Analyzing Flavonoids in Chocolate-based Beverages

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Abstract

Some amount of previous research has suggested that chocolate-based drinks are very effective post-exercise recovery beverages. The fluid, electrolyte, protein and carbohydrate content of chocolate milk has been credited with possibly providing this benefit. Antioxidants perhaps contribute to post exercise recovery. Flavonoids, also naturally occurring phytochemicals in cocoa, the chief ingredient in chocolate, may be capable of providing antioxidant benefits. Using high-performance liquid chromatography (HPLC) and colorimetry, the present research involved examining the presence of flavonoids (particularly catechin) in seven chocolate-based milk drinks, as they may serve some importance regarding post-exercise recovery. A similar protocol (which basically involved defatting samples, performing a colorimetric assay and an HPLC) was treated to all samples. The colorimetric protocol was helpful in quantifying the flavonoids that may exist in 3 samples ("Chocolate Lowfat Milk," "Unsweetened Chocolate Almondmilk," and "Chocolate Protein Shake" in μg/mL). The presence of catechin in these samples could not be definitively confirmed, since at its retention times (13 to 14 minutes), the peak did not appear or were very inconspicuous. The rest of the samples did not show the expected end-point pink color change during the colorimetry, and no peaks at the retention times of concern during the HPLC.

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A free radical is a molecular entity that exists with an unpaired electron in its atomic orbital. This state of instability renders the molecule profoundly reactive; it can donate or receive an electron, thereby becoming efficient species in redox reactions (Lobo et al., 2010). Their interference with the function of vital macromolecules like nucleic acids, proteins and lipids can lead to physical and chemical imbalances and destruction of cells (Lobo et al., 2010). They are believed to cause numerous clinical conditions like cancer (Aruoma, 1998), and their abundance can lead to oxidative stress induced by alcohol (Albano, 2006).

Production of free radicals in the body is incessant, and it is engineered by both internal (metabolic) and external (X-ray, ozone, air pollutants exposure) factors (Lobo et al., 2010). Sources of internally generated free radicals include exercise, phagocytosis, and inflammation (Lobo et al., 2010).

The damage sustained as a result of a disparity between defensive antioxidants and harmful free radicals develops into a condition known as oxidative stress. This antioxidant-radical imbalance is caused by detriment to lipids, proteins and nucleic acids or oftentimes trauma and excessive exercise (Lobo et al., 2010). Oxidative stress causes damage to body tissues, which, in response, generate radical-producing enzymes like xanthine oxidase and lipogenase that cause the release of free iron, copper ions, or more reactive oxygen species (ROS). The onset and progression of cancer, Parkinson's disease, diabetes, noise-induced hearing loss (Yamashita et al., 2004), and age-linked eye diseases have been traced to the imbalance between ROS and antioxidants. Vascular dysfunction, including modified permeability of the blood vessels can be an outcome of accretion of ROS (Bielli et al., 2015). Exercise-induced production of ROS also causes muscle soreness (Lobo et al., 2010).

Antioxidants are stable molecular species that are capable of donating electrons to unstable free radicals, thereby neutralizing them and reducing the extent of their damage to body tissues (Lobo et al., 2010). The body, through regular metabolism, can produce some antioxidants like uric acid and ubiquinol, but other vital antioxidants like vitamin E and C, and B-carotene must be consumed through diet.

After an intense or unaccustomed exercise, the free radicals developed by the radical-producing enzymes induce muscle soreness. This discomfort, referred to as delayed onset muscle soreness (DOMS) usually begins 8 to 10 hours after exercise (Close et al., 2005). Ingestion of foods with naturally occurring antioxidants (for example, fruits and vegetables) reduces the severity of the harm, and aids a speedy recovery (Lobo et al., 2010). Antioxidants, according to several studies mentioned in this article provide health benefits (Yao et al., 2004; De Carvalho et al., 2018; Sousa et al., 2013), but other in vitro and observational studies have suggested a potential health benefit provided by antioxidants in foods, pure antioxidants as supplements have not (Dilis et al., 2010). Also, when levels of antioxidants in human bodies are beyond normalcy, their effects could be detrimental, and physiological functions like insulin signaling or vasodilation could be inhibited (Peternelj, 2011). Essential antioxidants are comprised of manganese, flavonoids, and vitamins A, E and C.

Flavonoids, the antioxidants of interest, are phenolic compounds that exist as phytonutrients and behave as metabolites found in all body tissues and organs (Hernández et al., 2009). Their natural sources include all photosynthetic plants (Yao et al., 2004). Several researchers have predicted that they serve an antioxidant function in plants under extreme environmental stress (Agati et al., 2012). Their free “radical scavenger” property also allows dietary flavonoids to cause the secretion of protective enzymes which battle cancers,

cardiovascular and age-related diseases (Yao et al., 2004). Through observation, some research experiments have suggested that muscle soreness after strenuous exercise, caused by muscular inflammation and oxidative stress can be reduced by dietary flavanols in chocolate milk (De Carvalho et al., 2018).

Muscle damage is believed to be induced by mechanical and metabolic operations in the body, the extent of the damage depends on the manner, intensity, and the length of the physical activity (Sousa et al., 2013). Some researchers have concluded that proteins reduce muscle damage by facilitating muscle repair and adaptation from exercise-induced muscle damage (EIMD) (Sousa et al., 2013). Carbohydrate-only supplements (CHO) when co-administered with chocolate milk (CM) to teenage athletes and observed separately, relatively contributes insignificantly to subjects' recovery, but never anything detrimental (Born et al., 2019). Flavonoids like epigallocatechin gallate (EGCG) through experimentation, have also demonstrated a contribution to reduction of muscle soreness (Sousa et al., 2013).

Chocolate based beverages, with a healthy amount of naturally occurring flavonoids, have been suggested to be effective post-exercise recovery drinks (Spaccarotella, 2011). For example, researchers administered low fat chocolate milk to 13 collegiate athletes (5 males and 8 females) between Division III soccer practice sessions and compared with the effect of a carbohydrate–electrolyte beverage. The athletes were fed chocolate milk between their morning and afternoon practice sessions. After afternoon sessions, they participated in a shuttle run till exhaustion. It was observed that the males had developed a significant increased runtime and time to fatigue (Spaccarotella, 2011).

Also, other research has suggested that chocolate based beverages may work as well as or more effectively than placebo and other sport drinks in post-exercise recovery (Amiri, 2018) and

that antioxidants may help promote post-exercise recovery (Spaccarotella, 2011; Yi, 2014; Morillas-Ruiz, 2006; Jenkins, 2006). Catechin and epicatechin are the vital flavonoids found in dark chocolates, according to research by Meng et al (2009). Dark and milk chocolates also contained theobromine, which was absent in white chocolate. The findings suggested that dark chocolate may outperform milk and white chocolate in health enhancement and nutrition because of the higher concentrations of antioxidants. (Meng et al, 2009). It is therefore inferable that cocoa-based beverages may improve post-exercise recovery in part due to their flavonoid content.

The present experiment was conducted to identify the flavonoids (particularly catechin) that exist in chocolate-based beverages and the importance they serve in post-exercise recovery, which may make them more suitable than other drinks like Gatorade.

Method

All the milk samples used in the experiment were purchased by Dr. Spaccarotella, and other chemical reagents used were obtained from the chemistry labs at Kean University. A protocol similar to that previously employed by Meng et al. (2009) and Natsume et al. (2000) was followed. The seven different brands of chocolate milk followed the same protocol for flavonoid extraction and examination, with only a few variations, due to the quest to find more efficient ways to work because of time constraints. While trying to figure out what volume of sample would be adequate for the experiment, 100 mL was used first. After deciding that that was too much, it was reduced to 25 mL; but that was very little, so we doubled up to 50 mL and maintained this for the rest of the samples.

The entire study spanned 17 days, with about 14 days spent in the lab. The difference in the time the samples were left to stand is due to the research schedule. For example, after a third fat removal trial on Wednesday, samples would be attended to the following Monday, or maybe the next two days after, in case the labs were not open. The samples with variations in durations of treatment are listed below.

Table 1.

A table listing some descriptive characteristics of samples used.

Product	**Size (US fl oz)**	**Manufacturer**	**Amount of sample used in fat extraction (mL)**	**Time left to stand**
Chocolate Lowfat Milk	12	Lucerne Dairy Farms	100	8 days
Silk Almondmilk Dark Chocolate	8	WhiteWave Foods	50	9 days
Chocolate Almond Breeze Almondmilk	8	Blue Diamonds Almonds	50	9 days
Chocolate Protein Shake	11	Muscle Milk®	50	9 days
Nesquik 1% Lowfat Chocolate Milk	32	Nestlé	50	9 days
Unsweetened Chocolate Almondmilk	64	Blue Diamonds Almonds	25	7 days
Chocolate Silk Protein (almond &	64	Silk®	100	8 days

cashew)				

High-Pressure Liquid Chromatography

Packaged in plastic bottles or cartons, milk samples when not in use were stored in a refrigerator at 7 °C. Adequate amounts of the samples were poured into separate plastic containers and transferred to the chemistry lab.

A volume of 50 mL of each sample (refer to Table 1. for other volumes used) was poured into a graduated beaker and mixed with an equal amount of heptane to stand for at least 24 hours (Natsume et al., 2000). Two layers formed in the beaker. The top layer (presumably fat because it was gelatinous) was removed with a scoopula and stored in another beaker. This step was repeated three times to remove most of the fat in the samples, wherein during the final one, the sample was left to stand for days.

The bottom layer was pipetted into centrifuge tubes and centrifuged at 4700 RPM and 19 °C for 3 minutes. Three distinct layers formed. The top layer was a transparent liquid (heptane or hexane); the middle layer was brown (milky chocolate), and the bottom layer was more particulate and darker (solid cocoa particles).

The middle layer was extracted with a pipette and a few drops were deposited into two separate vials. Water was added to the sample in one vial and acetonitrile to the other.

The water mixed homogeneously with the sample in the vial, suggesting the absence of ample fat in the chocolate milk extract.

A faint yellowish color was observed in the vial with acetonitrile and milk extract. Two layers formed. The upper layer was extracted and injected into a high-pressure liquid chromatography (HPLC) instrument. The instrument was run for about 40 minutes.

Colorimetric Assay

To calculate the flavonoid content in the samples, colorimetry was used. The protocol performed was similar to that previously employed by Zhishen et al (1999). The process involved making solutions with known concentrations of catechin and creating a standard curve to find the concentrations of the chocolate milk samples in micrograms per milliliter (μg/mL). This method was performed twice, on two different days.

The following solutions were prepared and used in the colorimetric assay: 10% Aluminum Chloride ($AlCl_3$), 1M Sodium hydroxide (NaOH), 5% Sodium nitrite ($NaNO_2$), Catechin Standards (0, 1, 5, 10, 20 μg/mL).

A 1 mL sample of each chocolate milk extract in acetonitrile was measured and deposited in a 15 mL volumetric tube containing 4 mL distilled water. 0.3 mL of 5% Sodium nitrite ($NaNO_2$) was added to all the samples in each tube and incubated at room temperature for 5 minutes. The samples were incubated again for 6 minutes after the addition of 0.3 mL 10% Aluminum Chloride ($AlCl_3$). Finally, 2 mL of 1M NaOH was combined with each sample, and the volume was increased to 10 mL with distilled water. At 510 nm, the absorbance of the pink color change in the samples were measured with a spectrophotometer.

Results

Colorimetric Assay

On the first day, all the prepared catechin standards and the two samples tested, showed a pink end point color change. The samples on the first day were “Chocolate Lowfat Milk,” and “Unsweetened Chocolate Almondmilk.” The transmittance of the standard catechin solutions were used to generate a standard curve, and the equation of the line was used to determine the

flavonoid content in the samples (in µg/mL).

Colorimetry Day 1

Six catechin standards were prepared during the first colorimetry test. Their transmittances were measured by a spectrophotometer and used to generate a graph. The equation of the line was used to determine the flavonoid content in the "Chocolate Lowfat Milk" and "Unsweetened Chocolate Almondmilk" sample (in µg/mL).

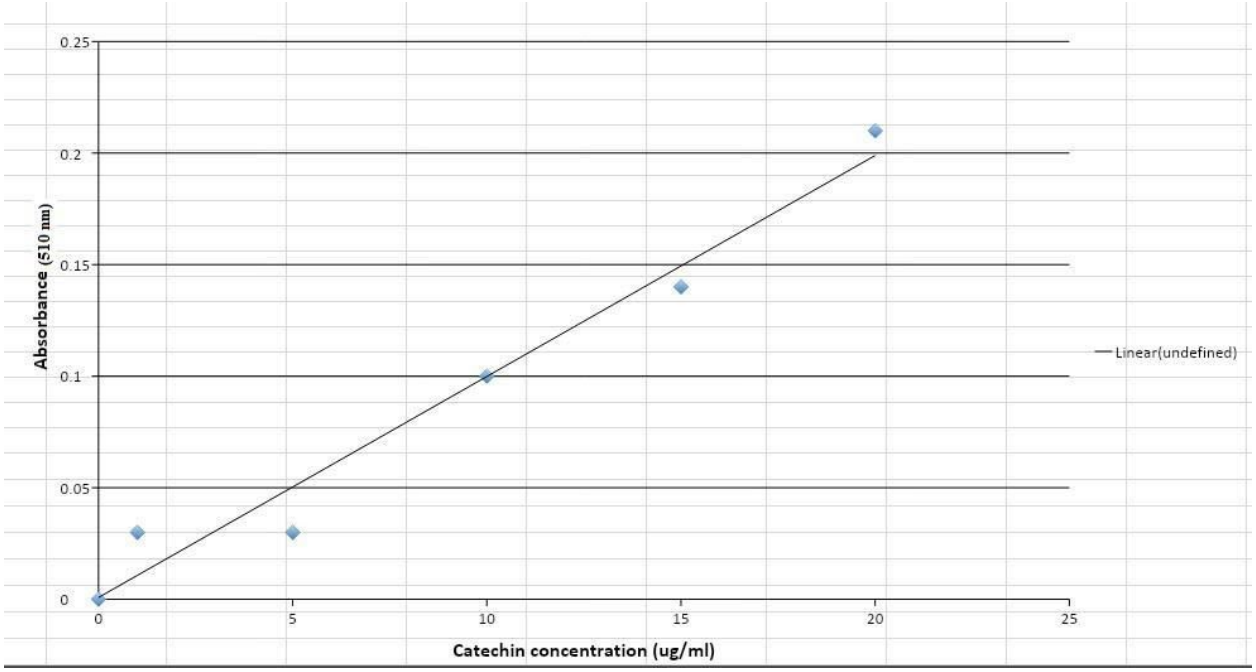
Table 2.

A table showing the results of the colorimetric assay on Day 1.

Sample / Standard (µg/mL)	Transmittance (510 nm)	Color Change (Pink)	Flavonoid Content (µg/mL)
0	0.00	Yes	-
1	0.03	Yes	-
5	0.03	Yes	-
10	0.10	Yes	-
15	0.14	Yes	-
20	0.21	Yes	-
Chocolate Lowfat Milk	0.12	Yes	12.04
Unsweetened Chocolate Almondmilk	0.14	Yes	14.06

Figure. 1.

Standard curve for Catechin standards prepared on Day 1.



Note. Equation of the line: Y = 0.0099x + 0.0008

Colorimetry Day 2

In the second colorimetric test, 6 catechin standards were prepared in addition to the 5 remaining chocolate milk samples. All the samples, but "Chocolate Protein Shake" by Muscle MIlk® and the catechin standards, did not show any pink end point color change. The transmittance of the standard catechin solutions were used to generate a standard curve, and the equation of the line was used to determine the flavonoid content in the "Chocolate Protein Shake" sample (in μg/mL).

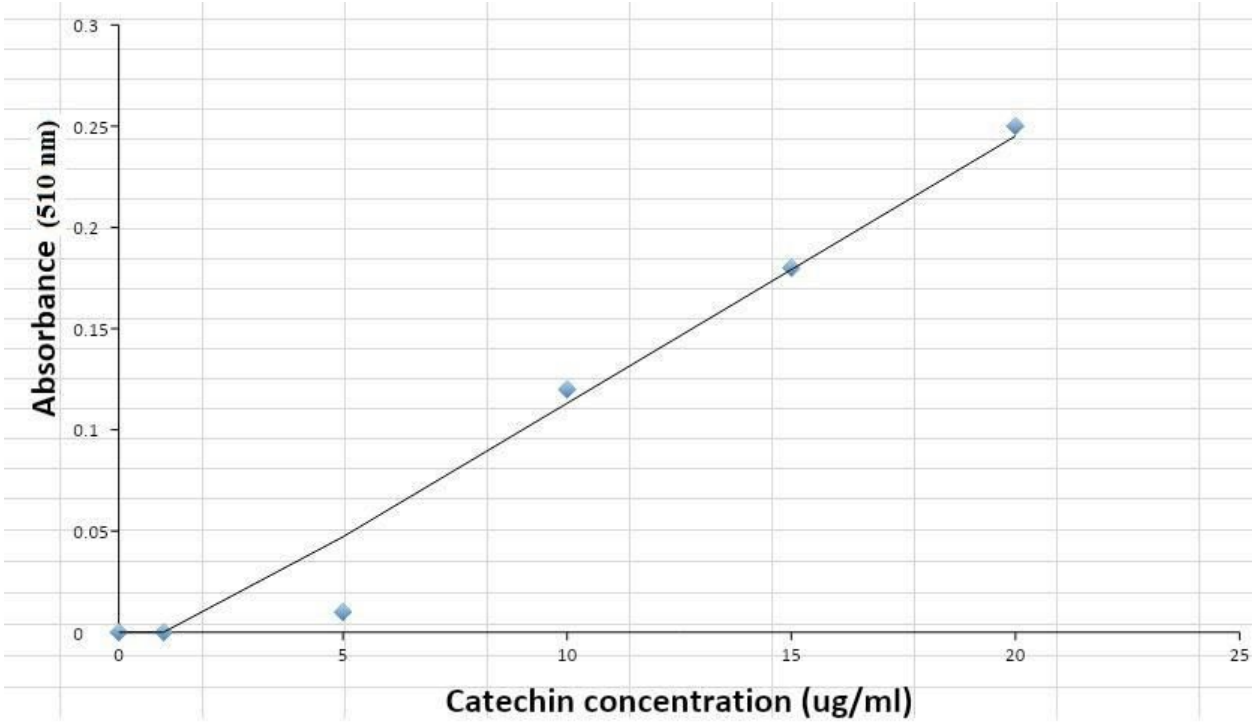
Table 3.

A table showing the results of the colorimetric assay on Day 2.

Sample / Standard (µg/mL)	Transmittance (510 nm)	Color Change (Pink)	Flavonoid Content (µg/mL)
0	0.00	Yes	-
1	0.00	Yes	-
5	0.01	Yes	-
10	0.12	Yes	-
15	0.18	Yes	-
20	0.25	Yes	-
Chocolate Protein Shake	0.59	Yes	10.6
Chocolate Silk Protein (almond & cashew)	2.30	No	-
Silk Almondmilk Dark Chocolate	2.16	No	-
Nesquik 1% Lowfat Chocolate Milk	1.89	No	-
Chocolate Almond Breeze Almondmilk	2.43	No	-

Figure 2

Standard curve for Catechin standards prepared on Day 2.



Note. Equation of the line: Y = 0.7474x - 7.35

High-Pressure Liquid Chromatography (HPLC)

Previous research that involved the analysis of polyphenols in cocoa-based products using HPLC identified a catechin peak from retention times 13 to 14 minutes in the HPLC spectrum (Natsume et al, 2000). This information was used as a reference for the peaks that showed in the HPLC spectra of the chocolate milk samples.

The HPLC spectra included in this current article are the ones of the samples that showed a pink color change in the colorimetric assay. They are: “Chocolate Lowfat Milk,” “Unsweetened Chocolate Almondmilk,” and “Chocolate Protein Shake.” The other samples did not show any significant peaks in the chromatogram, at the retention time of interest.

Figure 3

A HPLC spectrum of catechin in acetonitrile.

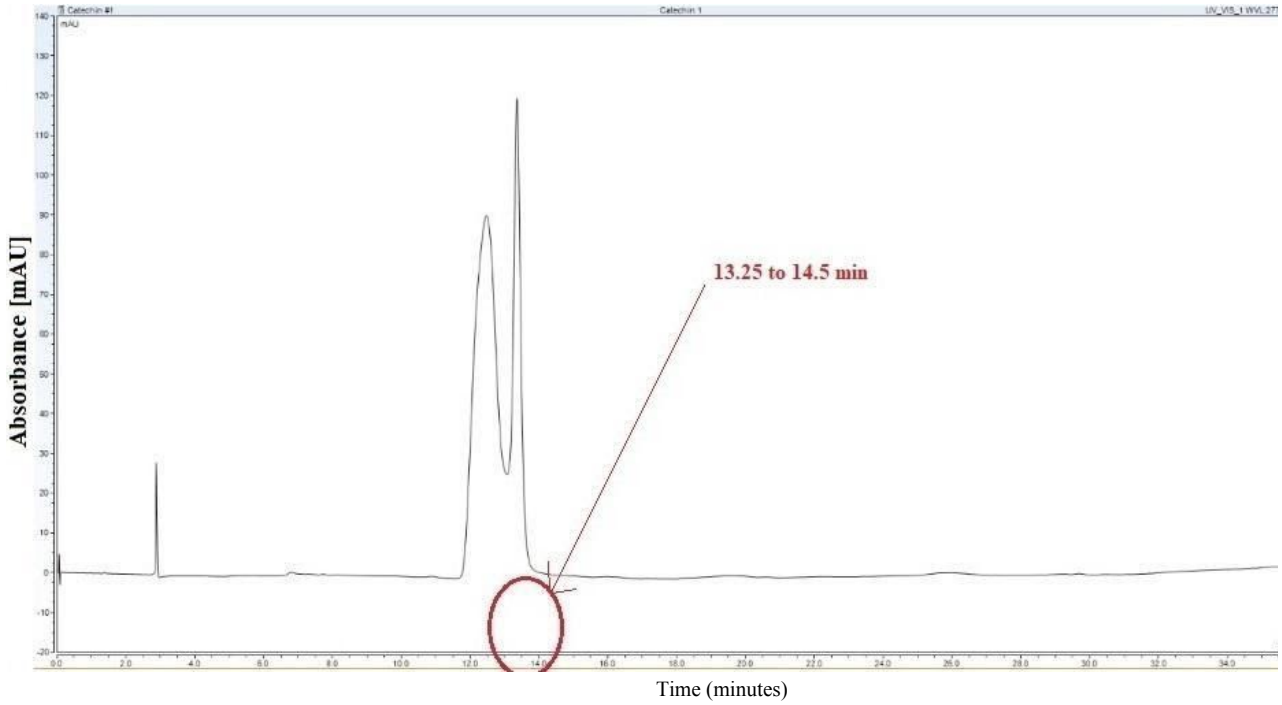


Figure 4

A HPLC spectrum of "Chocolate Lowfat Milk" extract in acetonitrile.

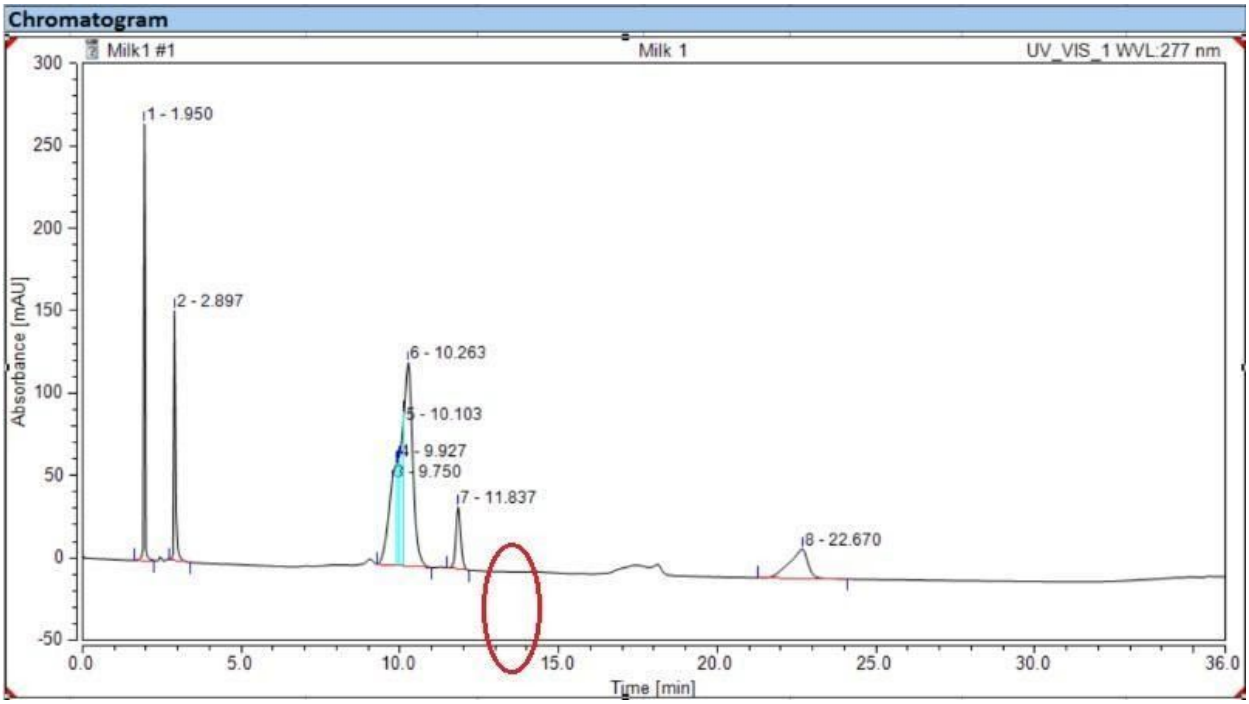


Figure 5

A HPLC spectrum of "Unsweetened Chocolate Almondmilk" extract in acetonitrile.

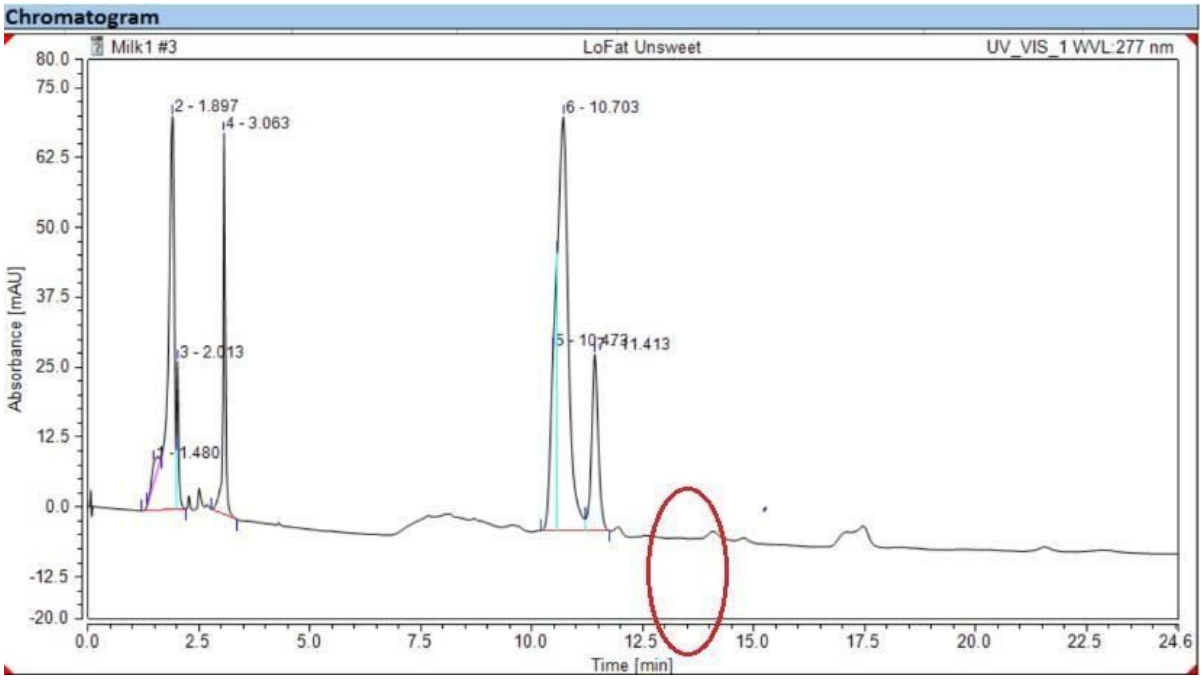
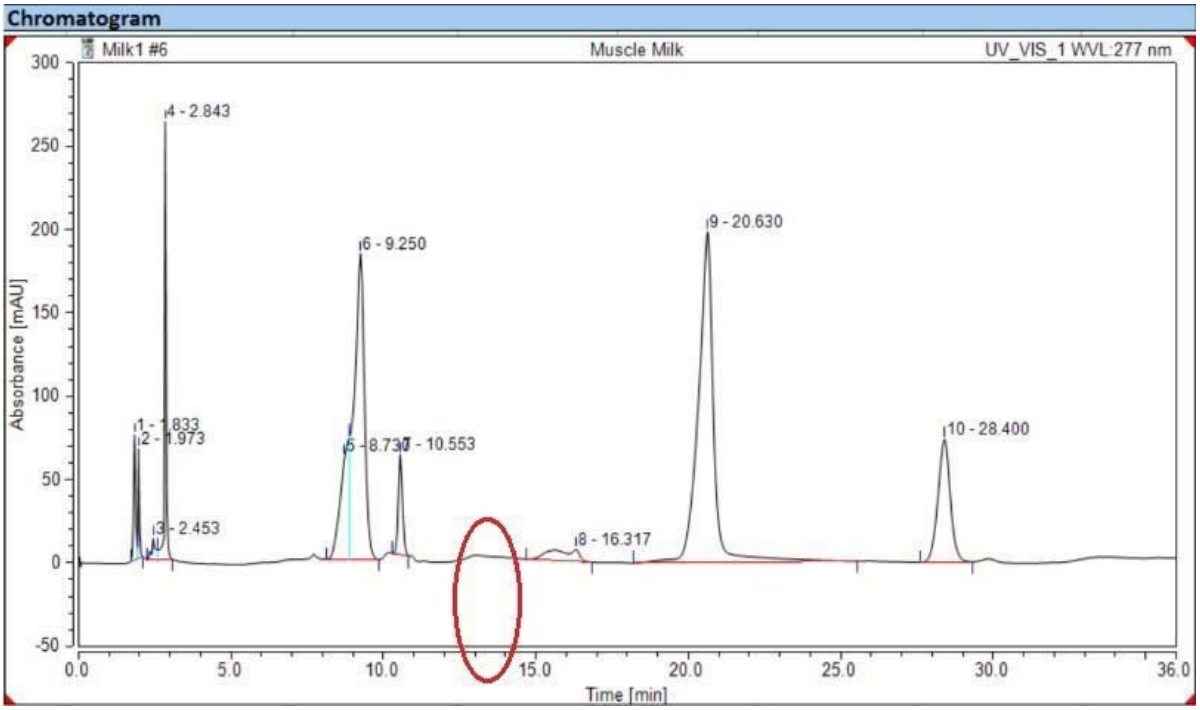


Figure 6

A HPLC spectrum of "Chocolate Protein Shake" extract in acetonitrile.



Discussion

Colorimetric Assay

The colorimetric assay was performed on two different days because there was little time available on the first day; also, all the samples in acetonitrile had not been prepared and run through the HPLC instrument. The results for the first colorimetric test were very consistent, because all samples and catechin standards showed an end point color change.

In the second colorimetric test, all the samples, but “Chocolate Protein Shake” by Muscle MIlk® and the catechin standards, did not show any pink end point color change, but turned very foggy. This may in part, be due to the presence of heptane or hexane in the samples still. In order to treat every sample to the protocol before time was due, the samples used on Day 2 of the Colorimetric assay were not centrifuged; this may have caused some heptane or hexane to remain in the samples and interfere with the outcome of the experiment.

High-Pressure Liquid Chromatography

The HPLC spectrum in Fig. 3. was generated after pure catechin dissolved in acetonitrile was injected into the instrument. Using the results from Natsume et al. (2000) for reference, it can be thought that catechin is present since there is a strong peak from retention times 13 to 14 minutes. It is unclear what caused the wider peak to the left of it, but the relatively smaller peak around retention time, 2.8 minutes may be due to the presence of acetonitrile in the solution. This peak appeared in all of the samples used in the experiment, even those that did not show any color change in the colorimetric assay.

In Fig. 4., the chromatogram of "Chocolate Lowfat Milk" did not show any peak between 13- and 14-minutes retention times. This may suggest that the product may lack the antioxidant of interest, catechin. There are other significant peaks that appear throughout the spectrum, most notably the highest peak at 2.8 minutes, which is possibly due to the presence of acetonitrile. The remaining peaks may be due to the presence of other flavonoids in the product.

At the retention times of concern, the chromatogram for "Unsweetened Chocolate Almondmilk" (Fig. 5.) shows a tiny ripple that suggests that catechin may be present, but possibly in minute amounts. The other peaks shown, including the one at retention time 2.8 minutes and thought to be caused by acetonitrile, may be due to the presence of other flavonoids in the milk sample.

The chromatogram for "Chocolate Protein Shake," by Muscle Milk® (Fig. 6.) shows a very inconspicuous peak from retention times 13 to 14 minutes and the absorbance is slightly above 0 mAU. Based on the findings from Natsume et al., it is likely that this peak is due to the presence of catechin. However, the peak is not so pronounced, and this creates a lot of uncertainty, regarding its presence and specific concentration. Also, the other peaks present may

be due to the presence of other flavonoids that are not analyzed in this study.

Conclusion

The results derived from the two parts of the methodology (Colorimetry and HPLC) are not strong enough to definitively conclude that catechin was extracted from the chocolate milk samples. The expected peaks for the phytochemical did not appear at all, or only showed up as a tiny ripple in the chromatogram. If any amount of catechin was detected during the HPLC protocol, they might have been in very small amounts, and may be responsible for the small peaks at 13 to 14 minutes retention times in the HPLC spectrum.

Throughout the experiment, the focus had been on extracting and analyzing catechin, but the emergence of many other peaks in the HPLC spectrum raised questions about what chemicals those peaks represented. It is likely that some of those peaks are caused by other flavonoids that are not of interest to this research.

Moving forward, researchers would find more ways to refine the protocol, in order to reduce errors that may hinder its efficiency. For example, they may develop ways to get rid of hexane or heptane accrued in the samples or perform HPLC at every step of the protocol to better identify catechin. Also, the sample size in this research could be reduced to maybe just the two samples that showed tiny ripples at retention times 13 to 14 minutes in the chromatogram, and then some other flavonoids could be included in the study.

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