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Bulk Process for Enrichment of Capsinoids from *Capsicum* Fruit

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Abstract: Various methods to synthesize capsinoids (the nonpungent analogs of capsaicinoids) from precursor molecules have been reported. Capsinoids are also naturally present, at typically low concentrations, in the fruit of many *Capsicum* species and genotypes. However, they are also present in the fruit of select genotypes at high concentrations. The fruit of high-capsiate genotypes represents a commercial source of these compounds. To date, no method has been published that efficiently extracts and purifies capsinoids from *Capsicum* fruit in a rapid and simple bulk process. This study evaluated the efficacy of various organic solvents for the extraction of capsinoids from dried *Capsicum annuum* fruit. Among the organic solvents evaluated, pentane appeared to provide a good combination of both recovery and purity. A subsequent liquid/liquid extraction step, utilizing pentane and acetonitrile, resulted in 26.3% (*wt*/*wt*) capsiate and 19.4% (*wt*/*wt*) dihydrocapsiate for a combined capsinoids yield of 45.7% (*wt*/*wt*). A third step, involving a rapid hp20ss chromatography column using a water/acetonitrile gradient, resulted in a combined capsinoids yield of 96.6% (*wt*/*wt*).

Keywords: capsiate; dihydrocapsiate; extraction; purification; fruit; *Capsicum*

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1. Introduction

Capsinoids, the non-pungent analogs of capsaicinoids, were first identified in fruit of the sweet pepper (*Capsicum annuum*) cultivar CH-19 by Kobata et al. [1] (Figure 1). Numerous subsequent studies have reported that many (but not all) of the types and degrees of biological activity characteristic of capsaicinoids are also characteristic of capsinoids [2], albeit with fewer of the adverse side effects associated with pungency [3,4]. In addition to analgesic and antioxidant activity [2,5], capsinoids enhance adrenal catecholamine secretion, inhibit angiogenesis and UV-induced skin inflammation, promote energy metabolism, and suppress body fat accumulation [6–11]. The thermogenic and metabolic effects of capsinoids have been noted as supportive of their potential role as an adjunct weight loss aid [12–14]. Macho et al. [15] noted the powerful chemopreventitive properties of nor-dihydrocapsiate. These and other reports on the properties of capsinoids suggest a potential for their pharmaceutical development [15].

It was initially assumed that capsinoids were unique to cv. CH-19 and unique to *C. annuum*. However, additional reports on the biological activities attributed to capsinoids, and the general lack of availability of purified capsinoids and seeds of cv. CH-19 [16] prompted efforts to identify additional high-capsiate genotypes. To this end, Tanaka et al. [17] screened 35 lines of *Capsicum* (including five species) and noted that all species evaluated had one or more varieties that produced capsiate, the highest concentrations being found in fruit of certain *C. baccatum* var. *praetermissum* and *C. chinense* genotypes. Singh et al. [18] screened 49 accessions in the USDA's *Capsicum* genebank that included a variety of species (*C. frutescens*, *C. chinense*, *C. baccatum* var. *pendulum*, and *C. annuum* var. *glabriusculum*). Concentrations of capsiate in fruit of these plant materials ranged from ~10 to ~370 ug.g^{-}1 fresh weight, while concentrations of dihydrocapsiate ranged from 0 (not detected) to ~85 ug.g^{-}1 fresh weight. These studies indicated that capsinoids are present

in the fruit of a wide variety of *Capsicum* species and genotypes—albeit at typically low concentrations [18].

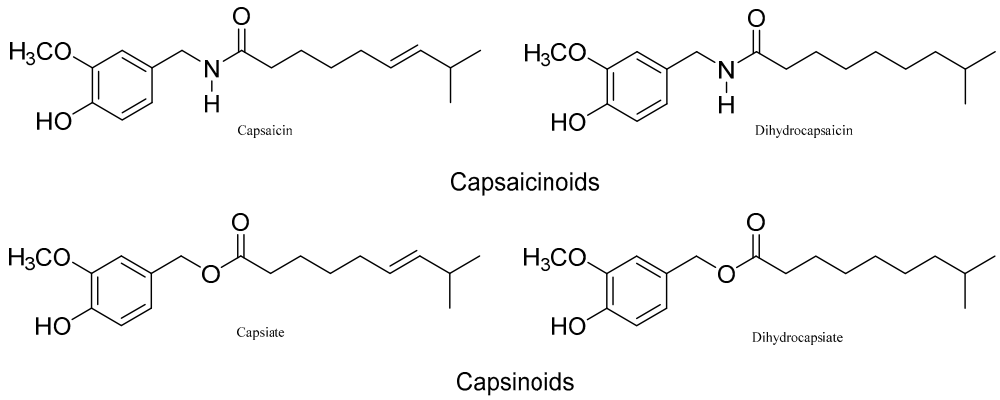

Figure 1. Chemical structures of major capsaicinoids and capsinoids.

Accessions of *C. annuum* in the USDA *Capsicum* germplasm collection were also screened for the presence of capsiate and dihydrocapsiate (Jarret—unpublished). Among these, extracts of bulked fruit of a single plant of accession PI 645509 was found to have a significantly higher concentration of capsiate among the 120 accessions examined. Fruit of PI 645509 are typically small and extremely pungent. However, individual plants of this accession also produced non-pungent fruit, and these were, upon analysis, found to contain high concentrations of capsinoids. Subsequent self-pollinations of and further selection within the progeny of these non-pungent plants resulted in the release of germplasm 509-45-1 [19]. Fruit of this germplasm can contain concentrations of capsinoids exceeding 1000 ug.g^{-1} dry weight. Concentrations of capsinoids were highest in immature fruit. The genetic mechanism accounting for the loss of pungency in fruit of 509-45-1 has yet to be determined. However, the functional loss of putative aminotransferase (pAMT) has been shown to result in the production of capsinoids in CH-19 [20], and a similar mechanism may be at work in 509-45-1.

The initial report of Kobata et al. [1] and subsequent reports [21,22] documented procedures for the synthesis of capsinoids. In an effort to improve upon the methodology described in these early reports, and to make capsinoids more readily available for research purposes, various authors have since reported alternative procedures for their synthesis. For example, Castillo et al. [16] synthesized capsiate using an enzymatic transacylation strategy, while Singh et al. [18] synthesized capsiate from the esterification of vanillyl alcohol onto (*E*)-8-methylnon-6-enoic acid. More recently, Kurosawa et al. [23] described a procedure for the large-scale synthesis of nordihydrocapsiate based on the copper-catalyzed cross-coupling of ethyl 6-bromohexanoate with isobuyl-magnesium bromide and subsequent hydrolysis.

In contrast to the procedures for synthesizing capsiate and its analogs from various precursors, procedures for the isolation and purification of capsinoids from a natural source are lacking in the peer-reviewed literature [24]. We suggest that extraction and purification of capsinoids from fruit of germplasm 509-45-1, and similar high-capsinoid genotypes, provides a practical and cost-effective alternative to the synthesis of capsinoids. However, to date, no protocol has been reported that provides for the large-scale isolation and purification of capsinoids from fruit. This report describes efforts to develop an extraction protocol and subsequent purification procedure for capsinoids derived from dried fruit of *Capsicum annuum* germplasm 509-45-1—a representative source of capsinoids.

2. Materials and Methods

2.1. Plant Materials

Seeds of germplasm 509-45-1 were obtained from the USDA genebank in Griffin, GA. These seeds were sown in peat pots in April 2016 and 2017 using standard germination and propagation mixes (Sungro Horticulture, Vancouver, Canada) and ultimately transplanted to the field in early June on the Georgia Experiment Station, Griffin, GA, USA. Plants were spaced 24" apart in rows six feet apart. Plants were subjected to periodic weed and pest control measures, fertilization and irrigation as required in order to maintain plant health and vigor. Plants of 509-45-1 are indeterminate and flower profusely until frost. Hence, plants were allowed to grow until October when entire plants were pulled from the field. All immature fruit were removed by hand, bagged and frozen at -20°C until shipped to University, MS, USA for processing.

2.2. Fruit Processing

Bags (approximately 1 kg of fruit each) were placed open in a Labconco FreeZone Legacy Freeze Dryer system. Fruits were allowed to dry for two weeks under vacuum at room temperature. Loss on drying was 81%, leaving approximately 190.0 g of dried fruit in each bag.

2.3. General Experimental

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer (Bruker, Billerica, MA, USA). NMR spectra were recorded in $CDCl_3$.

2.4. Extractions of Dried Fruit

Fruit were ground to a fine powder in a large Waring commercial stainless-steel blender. Powder was transferred to 50 mL amber glass jars with a total dried powder weight of 180.394 g. Approximately 1 g of powdered peppers was weighed into thirty 20 mL scintillation vials for extraction with 10 mL of the following solvents: hexane, ethyl acetate, chloroform, dichloromethane, methanol, pentane, heptane, iso-octane, acetone, and butanol. Extractions were repeated three times for 24 h each. Solvents were evaporated under a stream of N_2. Crude extract yields were determined by dividing the dry weight of the crude extract obtained by the total amount of powdered peppers extracted (typically 1 g) and multiplying by 100. Crude extract yields are reported in Table 1.

Table 1. Efficiency of solvents on the extraction and recovery of capsinoids from dried fruit of *Capsicum annum* (509-45-1) following triplicate extraction.

Extraction Solvent	Yield of Crude Extract (% ± SD, *wt*/*wt*) *	Purity in Crude Extract (% ± SD, *wt*/*wt*) *		Recovery (mg/1 g Peppers) *	
		Capsiate	Dihydrocapsiate	Capsiate	Dihydrocapsiate
hexane	6.717 ± 0.093 ^{b}	2.487 ± 0.456 ^{a}	1.968 ± 0.325 ^{a}	1.667 ± 0.285 ^{a,b,c}	1.32 ± 0.201 ^{b,c}
ethyl acetate	7.91 ± 0.243 ^{b}	2.603 ± 0.099 ^{a}	2.056 ± 0.033 ^{a}	2.061 ± 0.142 ^{a}	1.627 ± 0.076 ^{a,b}
chloroform	4.797 ± 1.024 ^{d}	2.241 ± 0.158 ^{a}	1.803 ± 0.162 ^{a}	1.072 ± 0.209 ^{b,c}	0.866 ± 0.196 ^{c,d}
methylene chloride	5.027 ± 0.599 ^{cd}	2.202 ± 0.461 ^{a}	1.699 ± 0.35 ^{a}	1.123 ± 0.355 ^{b,c}	0.867 ± 0.271 ^{c,d}
methanol	26.447 ± 1.135 ^{a}	0.027 ± 0.001 ^{e}	0.067 ± 0.004 ^{e}	0.07 ± 0.002 ^{f}	0.178 ± 0.016 ^{f}
pentane	7.433 ± 0.429 ^{b}	2.02 ± 0.258 ^{a}	1.607 ± 0.203 ^{a}	1.497 ± 0.148 ^{a,b,c}	1.19 ± 0.105 ^{b,c}
heptane	6.613 ± 0.227 ^{b}	2.221 ± 0.093 ^{a}	1.843 ± 0.08 ^{a}	1.47 ± 0.111 ^{a,b,c}	1.22 ± 0.094 ^{b,c}
iso-octane	6.16 ± 0.02b ^{b}	0.878 ± 0.075 ^{c}	0.953 ± 0.075 ^{c}	0.541 ± 0.045 ^{d,e}	0.587 ± 0.046 ^{d,e}
acetone	7.953 ± 0.117 ^{b}	1.191 ± 0.126 ^{b}	1.069 ± 0.162 ^{b}	0.946 ± 0.093 ^{c,d}	0.849 ± 0.12 ^{c,d}
n-butanol	7.41 ± 0.269 ^{b}	0.564 ± 0.164 ^{c,d}	0.611 ± 0.118 ^{c,d}	0.42 ± 0.137 ^{e}	0.455 ± 0.103 ^{e}

* Extracts not sharing a letter in common are significantly different based on the Tukey post hoc test.

2.5. Steam Distillation of Fresh Fruit for Essential Oil

Fresh fruit of 509-45-1 were steam distilled to obtain an essential oil. A total of 652.26 g of fruit were placed in 1.2 L of deionized water in a 3 L wide-mouth flask equipped with a Clevenger apparatus on a heating mantle at 45% power and distilled overnight. The resulting essential oil obtained was 116.8 mg. The oil was prepared at 10 mg/mL in methanol for analysis on HPLC.

2.6. Bulk Purification—Pentane Extraction and Liquid/Liquid Partitioning Using Pentane/Acetonitrile

Powdered dry fruit (1.02 kg) was transferred equally to three 4 L beakers. Plant material was covered with 1.5 L of pentane in each beaker. Extractions were repeated three times. Plant material was filtered using a Büchner funnel and a 4 L vacuum filtering flask. Pentane was removed by rotary evaporation yielding 83.33 g of pentane crude extract (8.17% yield). Only 81.05 g of this pentane crude extract was used for the liquid/liquid partitioning. Approximately 20 g of pentane extract was dissolved in 1 L of pentane (20 mg/mL) and partitioned with 1 L of acetonitrile in a 4 L separatory funnel. This liquid/liquid partitioning was repeated 4 times, and the pentane and acetonitrile partitions were dried by rotary evaporation providing 71.1 g of a pentane partition and 8.2 g of an acetonitrile partition.

2.7. HPLC Quantitative Analysis

Capsiate and dihydrocapsiate analyses were performed as follows: approximately 20 mg of each extract was weighed in a 4 mL vial and prepared at 10 mg/mL in dichloromethane, in duplicate. The extracts were filtered using a 1 mL plastic syringe and a Millex 13 mm, 0.20 µm PTFE filter. The solvent was evaporated under a stream of N_2, leaving the organic soluble material to be dissolved in methanol (100%) and analyzed by HPLC. Extracts were analyzed using an HPLC system (Agilent 1260 series consisting of a vacuum degasser, quaternary pump, ALS autosampler, a diode array detector, and an Agilent Zorbax SB-C_{18}, 4.6 mm × 250 mm, 5 µm column). The injection volume for all samples and for the capsiate standard was 20 µL. An analytical gradient method was used (1 mL/min, 50% acetonitrile: 50% deionized water to 100% acetonitrile over 30 min and held at 100% ACN for 5 min) followed by a 5-min re-equilibration. Analytes were detected at 280 nm.

Capsiate standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dihydrocapsiate standard was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Individual concentration gradients were prepared for capsiate and dihydrocapsiate to obtain a standard curve using six concentration points imposed by using response factors and regression coefficients independently. Response factors were calculated using the equation RF = PA/C, where PA is peak area and C was the analyte concentration. Confirmed integrated peaks were then used to determine the percentage of analyte in the extract. The RF of the target chemical constituent was used to determine the "percent" for each sample using the equation: PA/RF/C × 100 = % (peak area/response factor/concentration, *wt*/*wt*) in the plant tissue.

Percent purity calculations provided the percentage (*wt*/*wt*) of analyte present in the dry extract or fraction or partition. Essentially a measure of how pure the extract/fraction/partition is in the analyte on a dry weight basis. Recovery is the actual amount of analyte in milligrams obtained from the extraction experiment defined above in Section 2.4 above. This recovery is the mg of analyte from 1 g of powdered peppers. This is not normalized, but an actual recovery.2.8. HP20ss Column Chromatography

First, 1.07 g of the acetonitrile partition from the bulk liquid/liquid extraction (Section 2.6) was dissolved using 10 mL of isopropyl alcohol in a 500 mL round bottom flask. Then, 6.140 g of HP20ss resin was added to the flask with 200 mL acetone to adsorb the sample material to the resin. Solvent was removed by rotary vapor. Using a 200 mL glass fritted funnel, a column was prepared with 50 g of HP20ss resin. The column was conditioned by washing with 100 mL acetonitrile (ACN) and then 100 mL H_2O.

The dried acetonitrile partition (1.07 g) which had been adsorbed to 6.140 g of HP20ss resin was applied as a solid to the top of the column and eluted by vacuum filtration using 100 mL fractions of the following percentages: Fraction A- 100% H_2O, Fraction B- 75%:25% H_2O:ACN, Fraction C- 50%:50% H_2O:ACN, Fraction D- 25%:75% H_2O:ACN, Fraction E- 100% ACN, and Fraction F- 100% dichloromethane. Column fractions were dried by rotary evaporation and lyophilization.

2.8. Statistical Analysis

Data were subjected to an analysis of variance. The values of purity and recovery were log-transformed and expressed as proportions between 0 and 1. The marginal means for each extract were estimated and assessed differences between all pairs of extracts were determined using the Sidak adjustment for multiple comparisons. Analyses were conducted using R software, version 4.1.2, with the emmeans [25] and multcomp [26] packages.

3. Results and Discussion

An experiment consisting of a series of liquid extractions of dried powdered fruit with solvents from a range of polarities indicated which solvents provided good recovery and purity of capsiate and dihydrocapsiate in the resulting extracts. Solvents initially evaluated included hexane, ethyl acetate, chloroform, dichloromethane, methanol, pentane, heptane, iso-octane, acetone, and n-butanol.

The crude extract yield was highest in the methanol extract with a value of 26.447 $\pm$ 1.135 (% $\pm$ SD, *wt*/*wt*), far above the next highest yield of 7.953 $\pm$ 0.117 (% $\pm$ SD, *wt*/*wt*) for the acetone extract (Table 1). From the perspective of capsiate purity in the resulting dried crude extracts, the nonpolar and chlorinated solvents including hexane, ethyl acetate, chloroform, methylene chloride (DCM), pentane, and heptane provided significantly similar purities, ranging from 2.0% to 2.6% (Table 1 and Figure 2). The same solvents also provided the highest purity of dihydrocapsiate in the dried crude extracts with values ranging from 1.6% to 2.1% (Table 1 and Figure 3).

Analysis of the recovery of capsiate in the crude extracts indicated the highest amount recovered in the significantly similar hexane, ethyl acetate, pentane, and heptane extracts (Table 1 and Figure 2) and provided recoveries ranging from 1.5 to 2.0 mg from 1 g of dried fruit. Ethyl acetate provided the highest recovery of dihydrocapsiate (1.6 mg from 1 g of dried fruit), but it was not significantly different than hexane, ethyl acetate, pentane, and heptane with values ranging from 1.2 to 1.3 mg. An analysis of the essential oil produced from steam distillation of fresh fruit indicated no detectable capsiate or dihydrocapsiate.

The preferred crude extraction solvents, based on the analysis of recoveries and purity, were pentane, hexane, heptane, and/or ethyl acetate. Based on these results, we hypothesized that it should be possible to start with a nonpolar crude extract (such as pentane or hexane) and selectively extract the capsinoids from this nonpolar extract using a more polar solvent such as methanol or acetonitrile to obtain a higher purity capsinoid partition. As ethyl acetate is miscible with methanol and/or acetonitrile, this solvent was not appropriate for a liquid/liquid partitioning protocol; therefore, we evaluated the hydrocarbon-based solvents pentane, hexane, and heptane.

An analysis of solvent miscibility tables for common solvents [27] indicated dimethylformamide, dimethyl sulfoxide, acetic acid, acetonitrile, water, and methanol as possibilities for the polar solvent for liquid/liquid partitioning protocols appropriate for the nonpolar solvents pentane, hexane, and heptane. Dissolution experiments revealed that capsinoids were not soluble in acetic acid or water and hence these were omitted from further consideration. Dimethylformamide and dimethyl sulfoxide were possibilities; however, the problematic step of solvent removal of such high boiling point solvents on a large scale led us to not further pursue these as options. Liquid/liquid extractions were attempted using pentane as the nonpolar solvent and methanol as the polar solvent. However, the miscibility of the two solvents could not be overcome despite evidence that they should

not be miscible. Hence, this combination was not investigated further. Pentane, therefore, was chosen in combination with acetonitrile for liquid/liquid partitioning experiments.

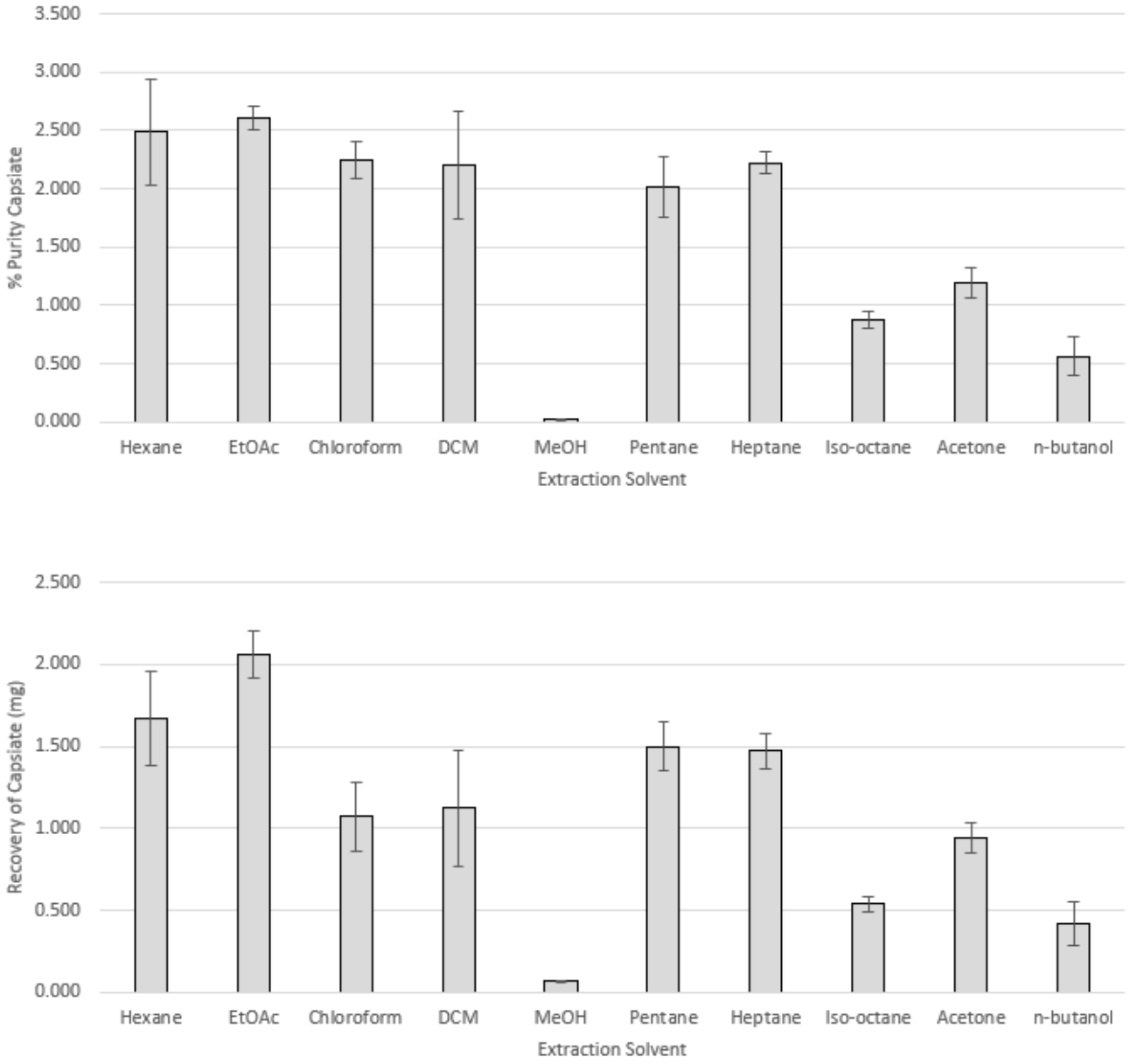

Figure 2. Extraction efficiency of solvents on the purity and recovery of capsiate from dried fruit of *Capsicum annuum* (509-45-1).

Liquid/liquid extraction was possible immediately following the crude extraction. This allowed for a quick transition to this step without the need to first dry the pentane crude extract. Additional non-polar solvents, that are not miscible with acetonitrile (such as hexane, heptane, and cyclohexane), could also be used successfully. Table 2 indicates the results from the liquid/liquid extractions using a pentane/acetonitrile combination at a concentration of 20 mg/mL in the starting pentane partition/extract. This resulted in 26.3% (*wt*/*wt*) capsiate and 19.4% (*wt*/*wt*) dihydrocapsiate for a combined capsinoids yield of 45.7% (*wt*/*wt*) (Table 2 and Figure S1). In contrast, the pentane partition contained only 0.06% (*wt*/*wt*) capsinoids. Supplemental Figures S2 and S3 provide the ^{1}H and ^{13}C NMR spectra for pure capsiate and the acetonitrile partition clearly indicating enrichment of capsiate.

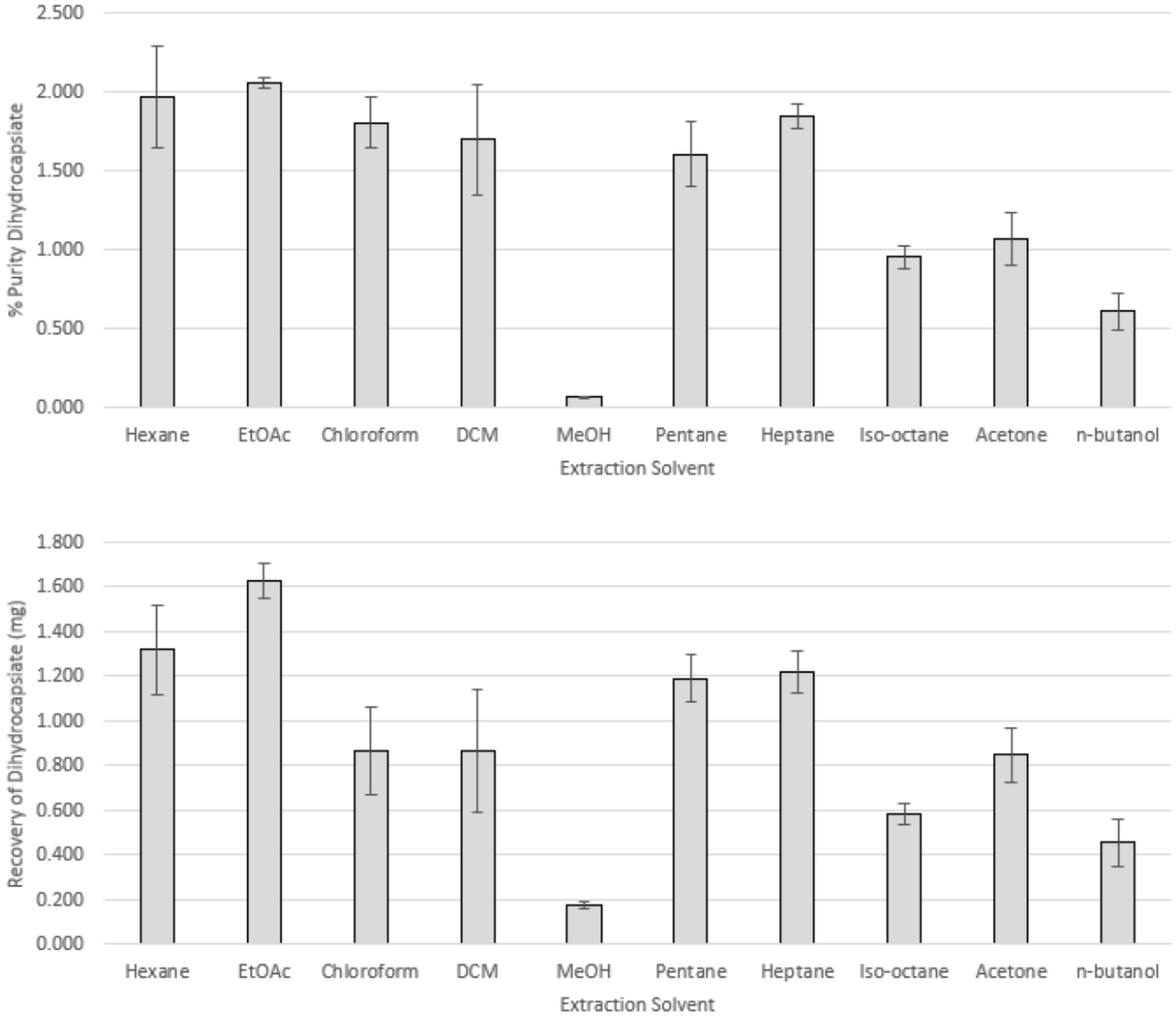

Figure 3. Extraction efficiency of solvents on the purity and recovery of dihydrocapsiate from dried fruit of *Capsicum annuum* (509-45-1).

Table 2. Capsinoid percent purity in the bulk liquid/liquid extraction experiment using pentane/acetonitrile.

Concentration	Partitions	% Purity (*wt*/*wt*) in Partition		Total % Purity of Capsinoids
		capsiate	dihydrocapsiate	
20 mg/mL	pentane	0.026 ± 0	0.03 ± 0.005	0.056
	acetonitrile	26.331 ± 2.579	19.358 ± 1.182	45.689

As a final option for bulk purification of capsinoids, column chromatography was performed using hp20ss [28] as the stationary phase and a water/acetonitrile step gradient. The starting material used for the column chromatography was the acetonitrile partition from the previous step. Solvent ratios in each step are given in Table 3 together with the composition of the capsinoids in each fraction. This stepwise elution system resulted in nearly all of the capsinoids being present in the starting acetonitrile partition eluting in the acetonitrile fraction. This resulted in 55.5% (*wt*/*wt*) capsiate and 41.2% (*wt*/*wt*) dihydrocapsiate for a combined capsinoids percent purity of 96.6% (*wt*/*wt*).

Table 3. Capsinoid percent purity in fractions generated from the bulk Diaion HP20ss column chromatography of the acetonitrile portion from the liquid/liquid extraction experiment.

Fraction	Solids Yield (mg)	Elution Solvents			% Purity (*wt*/*wt*) in Partition		Total % Purity of Capsinoids
		% water	% acetonitrile	% methylene chloride	capsiate	dihydro-capsiate	
A	0	100	0	0	n.d.	n.d.	-
B	0	75	25	0	n.d.	n.d.	-
C	0	50	50	0	n.d.	n.d.	-
D	5	25	75	0	n.d.	n.d.	-
E	494.6	0	100	0	55.464 ± 4.431	41.15 ± 3.817	96.614
F	633.5 *	0	0	100	0.106 ± 0.003	n.d.	0.106

n.d. = not detected. * Methylene chloride typically results in bleed from the stationary phase leading to increased weight in this fraction.

4. Conclusions

As additional research is completed on health-promoting properties associated with capsinoids, and as we better understand the biological activities of these compounds, it will be necessary to have adequate supplies of capsinoids such as capsiate and dihydrocapsiate [6–11]. Such studies and associated reports on the properties of capsinoids already suggest a potential for their nutraceutical and pharmaceutical development [15]. Lack of availability of purified capsinoids and seeds of cv. CH-19 prompted efforts to identify additional high-capsiate genotypes. With additional genotypes identified and the subsequent release of germplasm 509-45-1 [19], we now have a source of available biomass for producing capsinoids by agricultural means. What was lacking is a scalable and rapid procedure for producing capsinoids in high purity. We report here the successful effort to develop an extraction protocol and subsequent purification procedure for capsinoids derived from dried fruit of *C. annuum* germplasm 509-45-1—a representative source of capsinoids. In brief, the procedure begins with dried fruit of *C. annuum* germplasm 509-45-1, which is extracted with pentane to provide a pentane crude extract. This crude pentane extract can be concentrated to 20 mg/mL and partitioned with an equal volume of acetonitrile. This acetonitrile partition contains 45.6% capsinoids from these two simple steps. For added purity, this acetonitrile partition can be rapidly purified using an HP20ss resin as the stationary phase providing a 96.6% capsinoids enriched product.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr10020305/s1, Figure S1: HPLC analysis at 280 nm of the pentane and ACN portions resulting from the liquid/liquid extraction procedure, Figure S2: ^{1}H spectrum (400 MHz) of pure capsiate (1-lower) and the acetonitrile partition product enriched in capsinoids (2-upper), and Figure S3: ^{13}C NMR spectrum (100 MHz) of pure capsiate (1-lower) and the acetonitrile partition product enriched in capsinoids (2-upper).

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