

Influence of Extraction Time on the Total Phenolic Content of Hemp Seeds (*Cannabis sativa* Sb. Sativa) Water-Extracts

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Abstract

Water extraction of hemp seeds could be performed to isolate water-soluble compounds such as proteins, carbohydrates and potentially other bioactive compounds. The aim of this study was to determine the influence of extraction time on the content of total phenolic compounds in water extracts of hemp seeds. The extracts were made taking different extractions times and using a 1:100 ratio of hemp seeds-water. Total phenolic compounds were determined in microplate by the Folin-Ciocalteu method and a Gallic Acid (GA) calibration curve (R²=0.94). The results showed that GA equivalents were significantly higher at 60 min extraction time. The evaluation of the influences of time in extractions allow to have a better selection of parameters for a thermal process considering an objective response, in this case total phenolic content.

Keywords: Hempseeds; Water-extraction; Total phenolic content; Antioxidant capacity

Introduction

Water extraction (WE) is a common method used to isolate bioactive compounds, proteins and other components from various plant materials, including seeds. WE is a versatile and essential process in food science, impacting the flavor, texture, safety and quality of food products. It plays a vital role in ingredient preparation, preservation and the development of new food innovations while also contributing to sustainable food production practices. Several factors influence the efficiency and outcomes of water extraction processes in food science, mainly temperature and time. Time is a critical factor that influences extraction, including various extraction processes in chemistry, food science and other industries. The duration of the extraction process, often referred to as the extraction time or contact time, is critical. Longer contact times generally lead to more extensive extraction, but there is an optimal point beyond which further extraction may not yield significant improvements and could lead to the extraction of unwanted compounds. The extraction

time in water-based extracts can have a significant impact on the composition, concentration and quality of the extracted compounds.

Hemp seeds (*Cannabis sativa* Sb. sativa) are known for their nutritional value, containing essential fatty acids, protein, fiber and various vitamins and minerals. Likewise, hemp seed is also rich in phenols, as approximately 500 compounds have been identified, which may include bioactive compounds such as hydroxycinnamic acids, hydroxybenzoic acids, flavonoids and lignan amides [4]. The antioxidant capacity of these compounds is evident in several chemical and *in vitro* studies [5]. The antioxidant properties of hemp seed lignanamides have been documented in several studies and N-trans-cafeoyltyramine and its low-density lipoprotein oxidation suppressing ability, cannabisin B and its oxidation inhibitory effect have been reported., 3,3 dimethyl-heliotropamide and its potent DPPH radical scavenging activity at 81.5%, etc.

Moreover, apart from lignanamides, the abundance of flavonoids in the seed oil has been positioned with high antioxidant activity (695.2 and 3,690.6 µmol Trolox Equivalent (TE)/100 g) [6]. Hemp oil is predominantly derived from hemp seeds and is rich in essential fatty acids, notably alpha-linolenic acid (omega-3) and linolenic acid (omega-6), which offer various health benefits. The oil concentration in hemp seeds can fluctuate due to factors like hemp variety, cultivation conditions and processing techniques, typically falling within the range of 30% to 35% or potentially higher. It's worth noting that certain phenolic compounds found in hemp seeds may have solubility in oil. Consequently, if the oil is separated during processing, it might impact the phenolic content within the residual seed material [7,8]. The principal oil-soluble constituents in hemp seeds include lignans and phytosterols. Lignans represent a category of phenolic compounds, with notable examples like Secoisolariciresinol Diglucoside (SDG) present in hemp seeds, believed to offer potential health advantages. Phytosterols, while not genuine phenolic compounds, are frequently linked to the phenolic composition of plant-based foods. Hemp seeds contain a range of phytosterols, such as α, γ and δ-tocopherol and γ-tocotrienol. These compounds share a chemical structure akin to cholesterol [9,10].

Hemp seeds contain a diverse array of proteins, encompassing globulins, albumin, edestin and peptides. Among these, hempseed peptides, which are short amino acid chains derived from proteins, have the potential to enhance the antioxidant capacity of hemp seeds. These hempseed peptides may feature specific amino acid sequences with inherent antioxidant properties. These peptides can scavenge free radicals and reduce oxidative stress, contributing to the overall antioxidant capacity of the seeds [8,11]. Peptides can be more easily absorbed and utilized by the body than larger proteins. This improved bioavailability means that the antioxidant peptides in hemp seeds may have a more significant impact on reducing oxidative damage in the body compared to larger protein molecules. Peptides and phenolic compounds can interact in various ways, often leading to synergistic effects in terms of their antioxidant and health-promoting properties [12,13].

Water extraction of hemp seeds could be performed to isolate water-soluble compounds such as proteins, carbohydrates and potentially other bioactive compounds. Hempseed Water-Extracts (HWE) could contain various bioactive compounds, including phytosterols, terpenes and phenolic compounds, which have potential health benefits. Extracts enriched with these compounds might have antioxidant, anti-inflammatory and other beneficial properties. The aim of this study was to determine the influence of extraction time on the content of total phenolic compounds in water extracts of hemp seeds.

Methodology

Plant material

The hemp seeds hearts (*Cannabis sativa* L. ssp. *sativa*) were obtained through Manitoba Harvest, Inc., Manitoba, Canada.

Chemicals

6-hydroxy-2,5,7,8-tetramethylchroman-3-carboxylic acid (Trolox), ethanol reagent (V60), Folin and Ciocalteu's phenol reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade, sodium hydroxide (NaOH), sodium chloride, buffer solution pH4 and buffer solution pH7.

Hempseed water-extractions and water-extractions

The extracts were made according to Izzo et al. taking different extraction times and using a 1:100 ratio of hemp seeds-water, respectively (E1), 30 min (E2), 60 min (E3). After boiling process each extract was vacuum filtered using a 11 µm medium-flow filter paper (Whatman, Little Chalfont, Buckinghamshire, United Kingdom) to remove excess solids. Each extract was made by triplicate (Table 1).

Table 1: Hempseed water-extractions conditions.

	Extractions		
	E1	E2	E3
Temperature (°C)	30 ± 1	60 ± 1	90 ± 1
Time (min)	30 ± 1	60 ± 1	90 ± 1

Subsequently, in order to compare the extracts and relating it to its future applications, it was compared with extracts of black tea and chai tea. The tea extracts were made 1:100 ratio of tea-water, respectively. Then the extracts were cooled out at 98°C ± 2°C for 60 min.

After boiling process each extract was vacuum filtered using a 11 µm medium-flow filter paper (Whatman, Little Chalfont, Buckinghamshire, United Kingdom) to remove excess solids. Each extract was made by triplicate. All determinations were performed in duplicate and were statistically analyzed.

Proximate analysis

Hemp seeds proximate composition (moisture, crude protein, crude fat, total ash) was determined using the official methods of analysis of AOAC. Total carbohydrates were determined by difference. For estimate the protein content the 6.25 factor was used. Lipids were extracted from the seeds using the Soxhlet method and stored at -20°C for further analysis. The dietary fiber was calculated as default samples according to NMX-F-622-NORMA-2008. All assays were performed in triplicate.

Mineral and heavy metals content

The determination of the mineral composition was carried out with ICP-OES analysis (Inductively Coupled Plasma-Optical Emission Spectroscopy) according to Intema® internal method (Agilent 5110, ICP-OES). The minerals chosen as the focus of this study were sodium (Na), potassium (K), calcium (Ca), zinc (Zn), magnesium (Mg), copper (Cu), iron (Fe) and manganese (Mn). Briefly, an acid digestion of the samples was carried out. Mineral calibration standards for ICP-OES analysis were prepared by dilution of NIST-traceable 1,000 mg/L Ca, Mg, Na, Fe and K standards.

Total phenolic content

Determination of polyphenols was performed according to Ramírez-Rodriguez. Total phenolics were estimated using the Folin–Ciocalteu colorimetric method. Briefly, 200 µL of the sample was diluted with 1 mL of distilled water and 250 µL of undiluted Folin–Ciocalteu reagent. Immediately, 2.5 mL 7% (w/v) Na₂CO₃ was added. Then, a standing time was taken (90 min) in the dark.

The absorbance was measured at 760 nm using a UV-Vis spectrophotometer model Multiskan Sky Microplate (Thermo Fischer Scientific, Waltham, MA, USA) and compared to a pre-prepared gallic acid calibration curve (R²>0.94). Determinations were performed in triplicate. Results were expressed as milligram of Gallic Acid Equivalents (GAE) per mL of extract (0-800 mg/L of gallic acid).

Total soluble solids and pH of extracts

Total soluble solids (°Brix) were determined in the extracts with a hand refractometer (Reichert technologies AR 200) and the pH was measured with a potentiometer (Hanna Instruments, HI5522-02).

Statistical analysis

For the analysis of the experimental data, the mean and Standard Deviation (SD) of all the determinations were reported. The differences between sample were considered statistically different for $p < 0.05$ through the Analysis of Variance Test (ANOVA). All collected data were analyzed using Minitab statistical software. 17.2020 (Minitab Inc., State College, PA, USA). The differences between the means will be considered statistically different for $p < 0.05$ through Tukey's analysis.

Results

Proximate analysis

Table 2 shows the result for the proximate analysis of hemp seeds. It was found that hemp seeds contain approximately 30% of lipids, 29% of carbohydrates where 21% is dietary fiber (including cellulose, hemicellulose, lignin, soluble and insoluble fibers), 28% of crude proteins, 6% of ashes and finally 5.7% of moisture content. The results of the current study were consistent with the previously reported ranges by Callaway (2004) and Russo and Reggiani (2013) since hemp seeds exhibited an average content of 27%-39% for lipids, 28%-30% for carbohydrates where 19%-23% includes dietary fiber, 24%-30% for crude proteins, 6%-7.2% for ashes and finally 4.8%-56.7% moisture content [15-17].

Table 2: Proximate analysis of hemp seeds.

Compound	(%)
Moisture	5.70 ± 0.14
Ashes	6.1 ± 0.01
Proteins	28.11 ± 0.08
Fat	29.24 ± 0.06
Carbohydrates	29.61 ± 0.02
Fiber	21.18 ± 0.23

Note: The data shown correspond to means ± standard deviations from three independent measurements.

Lipids and proteins were two of the most abundant compounds in the samples which are known to have an important role in contribution of nutrients in the daily diet [18]. Regarding lipids, on the one hand the abundance of flavonoids in the seed oil has been positioned with high antioxidant activity and on the other side the oil extraction process can indirectly influence the phenolic content. Certain phenolic compounds in hemp seeds might be oil-soluble, so the removal of oil during processing could potentially alter the phenolic content in the remaining seed material. Nevertheless, the degree of this

influence would be contingent upon the particular processing method employed [6,7,10].

High temperatures can promote the hydrolysis of proteins, leading to the breaking of peptide bonds. As the temperature increases, the kinetic energy of molecules rises and this can result in the cleavage of peptide bonds [13]. Consequently, proteins may fragment into smaller peptides or eventually into individual amino acids. Although hempseed peptides, formed from short amino acid chains derived from hemp seed proteins, do not have a direct impact on the phenolic content of hemp seeds, they are intricately linked to bolstering the seed's antioxidant capacity [12]. The interaction of different components in hemp seeds, including peptides and other antioxidants like phenolic compounds and vitamin E, can result in synergistic effects that enhance the overall antioxidant capacity. Combining various antioxidants may provide a more potent protective effect against oxidative stress [19,20]. The way hemp seeds are processed and applied can influence the release and bioavailability of antioxidant peptides.

Minerals and heavy metal determination

For mineral determination ash represents an overview of the results where this parameter can be interpreted as the total mineral content of a sample, made by inorganic components. **Table 3** shows the more abundant minerals in the sample: phosphorus, potassium and magnesium that agreed with results reported by Callaway [15] and García [21]. Findings evidenced that a consume of 100 g of hempseed could cover around the 235% (P), 32% (K) and 16% (Mg) of the daily reference intake for adult humans, respectively [5,16,22,23]. Besides the results of sodium and copper were lower than reported by Callaway, Russo and Reggiani which is interpreted as an ideal due to the recommended consumption is lower than the other minerals and heavy metals [15-17].

Table 3: Mineral and heavy metals content of hemp seeds.

Compound	(mg/100 g)
Sodium (Na)	5.807 ± 0.01
Phosphorus (P)	1649.669 ± 0.43
Potassium (K)	1127.347 ± 0.18
Calcium (Ca)	63.688 ± 0.01
Magnesium (Mg)	660.439 ± 0.13
Iron (Fe)	15.914 ± 0.02
Zinc (Zn)	7.754 ± 0.01
Manganese (Mn)	5.466 ± 0
Copper (Cu)	1.391 ± 0.01

Note: The data shown correspond to means ± standard deviations from three independent measurements.

Studies have suggested that certain minerals, such as selenium and zinc, have antioxidant abilities of their own. They can help protect plant tissues from oxidative damage. In response, the plant may make fewer phenolic compounds if it doesn't need as many antioxidants. Furthermore, other minerals, including copper and iron, serve as cofactors for enzymes responsible for producing phenolic compounds [24-26].

Total phenolic content

Phenolic compounds are valued for their potential health benefits, as they are known to have antioxidant properties and may contribute to the overall health benefits of consuming tea. The results showed that when the extraction time is 60 min, the measurement from GA equivalents was significantly higher ($p < 0.05$) than E1 and E3. However, if the time of the water extractions was 90 min, the values start to decrease. The findings of the study could be attributed to the fact that the best extraction time varies depending on affinity of the compounds to be extracted and which extraction method is used. Related results were found by Ferrante et al. where they evaluated hemp lower water extracts as potential source of antioxidants with potential efficacy in managing clinical symptoms related to ulcerative colitis [27]. The finding showed that at longer exposure time during extraction, the target responses increased until a latent point was reached where they began to decrease [27].

Similarly, Nuapia et al. applied response surface methodology to investigate the influence of extraction time (5 min, 60 min), extraction temperature (50°C-200°C) and collection vessel temperature (25°C-200°C) on the recovery of cannabinoids compounds (THC: Indelta-9-Tetrahydrocannabinol; CBG: Cannabinol; CBD: Cannabidiol; CBG: Cannabimene and CBN: Cannabigerol) of hemp seeds (*Cannabis sativa*) [31]. In the same line, they found that extraction temperature and time have a significant negative influence on the extracted amount with a $p = 0.003$, however just extraction time has shown a significant positive influence on the amount extracted of most all target compounds. As well, they reported an increase in extraction between 45 min and decrease in values of the extracted compounds when exceeding the time [31].

As shown in Figure 1, the sample E2 got the highest value of 693.63 mg GAE/mL. In order to contrast the extract and relating it to its future application, it was compared with extracts of black tea and chai tea. Figure 2 shows the result of total phenolic compounds for all samples, where the best extract of hemp seeds got more than black tea extract. However, the GAE of E2 were lower than chai tea extract. These may be due to the fact that to chai tea, a traditional Indian beverage, is often found to have a relatively high total phenolic content compared to other types of teas, such as black or green tea [28]. There are several factors for this difference: a) Chai tea is typically made from a combination of black tea leaves and various spices like cinnamon, cloves, cardamom and ginger [29]; b) These spices are known for their high phenolic content where phenolic compounds not only add flavor and aroma but also contribute significantly to the antioxidant capacity of the tea [28,30]; c) The

addition of spices to the existing black tea has a cumulative effect and d) Combination of tea leaves and spices in chai may result in synergistic effects, where the phenolic compounds work together to provide greater antioxidant capacity than they would individually [2,28,31].

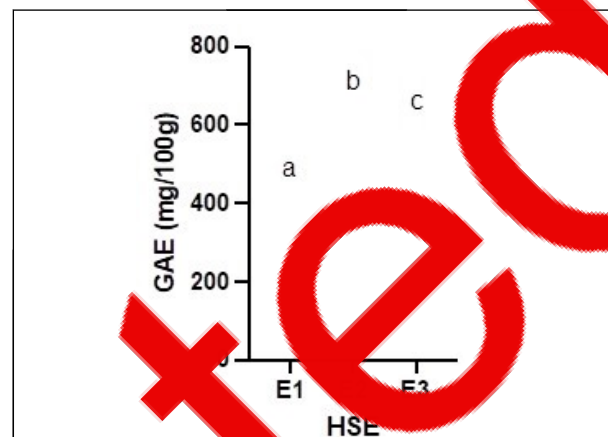


Figure 1: Total phenolic content of hempseed water-extracts. The data shown correspond to means \pm standard deviation from three independent measurements. Different letters indicate significant differences ($p < 0.05$) between samples where extractions time were 30 min (E1), 60 min (E2) or 90 min (E3) and GAE is gallic acid equivalents.

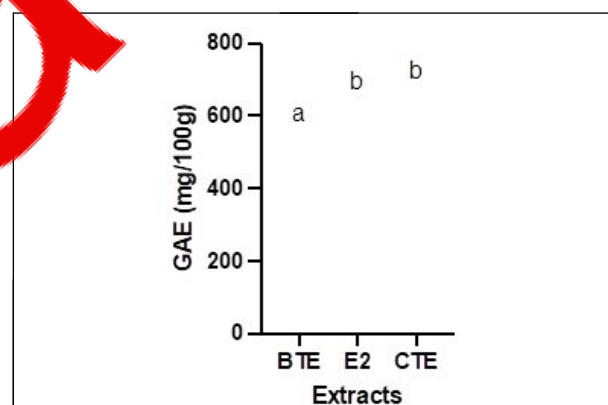


Figure 2: Comparison of total phenolic content to hempseed water-extract, black tea and chai tea extracts. The data shown correspond to means \pm standard deviations from three independent measurements. Different letters indicate significant differences ($p < 0.05$) between samples where BTE is Black Tea Extract, E2 is 60 min hemp seeds water-extraction, CTE is Chai Tea Extract and GAE is Gallic Acid Equivalents.

Total soluble solids and pH of extracts

Table 4 shows the results of pH and Total Soluble Solids (TSS) for all hemp seeds water-extractions. No sample got a significant difference. However, the values were close to a neutral pH and it is known that pH of water-extractions can influence of polyphenols from various sources, such as tea, coffee, fruits and vegetables [32]. The pH influences the structure, stability and

solubility of the source material, like plant cell walls and membranes, which can make it easier or harder for the polyphenols to be released into the water. The pH of the environment has an effect on polyphenols in that they can take up different forms (as acids or bases) depending on the acidity or alkalinity. This is because polyphenols can exist in various forms according to the surrounding pH level [3,33,34].

Table 4: Results of pH and total soluble solids of hemp seeds water-extractions.

	E1	E2	E3
pH	6.55 ± 0.32 ^a	6.64 ± 1.03 ^a	6.85 ± 0.93 ^a
TSS (°Brix)	0.50 ± 1 ^a	0.61 ± 1 ^a	0.55 ± 1 ^a

Note: The data shown correspond to means ± standard deviations from three independent measurements. ^aIndicate significant differences (p<0.05) between samples. Where TSS are Total Soluble Solids.

Conclusion

This study has shed light on the dynamic relationship between the extraction time and the total phenolic content in hempseed water-extracts. It is evident from the research that a longer extraction time generally leads to a higher total phenolic content, as more time allows for the efficient breakdown and release of these bioactive compounds from the plant material. The results showed that GA equivalents were significantly higher at 60 min extraction time which translates into a higher content of phenolic compounds with possible antioxidant activity. The evaluation of the influences of time in extraction allows to have a better selection of parameters for a thermal process considering an objective response in this case the phenolic content. To identify the most effective extraction time for each kind of plant material or compound of interest, it is essential to conduct comprehensive tests. Temperature, the amount of solvent used relative to the plant matter and agitation can also affect the process. Knowing the importance of timing extraction correctly can result in improved yields and better-quality extracts from optimization of the extraction process. The duration for which the extraction process is allowed to proceed plays a significant role in determining the efficiency, selectivity and overall outcome of the extraction.

In summary, the effect of extraction time on the total phenolic content of hemp seeds water-extracts is a critical factor that researchers and industries should consider when aiming to harness the full potential of this versatile plant. Further research and experimentation can continue to refine the extraction process, ultimately providing a range of applications that benefit from the unique phenolic profile of hemp seeds.

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