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Identification of Phenolic Compounds in Processed Cranberries by HPLC Method

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Abstract

Cranberries contain significant amounts of flavonoids and polyphenolic compounds that provide health benefits including antioxidants functions, protection against cardiovascular diseases and anti-adhesive properties that help to prevent urinary tract infections and stomach ulcers. In this study, phenolic compounds in different cranberry products were extracted using methanol and acetone. The phenolic extracts were then analysed using high performance liquid chromatography (HPLC). The results showed that the different cranberry products had different phenolic types (including tannic acid, (+) catechin, (-) epicatechin, procyanidin A2 and p-coumaric acid) in different amounts. Moreover, the results varied depending on the extraction methods. Generally, the methanol extracts had higher total phenolic in comparison to acetone extracts of all the different cranberry products studied. For methanol extraction, frozen cranberry had the highest total phenolic content (616.65 ± 0.14 mg/g), followed by sauces (360.80 ± 0.09 mg/g), then 17% juices (237.84 ± 0.03 mg/ml) and lastly, dry cranberry (142.13 ± 0.05 mg/g). However, for acetone extraction, the total phenolic content were in the order: frozen (609.92 ± 0.12 mg/g) > 17% juices (269.27 ± 0.06 mg/ml) > sauce (94.03 ± 0.06 mg/g) > dry cranberry (0.57 ± 0.01 mg/g). The results of the present study showed that methanol serves as a better solvent than acetone for extracting phenolic compounds for both qualitative and quantitative HPLC analysis of phenolic compounds in cranberry products.

Keywords: Cranberry; HPLC; Flavonoid; Procyanidin; p-coumaric acid; Epicatechin; Catechin

Introduction

Flavonoids are the most common group of polyphenolic compounds in the human diet and are found naturally in plants. They have recently received much public and medical attention owing to their antioxidant activity and health benefits such as protection against cardiovascular diseases and cancers [1,2].

Cranberry fruit has gained considerable attention for its putative health benefits. Most of the focus is on the flavonoid constituents due to their relatively high biological activity in various assays. The cranberry phenolic classes found of phenolic acids [3,4], anthocyanins [5], flavonols [6,7] and flavan-3-ols, which consist of both monomers and the polymer classes of procyanidins and proanthocyanidins [8]. A type linkage in cranberry have been recognized for their antiadherence activities against uropathogenic P type *Escherichia coli* and may play a role in urinary tract health [9].

A growing body of evidence has linked the phytonutrients found in cranberries to a number of conditions including the promotion of urinary tract health and the prevention of cardiovascular disease, certain stomach ulcers and even cancer [10,11]. Epidemiological studies have shown the benefits of a diet rich in flavonoids that includes the regular consumption of vegetables and cranberry fruits. Another study also showed that cranberry proanthocyanidins (PACs) are the novel compounds responsible for blocking the bacterial adhesion to stomach cell wall [12].

Same study showed that the PACs in 250 ml of cranberry juice cocktail containing 25% cranberry juice or equivalent can prevent bacteria from attaching to host cells, thereby preventing their growth and ulcer formation [12]. Therefore, cranberry juice can be used for the prevention of bacterial infections.

The order of amount of total polyphenols in cranberry foods on a fresh weight basis is as follows: dried > frozen > sauce > jellied sauce. On a serving size basis for all cranberry products, the order is as follows: frozen > 100% juice > dried > 27% juice > sauce > jellied sauce. It was also shown that cranberry juice produced an increase in plasma antioxidant and is an excellent source of high quality antioxidants [13].

Flavonoids are most commonly known for their antioxidant activity. Additionally, at high experimental concentrations that would not exist *in vivo*, the antioxidant abilities of flavonoids *in vitro* are stronger than those of vitamin C and E [14].

However, polyphenolic compounds including flavonoids can undergo various reactions in the course of food processing and storage. Processing and prolong storage can also promote or enhance enzymatic or chemical oxidation of phenolic compounds. Stability of polyphenols is also influenced by pH [15] and properties of polyphenols are greatly affected by their interactions with the food matrix, such as in the presence of sugars and ascorbic acid [16,17]. Phenolics can be affected by storage conditions such as temperature, atmosphere and light. Oxygen has been reported to be an important factor in destabilizing polyphenols, especially anthocyanins, and antioxidant capacity of processed products [18].

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The determination of bioactive compounds in cranberries has been of increasing interest nowadays. Keeping this in mind, the objective of the present study is to modify the current available extraction methods and to compare the phenolic content in cranberries, including the storage conditions that affect the content in cranberries phenolic compounds.

Materials and Methods

Chemicals

Standards of (+) catechin, (-) epicatechin, quercetin, flavone were purchased from Sigma Aldrich Chemicals Limited, UK. Procyanidin A2 and Procyanidin B1 were purchased from Extrasynthese Genay France. P-coumaric acid was obtained from Koch Light Laboratories, UK. Methanol, acetone and sodium hydroxide were acquired from Thermo Fisher Scientific, UK.

Preparation of standard solutions

Standard stock solutions of (+) catechin, (-) epicatechin, quercetin, ,flavones, p-coumaric acid, procyanidin A2 and procyanidin B were prepared in methanol at a concentration of 1 mg/ml and stored in the refrigerator at 5°C until use. All stock solutions were further diluted 10 times with methanol before injection. In addition, seven standard stock solutions (100 ppm) of 1 ml each were combined into a 10 ml vial at concentration 14.30 ppm before HPLC injection.

Sample collection

Commercially canned cranberry juice cocktail (containing 17% cranberry juice), cranberry sauce, dry cranberry and frozen cranberry were purchased from Sainsburry's Supermarket of Huddersfield in UK.

Extraction of phenolic compounds

Methanolic extraction: Samples of dry cranberry (1.882 g), frozen cranberry (5.644 g) were cut into small pieces with a sharp knife and cranberry sauces (4.571 g), were homogenized and extracted with 10 ml of methanol. All extracts were mixed by vortexing and then sonicated for 10 minutes on an ultrasonic water bath. The supernatants were centrifuged for 15 minutes at 3000 rpm. Then, 7 ml of the extract were transferred to 10 ml volumetric flask and the solution was redissolved in 3 ml of methanol to provide a total volume of 10 ml. 5 ml of cranberry juice (containing 17%) was combined with 5 ml of methanol into a 10 ml volumetric flask.

Acetonic extraction: Dry cranberry (1.007 g) and frozen cranberry (5.259 g) were cut into small pieces with a sharp knife and cranberry sauce (5.008 g), were homogenized smoothly with 10 ml acetone followed by vortexing, then sonicated for 10 minutes on an ultrasonic water bath. All extracts were centrifuged for 15 minutes at 3000 rpm. Then, 8 ml of the extract were transferred to 10 ml volumetric flask and the solution was mixed with 2 ml of methanol to make a total volume of 10 ml. 17% containing cranberry juice (5 ml) was combined with 5 ml of acetone into a 10 ml volumetric flask.

All of the methanolic and acetonic extracts were further diluted 10 times with methanol and then filtered through a 0.45 µm pore size syringe filter before HPLC analysis.

HPLC analysis: HPLC analysis was performed with Beckman HPLC with a Model 127 pump, a Model 166 UV detector and 32 KARAT Software operating system. The phenolic compounds were detected at 280 nm with a flow rate of 1 ml/min. The column was operated at a temperature of 25°C. Separations were carried out in a dual pumping system by varying the proportion of 2.5% (v/v) acetic acid in water (mobile phase A) and 70% methanol in water (mobile phase B). The solvent gradient elution program was as follows: 10% to 26% B (v/v) in 10 min, to 70% B at 20 min and finally to 90% B at 25 to 31 min. The injection volume for all samples was 100 µL. The phenolic compounds were analysed by matching the retention time and their spectral characteristics against those of standards.

Statistical Analysis: Data were statistically elaborated by analysis of variance using the multiple comparison test used in SPSS Version 18.The significance differences were considered at $p<0.05$.

Results and Discussion

Qualitative analysis of Phenolic compounds in cranberry products

The retention times of peaks in the HPLC chromatograph of the samples studied were compared with that of the standards to identify the unknown (Figure-1-9). The qualitative results of flavonoids detected in cranberry products after methanol and acetone extraction of Frozen, Dry, Sauce and 17% containing cranberry juice are as presented in table 1. The results obtained indicated that (+) Catechin, p-coumeric acid, (-) epicatechin and procyanidin A2 compound were found in

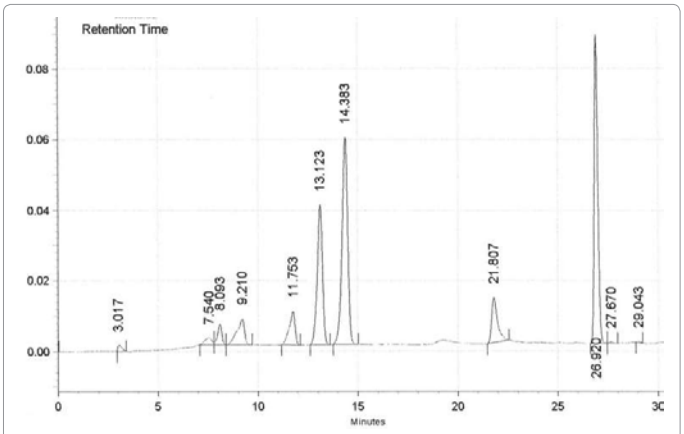

Figure 1: Separation of standard mixture of phenolic compounds by HPLC (280 nm). 8.093= Procyanidin B1, 9.210= (+) Catechin, 11.753= (-) Epicatechin, 13.123= Procyanidin A2, 14.383= p-Coumaric acid, 21.807= Quercetin, 26.920= Flavone.

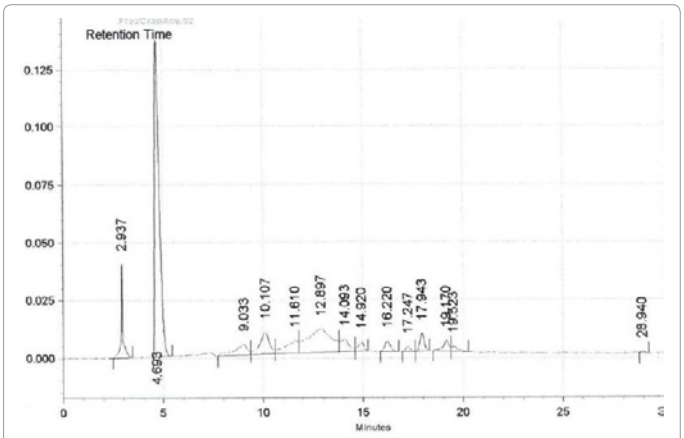

Figure 2: HPLC chromatogram for methanolic extraction of frozen cranberry.2.937= Tannic acid, 9.033= (+) Catechin, 11.610= (-) Epicatechin, 14.093= Procyanidin A2, 14.920= p-Coumaric acid.

cranberry products to methanol and acetone extraction methods. Flavone was also found in acetonic extraction procedure but it was not present in methanolic extraction procedure. The presence of quercetin has been reported in cranberry fruits [19]. However, no quercetin and procyanidin B1 could be detected in the samples of cranberry products of both the extraction procedures because methanol and acetone were not suitable solvent to extract these flavonoids. In addition, the other reason may be that HCl did not add throughout the sample extraction. It has been shown that HCl can release quercetin and procyanidin compounds during extraction [20, 21].

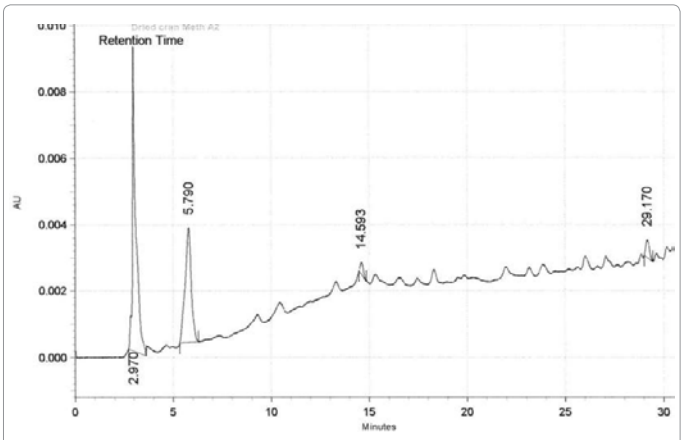

Figure 3: HPLC chromatogram for methanolic extraction of dry cranberry. 2.970= Tannic acid, 14.593= Procyanidin A2.

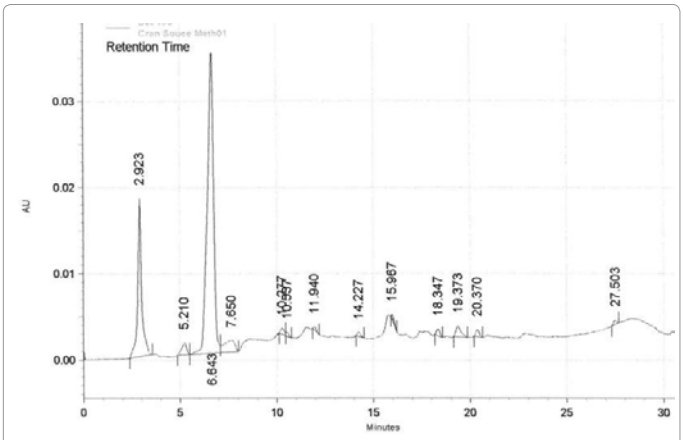

Figure 4: HPLC chromatogram for methanolic extraction of sauce cranberry. 2.923= Tannic acid, 10.07= (+) Catechin, 14.227= Procyanidin A2.

Tannic acid is one kind of phenolic acid which has retention time of 2.90 minutes [22]. It can be seen that tannic acid was also present in cranberry products following both extraction methods because the retention time is close to the peak of interest to all the samples. The methanolic extraction was better, but not for all processed cranberries, whereas authors stated that the methanolic extraction obtained the better result in general.

Quantitative analysis of phenolic compounds in processed cranberries

The flavonoids compound in cranberry products was estimated. The flavonoid content was calculated using the following equation-

Flavonoid content (mg/g, mg/ml for juice) =

$PA_{sample}/PA_{standard}$ × concentration of standards × volume of extract × DF × 1/weight of sample

Where, PA=Peak Area, Concentration of standards = 0.1 mg/ml (100 ppm), Volume of extract=10 ml, Dilution factor (DF)=10 (1 ml of sample extract + 9 ml of methanol), Weight of sample=cranberry samples weight (g, ml for Juice).

The total flavonoids content was obtained by adding the quantity of each flavonoid type present in the sample.

The calculated flavonoids amount of methanol extraction in the various cranberry products are presented for frozen (616.65 ± 0.14 mg/g), dry (142.13 ± 0.05 mg/g), sauce (360.80 ± 0.09 mg/g) and juice (237.84±0.03 mg/ml) in table 1 and figure 3. However, the results of total flavonoids content of acetone extraction in cranberry products are found for frozen (609.92 ± 0.12 mg/g), dry (0.57 ± 0.01 mg/g), sauce (94.03 ± 0.06 mg/g) and juice (269.27 ± 0.06 mg/ml) in table 2 and figure 4. The figure 5 depicts that the methanol and acetone extraction of frozen cranberries were compared for tannic acid (516.31 ± 0.02 mg/g ; 516.27 ± 0.02 mg/g), (+)catechin (36.93 ± 0.03 mg/g ; 37.44 ± 0.04 mg/g),(-)epicatechin (35.31 ± 0.03 mg/g ; 31.81 ± 0.02 mg/g),procyanidin (26.44 ± 0.02 mg/g ; 23.24 ± 0.01 mg/g) and p-coumaric acid (1.66 ± 0.04 mg/g ; 1.16 ± 0.03 mg/g) respectively and illustrated to have the high and low amounts of flavonoids. The amount of tannic acid from methanol and acetone extraction methods were compared and illustrated for frozen (516.31 ± 0.02 mg/g ; 516.27 ± 0.02 mg/g), dry (134.26 ± 0.01 mg/g ; not detected), sauce (348.43 ± 0.04 mg/g ; 92.63

Compound (mg/g),(mg/ml for Juice)	Cranberry Products							
	Frozen		Dry		Sauce		Juice	
	M	A	M	A	M	A	M	A
Tannic acid	516.31 ± 0.02	516.27 ± 0.02	134.26 ± 0.01	ND	348.43 ± 0.04	92.63 ± 0.02	237.68 ± 0.02	267.84 ± 0.01
Procyanidin B1	ND	ND	ND	ND	ND	ND	ND	ND
(+) Catechin	36.93 ± 0.03	37.44 ± 0.04	ND	ND	7.615 ± 0.03	ND	ND	ND
(-) Epicatechin	35.31 ± 0.03	31.81 ± 0.02	ND	ND	ND	ND	ND	1.27 ± 0.01
Procyanidin A2	26.44 ± 0.02	23.24 ± 0.01	7.87 ± 0.04	ND	4.75 ± 0.02	1.4 ± 0.04	ND	ND
p-Coumaric acid	1.66 ± 0.04	1.16 ± 0.03	ND	ND	ND	ND	0.16 ± 0.01	ND
Quercetin	ND	ND	ND	ND	ND	ND	ND	ND
Flavone	ND	ND	ND	0.57 ± 0.01	ND	ND	ND	0.16 ± 0.04
Total flavonoids (mg/g, mg/ml for Juice)	616.65 ± 0.14	609.92 ± 0.12	142.13 ± 0.05	0.57 ± 0.01	360.80 ± 0.09	94.03 ± 0.06	237.84 ± 0.03	269.27 ± 0.06

*ND=not detected, M= Methanolic extraction, A= Acetonic extraction

Table 1: Flavonoid and Phenolic content of methanol and acetonic extraction in cranberry products.

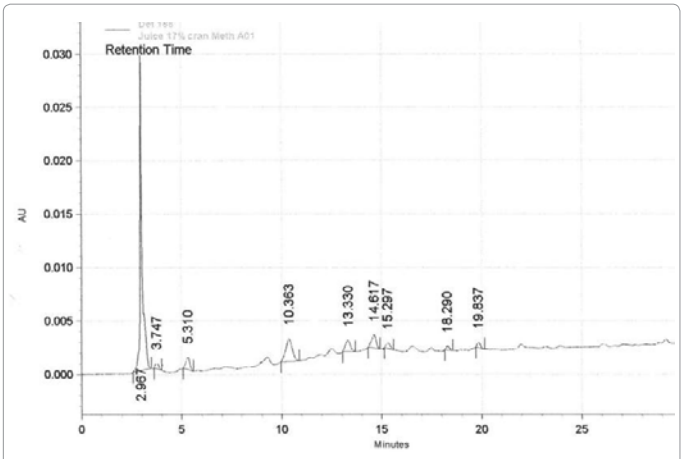

Figure 5: HPLC chromatogram for methanolic extraction of juice cranberry.2.967= Tannic acid,14.617= p- Coumaric acid.

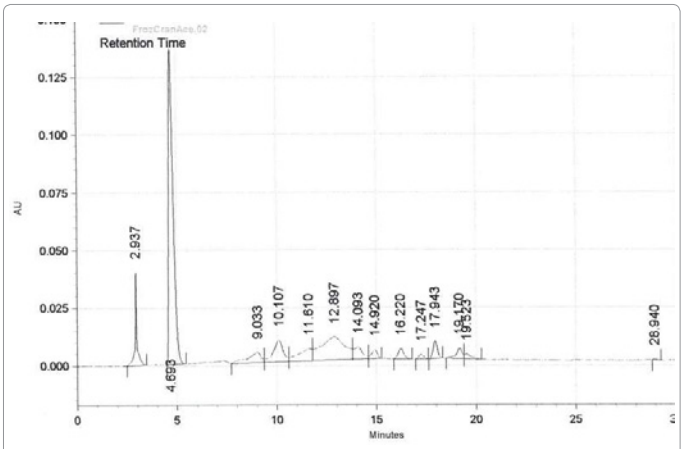

Figure 6: HPLC chromatogram for acetonic extraction of frozen cranberry.2.937= Tannic acid, 9.033= (+) Catechin, 11.610= (-) Epicatechin, 14.093= Procyanidin A2, 14.920= p-Coumaric acid.

± 0.02 mg/g) and juice (237.68 ± 0.02 mg/ml ; 267.84 ± 0.01 mg/ml) respectively as shown in table 1.

The total flavonoids content of the two extraction methods in cranberry products were found to be significantly different. The quantification of each compound was done according to the peak area measurements which were reported with corresponding standards. The result of methanol extraction revealed that cranberry products consist of total flavonoid of 616.65 mg/g in frozen samples, 142.13 mg/g in dry samples, 360.80 mg/g in sauce samples and 237.84 mg/ml in juice samples. It can be seen that frozen cranberries had the highest amount of total flavonoids followed by dry, sauce and juice cranberries (i.e. frozen>sauce>juice>dry cranberry) at $p<0.05$.

On the other hand, from the acetone extraction results, the content of total flavonoids estimated were 609.92 mg/g for frozen, 0.57 mg/g for dry, 94.03 mg/g for sauce and 269.27 mg/ml for juice. The amount of total flavonoids was the highest in frozen samples and was the lowest in dry samples ($P<0.05$). The results revealed that the values for the frozen and juice samples were quite close whereas that of the dry and sauce samples were significantly higher ($p<0.05$) when compared based on the extraction methods in table 1.

The separation and quantitation of phenolic compounds in cranberry products are difficult because of their similar chemical structures and complexity of cranberry samples [23]. The content of tannic acid was the highest of 516.31 mg/g for frozen cranberries to methanol extraction procedure; however it was nearly same amount of 516.27 mg/g to acetone extraction procedure in table 1. Therefore,

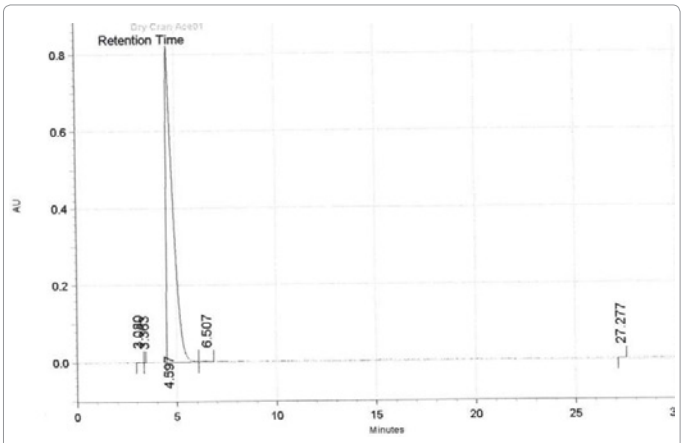

Figure 7: HPLC chromatogram for acetnic extraction of dry cranberry.27.277= Flavone.

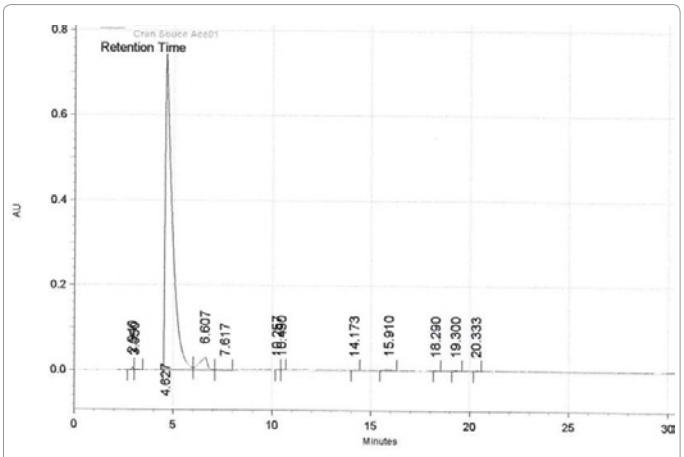

Figure 8: HPLC chromatogram for acetnic extraction of dry cranberry. 2.09= Tannic acid, 14.173= Procyanidin A2.

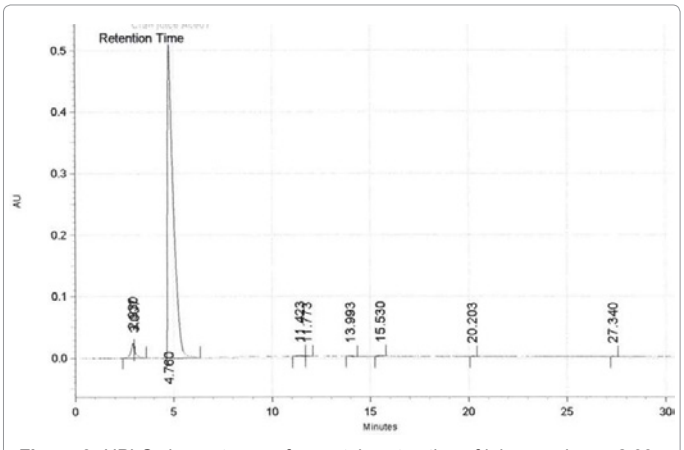

Figure 9: HPLC chromatogram for acetnic extraction of juice cranberry. 2.03= Tannic acid, 11.773= (-) Epicatechin, 27.340= Flavone.

it can be said that both extraction procedure are sufficient to identify it. Moreover, for sauce sample results, the amount of tannic acid in the methanol extraction was about four times higher (348.43 mg/g) than other method's result of 92.63 mg/g. It was also noted that in dry samples for methanol extraction procedure, the tannic acid content estimated was 134.26 mg/g but not detected in acetone extraction procedure. Therefore, acetone could not be a suitable extraction procedure for dry samples. In addition, in acetone extraction procedure for juice samples, the amount of tannic acid was estimated by 267.84 mg/ml which is slightly higher than other method by 237.68 mg/ml ($p<0.05$). So, authors stated from this study that acetone might be a good solvent for tannic acid extraction. In this study and previous finding report are compared that acetone extraction method utilizes an aqueous acetone system to extract tannic acid from cranberries. Acetone was preferred over other extraction solvent systems i.e., methanol due to its ability to solubilise tannic acid containing material that is insoluble in methanol [24].

Procyanidin (PC) A2 was found as one of flavonoids in cranberry extraction of both studied while it was determined at 26.44 mg/g for frozen sample, followed by dry cranberry (7.87 mg/g), sauce cranberry (4.75 mg/g) to methanol extraction, while for acetonic extraction this compound was quantified at 23.24 mg/g for frozen and 1.4 mg/g for sauce sample in figure 5. Although procyanidin A2 was taken to identify for all processed cranberries, in our experiment was not found this compound. In this study, authors has obtained the comparison of this compound in cranberries under storage conditions was different. Procyanidins are one of the most abundant subclasses of phenolic compounds in human diet [25]. Intervention studies with PC rich extracts and products like cranberry suggest protective effects of PC against cardiovascular diseases [26, 27].

There was no significant difference in the amount of various flavonoids (tannic acid and (+) catechin) content of the frozen cranberries for the two extraction methods in table 1. On the contrary to (-) epicatechin, the amount was estimated 35.31 mg/g to methanol extraction of frozen sample that is higher than by 31.81 mg/g to other method ($p<0.05$). Besides, p-coumaric acid was measured at 1.66 mg/g for the same sample at both methods in table 1.

In frozen fruits, flavonoids were reported to be comprised of mainly tannic acid, (+)catechin, (-)epicatechin, procyanidin A2 and p-coumaric acid. (+) Catechin was the second highest in concentration in frozen fruits that was the second major flavonoid in whole cranberries [4].

All phenolic compounds were identified in cranberry products occurring as free form which is in good agreement with previous findings reported in literature [28]. The results presented indicates that the content of tannic acid is higher than previous findings reported in literature [20]. This may be due to differences in fruit source, ripeness and length of storage time as well differences in the procedures used for obtaining samples. The consumption of cranberry fruit products can represent a significant amount of the total dietary intake of phenolic anti-adhesion properties [9]. Moreover, myricetin, benzoic acid and chlorogenic acids in cranberry products have been identified according to their retention times and the spectral characteristics of their peaks against those of standards [20], while the absence of this flavonoid and phenolic compounds were found in this experiment due to error of extraction procedure.

The techniques of UV-Vis spectrophotometry used to compare with this experimental method. So, authors obtained that isolation and quantification was difficult because of great variety of species present and the wide variations in their levels.

The amount of total flavonoids in cranberry is one of the highest among fruit and berry crops [29,30]. It is comparable with that of wolfberry leaves (547.0 mg/kg), guava (579.0 mg/kg), belmbi fruit (458.0 mg/kg), kale (110 mg/kg) and onions (486 mg/kg) which are known to be rich in total flavonoids [31]. The information obtained in the present study extends researchers knowledge on flavonoids in cranberry products which are widely utilized in cranberry derived products and may serve as the basis for future research of cranberry bioactive flavonoids with potential human health benefits.

There are some limitations in this study. For acetone extraction method, some present were not identified as they do not correspond to any of those of the standards used in table 1. However, some peaks like 4.5 minutes retention time showed that it might be contaminated during sample extraction. The lack of appropriate standards is the single most important factor that limits the aforementioned analyses for all peaks in the chromatograms. On the other hand, UV detection is not specific for proanthocyanidins relative to other polyphenolic compounds [24]. In addition, for quercetin and procyanidin B1, these compound might not be detected because both extraction methods are not suitable for bound forms of these compounds thus both extraction methods have limitations for conjugated forms of flavonoids. Most of the procedures described in the literature to analyse bound phenolic compounds in food take advantage of acid hydrolysis to release bound phenolic compounds using a modified Hertog method [20, 32].

Flavonols and phenolic acids are attracting tremendous interest in the field of health and nutrition, especially with regard to their potential UTIs health benefits. Given their ubiquitous occurrence in cranberries, it is critical that appropriate analytical methodologies be developed and used to characterize and quantify present in commonly consumed foods and drinks.

Procyanidin A2, tannic acid, (+) catechin, p-coumaric acid and (-) epicatechin were the major phenolic compounds detected in the methanol and acetone extracted cranberry frozen samples in the whole cranberry products. This study showed that the results were compared the phenolic content in cranberries under storage conditions. Two extracts analysed, the cranberries methanolic extract was found to be the most effective and the extract with the highest content of phenolic compounds. This suggests that methanol is an efficient solvent in the extraction. The authors also failed to characterise the bioactive compounds since not every compound is identified due to the lack of reference substances. The further research can be done to ascertain the findings of this study and possible modify the extraction methods and determination of the phenolic compounds in the cranberry products [33].

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