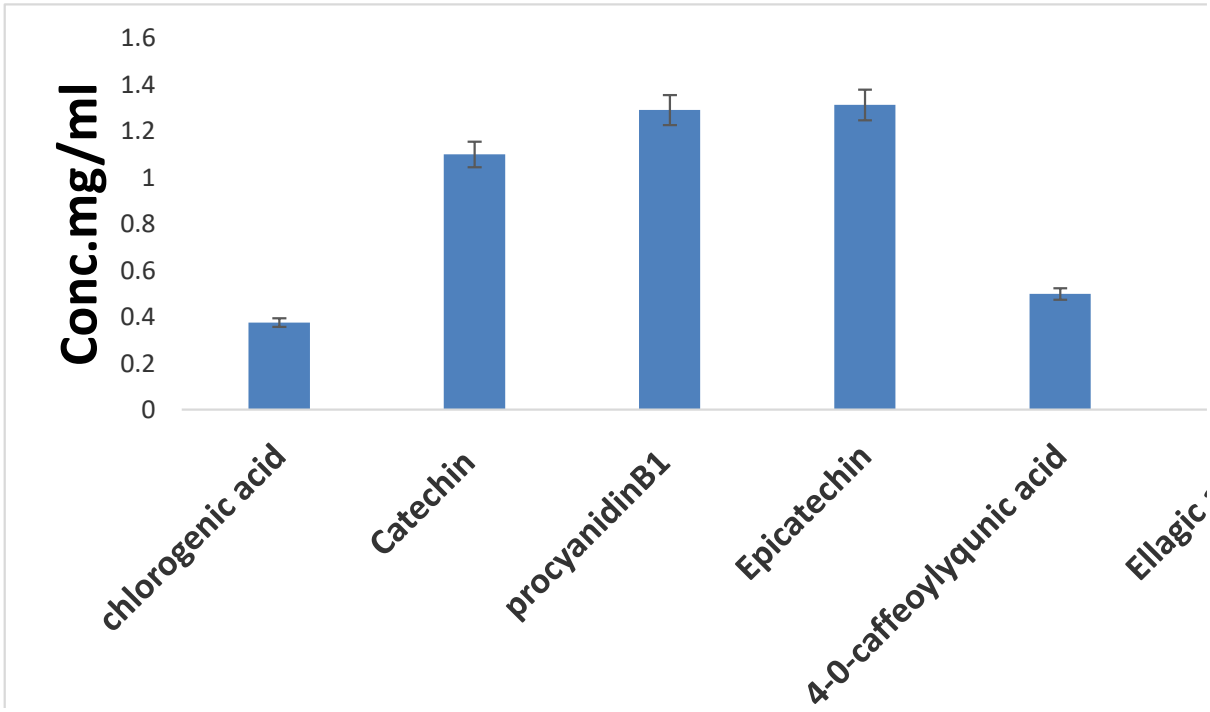

Figure 5: The concentrations of different NEPP in Apple

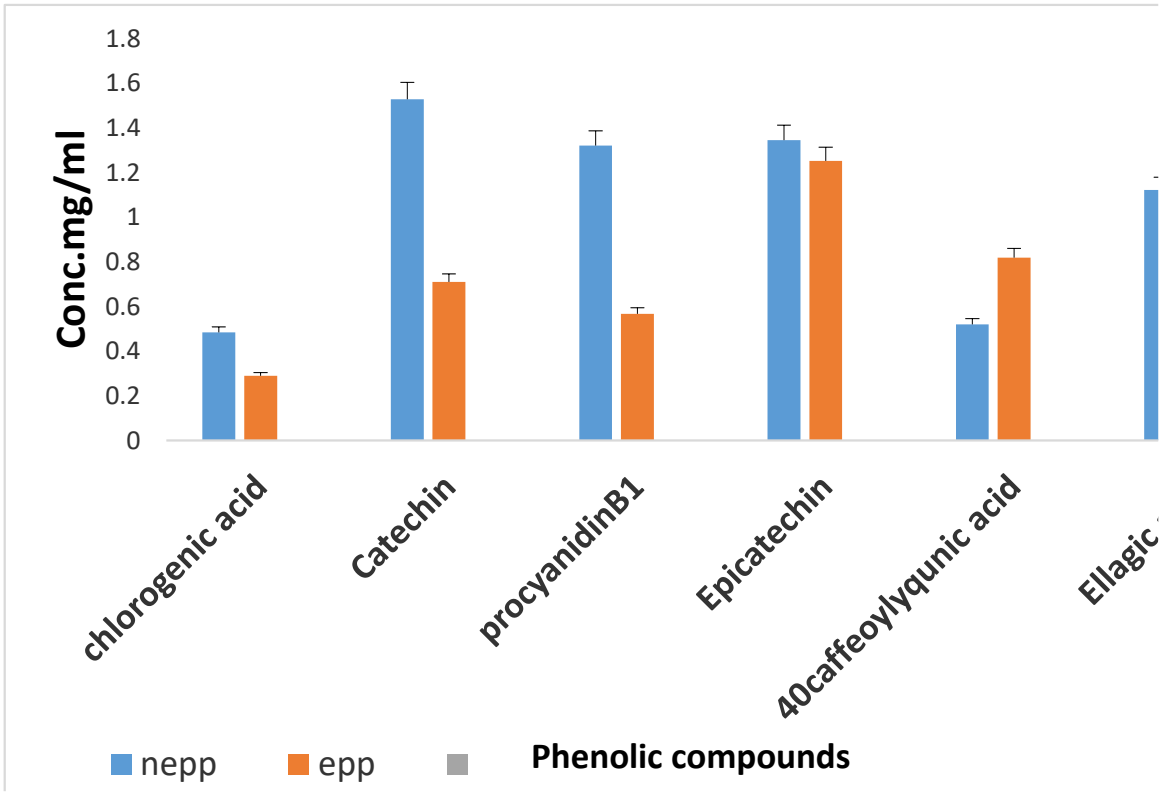

Figure 6: Comparison of concentrations of EPP and NEPP in apple

CONCLUSION

Based on this result, NEPP is a good source of natural antioxidants (phenolic compounds) and contains a higher concentration of proanthrocyandins than phenolic acid. Varieties such as WSU5, CRISPINK are rich in phenolic acids, while Alert, Honeycrisp, Aurogolgal and WSU7 are good sources of proanthrocyandins in

apples. The content of EPP in apples is lower than that of NEPP, so NEPP actually represent a major fraction of dietary polyphenols that has usually been overlooked in the study of polyphenols. However, NEPPs contribute significantly to polyphenol intake, which is usually assumed to come only from EPP

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