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Dispersive liquid-liquid microextraction for the isolation and HPLC-DAD determination of three major capsaicinoids in *Capsicum annuum* L.

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Abstract: Dispersive liquid-liquid microextraction (DLLME) was combined with high-performance liquid chromatography-diode-array detector (HPLC-DAD) for the extraction and quantitation of three major capsaicinoids (i.e. capsaicin, dihydrocapsaicin and nordihydrocapsaicin) from pepper (*Capsicum annuum* L.). Chloroform (extraction solvent, 100 µL), acetonitrile (disperser solvent, 1250 µL) and 30 s extraction time were found optimum. The analytes were back-extracted into 300 µL of 50 mM sodium hydroxide/methanol, 45/55% (v/v), within 15 s before being injected into the instrument. Enrichment factors ranged from 3.3 to 14.7 and limits of detection from 5.0 to 15.0 µg g^{-1}. Coefficients of determination (R^2) and %RSD were higher than 0.9962 and lower than 7.5%, respectively. The proposed method was efficiently applied for the extraction and quantitation of the three capsaicinoids in six cultivars of *Capsicum annuum* L. with percentage relative recoveries in the range of 92.0%–108.0%. DLLME was also scaled up for the isolation of the three major capsaicinoids providing purity greater than 98.0% as confirmed by liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) analysis, which significantly reduced the extraction time and organic solvent consumption.

Key words: Capsaicin, *Capsicum annuum* L., dihydrocapsaicin, dispersive liquid-liquid microextraction, nordihydrocapsaicin

1. Introduction

Peppers (*Capsicum annuum* L.), originating in the Americas, are popularly known for their spicy and pungent taste, which are caused by a group of molecules known as capsaicinoids from the genus capsicum, making them a popular spice in foods around the world [1]. Although over twenty capsaicinoids have been identified in various species of pepper [2], only two of them are responsible for up to 90% of the pungency of pepper, namely, capsaicin (CAP) and dihydrocapsaicin (DHC) [3]. The other capsaicinoids include nordihydrocapsaicin (NDHC), homodihydrocapsaicin and homocapsaicin [4]. The level of pungency depends on the species and cultivars, in which the concentration of capsaicinoids is influenced by certain variables such as the type of soil, climate and other growth conditions [5]. Capsaicinoids, especially CAP, are primarily used as coloring and/or flavoring agents in the food and pharmaceutical industry. Studies revealed that CAP can suppress carcinogenesis in the breast, prostrate, colon, lungs and human bladder [6]. It is used for topical applications in analgesic therapy for some neuropathic and osteoarthritic pain states [7] and has recently, been found to alleviate rheumatoid arthritis [8]. In addition, capsaicinoids have been reported to possess antimicrobial effects against disease-causing bacterial and aquatic microorganisms that cover underwater areas of ships [9]. Despite the important pharmacological, clinical and industrial uses of CAP, high doses (above 100 mg per body weight) administered for a long time might cause peptic ulcers, increase the chances of developing liver, duodenal, stomach and prostate cancer together with the enhancement of breast cancer metastasis [10].

The concentration of CAP in pepper is one of the essential factors that define its competitive value [11] and thus the need for rapid, high throughput analysis at a reduced cost has placed a high demand for developing efficient analytical methods for food analysis [12]. The short analysis time and reliability of high-performance liquid chromatography (HPLC) made it the favored technique for separating capsaicinoids. Recently, several HPLC methods with ultraviolet (UV) [13], fluorescence [14], electrochemical [15] and mass spectrometry (MS) [16] detectors have been reported for this purpose. Despite the substantial technological achievements made in HPLC instrumentation, it is still challenging to

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achieve rapid, highly efficient and highly selective separation of food matrices without an efficient sample preparation step [17] owing to the high complexity of such samples. The drawbacks of conventional sample preparation techniques such as liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are well known and documented in the literature, some worth mentioning are the tedious procedures and large consumption of toxic organic solvents involved in LLE, which are harmful to the researcher, living organisms and the environment. SPE uses smaller volumes of organic solvents, but it is still considered significant. In addition, SPE cartridges are cost-prohibitive and generate chemical waste [18].

The emphasis has recently been diverted towards the design of reliable miniaturized sample preparation techniques. Dispersive liquid-liquid microextraction (DLLME) [19] has achieved broad acceptance and recognition in food analysis [20–22] due to its rapidness, environmental friendliness, high extraction efficiency and affordability. In DLLME, the maximum extraction efficiency is maintained almost instantly thanks to the extremely large surface area of the extraction solvent available for the analyte. Although gas chromatography (GC) was the first instrument to be used with DLLME [19], in which the extract can be directly injected due to the compatibility of the final extract with the instrument, HPLC is so far, the most frequently used one after DLLME [23]. Evaporating the solvent to dryness and reconstituting in the mobile phase or back-extraction into a compatible solvent are common sample introduction methods of the final extract into reversed-phase HPLC after DLLME [24]. Back-extraction of ionizable compounds is a faster choice that can also provide additional selectivity, improved sample clean up and better regulation of the ionic strength in the final extract.

The objective of this research was to design a fast, robust and efficient extraction method using DLLME followed by back-extraction for preconcentration and determination of the three major capsaicinoids (i.e. NDHC, CAP and DHC) from different cultivars of *Capsicum annuum* L. using HPLC-diode-array detector (DAD). To date, this is the first study on DLLME of capsaicinoids from pepper samples. Influential experimental parameters on the extraction and back-extraction efficiencies were optimized. This was also the first attempt to scale up DLLME for the isolation of the three major capsaicinoids from *Capsicum annuum* L.

2. Experimental

2.1. Chemicals and reagents

Analytical grade standards of CAP (logP = 3.75), DHC (logP = 4.11) and NDHC (logP = 3.67) were isolated on a preparative scale with purity above 98% from *Capsicum annuum* L. in our pharmacognosy laboratory, as confirmed by LC-MS and NMR analysis (supplementary material). The chemical structures of the three analytes are shown in Figure 1. HPLC-grade methanol,

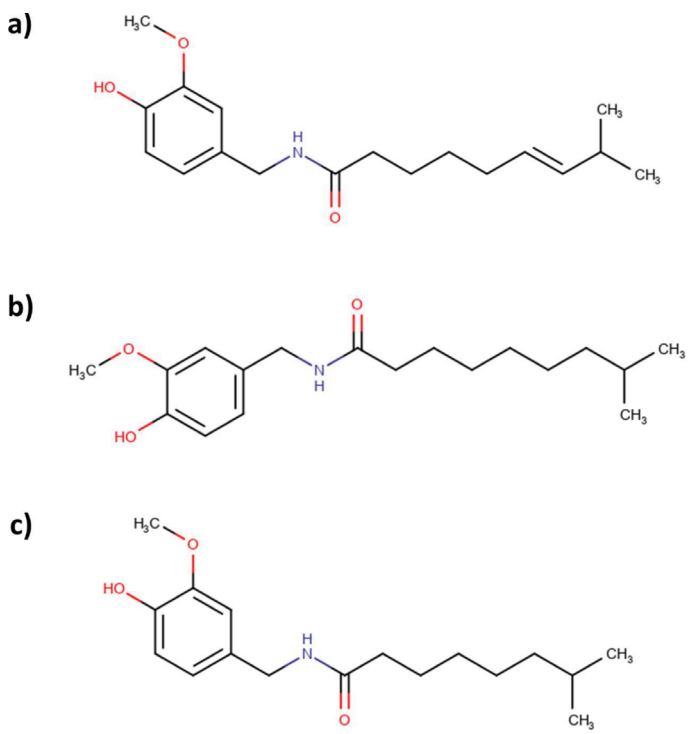

Figure 1. Chemical structures of the analytes: (a) capsaicin, (b) dihydrocapsaicin, and (c) nordihydrocapsaicin.

acetonitrile, tetrahydrofuran, chloroform, dichloromethane, sodium chloride, sodium hydroxide (NaOH) and ethyl acetate were acquired from Sigma-Aldrich (Darmstadt, Germany). Acetic acid, n-hexane and toluene were obtained from Honeywell Riedel-de Haën (Seelze, Germany). Silica gel was obtained from Merck (Søborg, Denmark). All reagents used were of analytical-grade. Deionized (DI) water (18.2 MΩ.cm), treated with Purelab Ultra Analytic (ELGA LabWater, Leicestershire, UK), was used for the preparation of aqueous solutions. MarvinSketch (Version 20.11.0, ChemAxon, Boston, MA, USA) was used for logP and pK_a calculations.

2.2. Standard solutions of capsaicinoids

Individual standards of the three capsaicinoids were prepared at 1000 µg mL^{-1} in methanol and stored at –4 °C. Mixed standard solutions were freshly prepared by diluting the stock solution with 50 mM sodium hydroxide/ methanol (45/55%, v/v), hereafter, referred to as the back-extraction solution (BES). Mobile phases and aqueous solutions were filtered using a vacuum solvent filtration system through 0.20 µm regenerated cellulose-membrane filters (Isolab, Eschau, Germany), whereas, samples were filtered through 0.22 µm nylon syringe filters (Microlab Scientific, Shanghai, China).

2.3. Instrumentation

An HPLC system (1200 series, Agilent Technologies, Santa Clara, CA, USA) with an online degasser, a quaternary pump, an autosampler, a column oven and DAD set at a wavelength of 280 nm was used for separations. Maximum absorption wavelength was selected using the 'Isoabsorbance' and '3D' plot functions available in the Agilent ChemStation for LC 3D systems (Rev. B.03.01) software. A reversed-phase column (i.e. Agilent Zorbax SB-Aq 150 mm × 4.6 mm × 5 µm) was used to separate the analytes using an optimum isocratic mobile phase composition of 0.5% acetic acid/ methanol (55/45%, v/v) at a flow rate of 1.2 mL min^{-1} and 22 °C. A medium-pressure liquid chromatograph (MPLC) (BÜCHI Labortechnik AG, Flawil, Switzerland), equipped with a binary pump, a fraction collector and a reversed-phase column (i.e. LiChroprep RP-18 460 mm × 26 mm × 25–40 µm) (Millipore, Dachstein, France), was used for isolation of the analytes. LC-MS with electrospray ionization (ESI) was used for mass analysis (Waters Alliance HPLC and ZQ micromass, Waters Corp., Milford, MA, USA). For structural elucidation, spectral analysis was performed using a Varian Mercury Fourier-transform NMR (FT-NMR) spectroscopy (^{1}H 400 MHz, ^{13}C 100 MHz) (Agilent Technologies). Deuterated dimethyl sulfoxide (DMSO-d_6) and chloroform ($CDCl_3$) (Sigma-Aldrich, Darmstadt, Germany) were used as the solvents. Tetramethylsilane (TMS, Sigma-Aldrich, Darmstadt,Germany) was used as the internal standard.

2.4. Sample preparation

Fresh cultivars of *Capsicum annuum* L. and green pepper pickles were collected from local markets in Nicosia, TRNC. The samples were washed with plenty of DI water, left to dry at room temperature, and cut into small pieces with a stainless steel knife. Then, they were dried overnight in the oven at 60 °C. Dry samples were powdered using a blender with stainless steel blades and stored in well-sealed glass bottles in a dark place till analysis. An initial solid-liquid extraction (SLE) was carried out by weighing 1.00 ± 0.01 g of the sample and extracting it with 50.0 mL of 50/50 (%, v/v) acetonitrile/ water through ultrasonication for 30 min at 25 °C. The mixture was filtered through a cotton wool and a 0.45 µL syringe filter and the filtrate was transferred into a 50 mL volumetric flask and made up to the mark with 50/50 (%, v/v) acetonitrile/water (referred to hereafter as the sample solution). A portion of 5.0 mL of the sample solution was mixed with 2.0 mL of saturated sodium chloride solution. The mixture was vortexed for 30 s and centrifuged for 3 min at 6000 rpm to induce salting-out extraction (SOE). Out of the 1.2 mL of acetonitrile that salted out, 1.0 mL was used in DLLME.

2.5. Dispersive liquid-liquid microextraction

One milliliter of acetonitrile, resulting from SOE, was completed to 10.0 mL with DI water into a 15 mL centrifuge tube after adding an extra volume of 250 µL of acetonitrile, which acted as the disperser solvent in DLLME. Upon the addition of chloroform and acetic acid (100 µL, each), the mixture was vortex mixed for 30 s and centrifuged (3 min at 6000 rpm). Chloroform, which settled to the bottom, was transferred completely into a snaplock microtube and the analytes were back-extracted into 300 µL of BES through vortexing for 15 s and centrifugation (3 min, 6000 rpm). A portion of the aqueous extract (i.e. 5 µL) from the upper layer was injected into HPLC-DAD for analysis.

3. Results and discussion

3.1. Optimization of extraction conditions

3.1.1. Type and volume of the extraction solvent

Selecting a suitable extraction solvent for DLLME is based on its ability to extract the analyte(s) from the sample solution and its immiscibility with the aqueous phase [25]. In conventional DLLME, extraction solvents denser than water, such as chloroform, dichloromethane and tetrachloromethane, are commonly used [19]. However, the use of less dense solvents has also been proposed, which include toluene, n-hexane, 1-undecanol and 2-dodecanol [26]. Chloroform, dichloromethane, toluene and n-hexane were tested as the extraction solvents considering extractability and solvent recovery. Although dichloromethane gave the highest extraction efficiency (Figure 2a), the recovered volume of chloroform (Figure 2b) was much higher (i.e.

170 ± 5 µL), making it an optimum extraction solvent to proceed with. Volume of the selected solvent can significantly influence the extraction efficiency [21]. The effect of chloroform volume was monitored starting from 100 to 300 µL within 50 µL intervals. Peak areas rapidly decreased as the volume of chloroform was increased due to dilution (Figure 2c). An optimum volume would ensure the highest enrichment factor (EF) and high solvent recovery after extraction [27]. When lower volumes of chloroform below 100 µL were tested, phase separation was not observed. Hence, 100 µL of chloroform was taken as optimum for subsequent experiments.

3.1.2. Type and volume of the disperser solvent

Due to their high miscibility with the extraction solvent, acetonitrile, methanol, acetone and ethanol are common disperser solvents in DLLME [23]. Since SOE was used prior to DLLME, it was decided to use acetonitrile as the disperser solvent

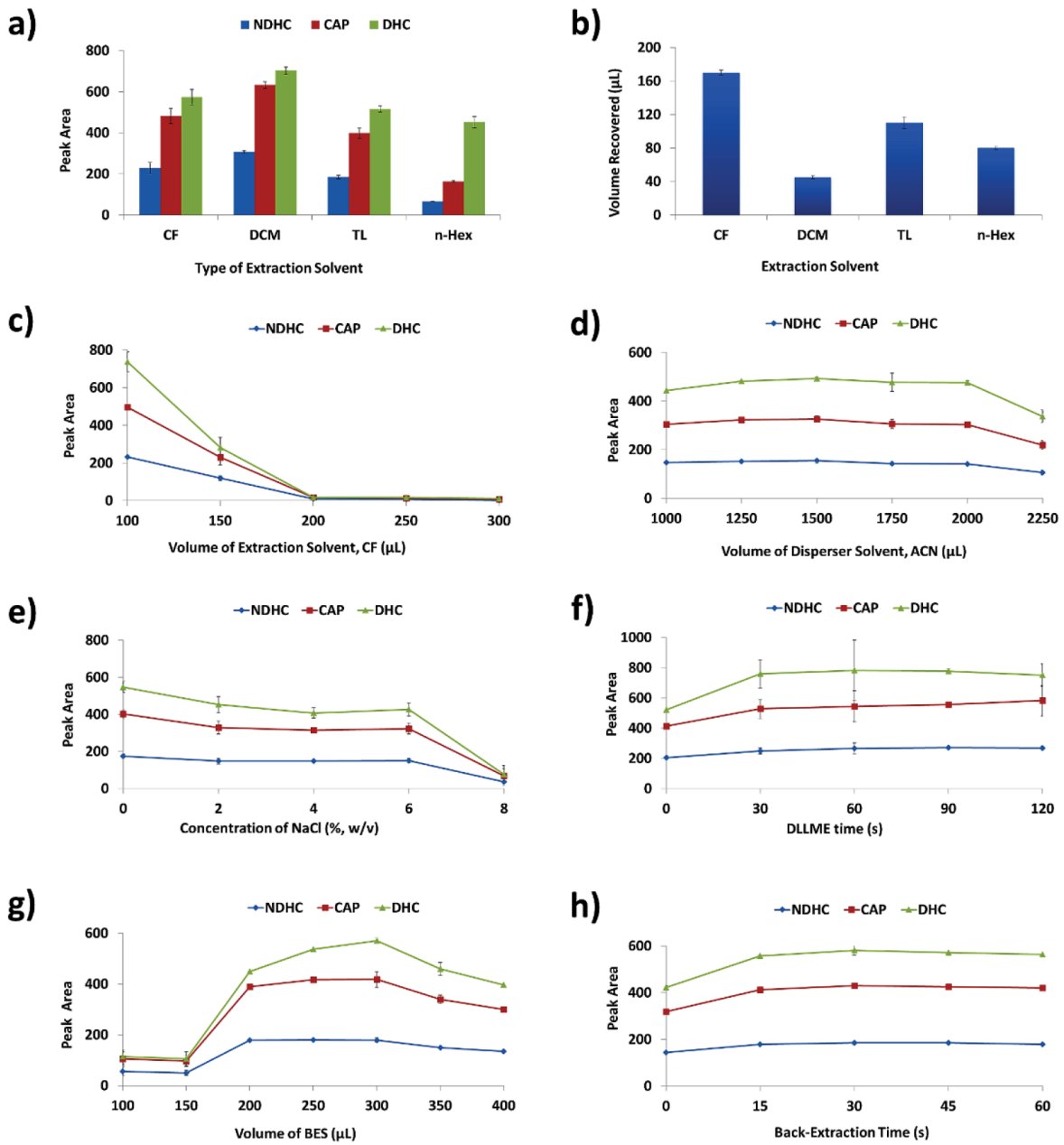

Figure 2. Optimization of DLLME parameters: (a) type of extraction solvent, (b) recovered volume of extraction solvent, (c) volume of extraction solvent, (d) volume of disperser solvent, (e) concentration of sodium chloride, (f) DLLME time, (g) volume of back-extraction solvent (BES), and (h) back-extraction time. CF: chloroform; DCM: dichloromethane; n-Hex: n-hexane; TL: toluene.

Table 1. Figures of merit of conventional HPLC-DAD and DLLME-HPLC-DAD for pepper samples.

Method	Sample[a]	Analyte	Regression equation[b]	R^{2c}	%RSD[d]		LOD[e]	LOQ[f]	LDR[g]	EF[h]
					Intraday	Interday				
Conventional HPLC-DAD	Aqueous standards	NDHC	y=3.5(±0.1)x+1.3(±4.7)	0.9957	2.7	3.5	5.4	18.0	18.0–150	-
		CAP	y=2.1(±0.1)x-4.6(±1.5)	0.9991	1.5	2.6	2.7	9.0	9.0–150	-
		DHC	y=2.6(±0.1)x-1.5(±1.5)	0.9987	2.3	3.4	3.4	11.3	11.3–150	-
DLLME-HPLC-DAD	SGC	NDHC	y=16.9(±0.2)x-1.4(±1.1)	0.9967	5.4	7.1	0.3	1.0	1.0–15.0	4.8
		CAP	y=19.2(±0.2)x-1.6(±1.1)	0.9974	4.7	6.2	0.3	1.0	1.0–15.0	9.1
		DHC	y=18.1(±0.2)x-1.4(±1.3)	0.9962	5.8	7.5	0.3	1.0	1.0–15.0	7.0
	GPP	NDHC	y=23.2(±0.2)x-0.8(±1.1)	0.9982	3.9	4.8	0.2	0.7	0.7–15.0	6.6
		CAP	y=29.2(±0.3)x+0.4(±1.4)	0.9982	2.8	3.2	0.2	0.7	0.7–15.0	13.9
		DHC	y=38.1(±0.5)x-4.5(±2.4)	0.9973	4.9	6.3	0.3	1.0	1.0–15.0	14.7
	LGP	NDHC	y=14.0(±0.1)x-0.4(±0.3)	0.9996	1.9	2.5	0.1	0.3	0.3–15.0	4.0
		CAP	y=16.0(±0.1)x-1.0(±0.7)	0.9987	3.3	4.3	0.2	0.7	0.7–15.0	7.6
		DHC	y=22.0(±0.2)x+1.5(±1.1)	0.9979	4.1	5.4	0.2	0.7	0.7–15.0	8.5
	YP	NDHC	y=40.5(±0.3)x-1.8(±1.4)	0.9990	2.8	3.7	0.2	0.7	0.7–15.0	11.6
		CAP	y=25.1(±0.3)x-1.9(±1.8)	0.9981	4.0	5.4	0.2	0.7	0.7–15.0	12.0
		DHC	y=15.2(±0.1)x+0.9(±0.7)	0.9984	2.1	2.7	0.2	0.7	0.7–15.0	5.8
	LRP	NDHC	y=16.8(±0.2)x-2.1(±1.1)	0.9969	5.6	7.0	0.3	1.0	1.0–15.0	4.8
		CAP	y=18.7(±0.2)x-1.2(±0.8)	0.9986	2.9	3.3	0.2	0.7	0.7–15.0	8.9
		DHC	y=26.1(±0.4)x-3.5(±2.0)	0.9963	6.1	7.4	0.3	1.0	1.0–15.0	10.0
	BRP	NDHC	y=11.7(±0.1)x+0.7(±0.5)	0.9989	3.0	4.0	0.2	0.7	0.7–15.0	3.3
		CAP	y=12.8(±0.1)+0.1(±0.1)	0.9999	0.3	0.4	0.1	0.3	0.3–15.0	6.1
		DHC	y=17.6(±0.1)x-1.2(±0.8)	0.9986	3.3	4.5	0.2	0.7	0.7–15.0	6.8

a. SGC: small green chili; GPP: green pepper pickle; LGP: light green pepper; YP: yellow pepper; LRP: long red pepper; BRP: big red pepper.
b. Peak area = slope (±SD)× [analyte concentration (μg mL^{-1})]+ intercept (± SD).
c. Coefficient of determination.
d. Percentage relative standard deviation, n = 3.
e. Limit of detection (μg mL^{-1}).
f. Limit of quantitation (μg mL^{-1}).
g. Linear dynamic range (μg mL^{-1}).
h. Enrichment factor: ratio of calibration slope with DLLME-HPLC-DAD to that with external aqueous calibration (conventional HPLC-DAD).

being the most suitable solvent for SOE [28]. Volume of the disperser solvent has a significant impact on the formation of a cloudy solution, which in turn determines the extraction efficiency [23]. The effect of this parameter was investigated by using 1000 μL of the salted-out acetonitrile and adding increasing volumes of pure acetonitrile. The optimum volume of acetonitrile was found as 1250 μL (Figure 2d), beyond which peak areas decreased due to increased miscibility and solubility of chloroform and the analytes in the aqueous phase, respectively.

3.1.3. Ionic strength and extraction time

In LLE, the addition of inorganic salts that increase the ionic strength has been reported to reduce the solubility of CAP in aqueous solutions [29]. However, in DLLME, such salts can also act as demulsifiers, which can eventually lead to faster phase separation, yet with reduced extraction efficiency [30]. The effect of ionic strength was evaluated by adding increasing concentrations of sodium chloride to the sample solution. It was observed that its addition caused a negative effect on the extraction efficiency (Figure 2e). Therefore, no salt was added to the sample solution in subsequent experiments. Extraction, or vortex, time corresponded to the period between the injection of the mixture of disperser/extraction solvent into the sample solution to the moment the mixture was centrifuged [19]. The effect of vortex time was monitored from 0 to 120 s. It was noticed that equilibrium was achieved within 30 s such that this value was considered optimum (Figure 2f).

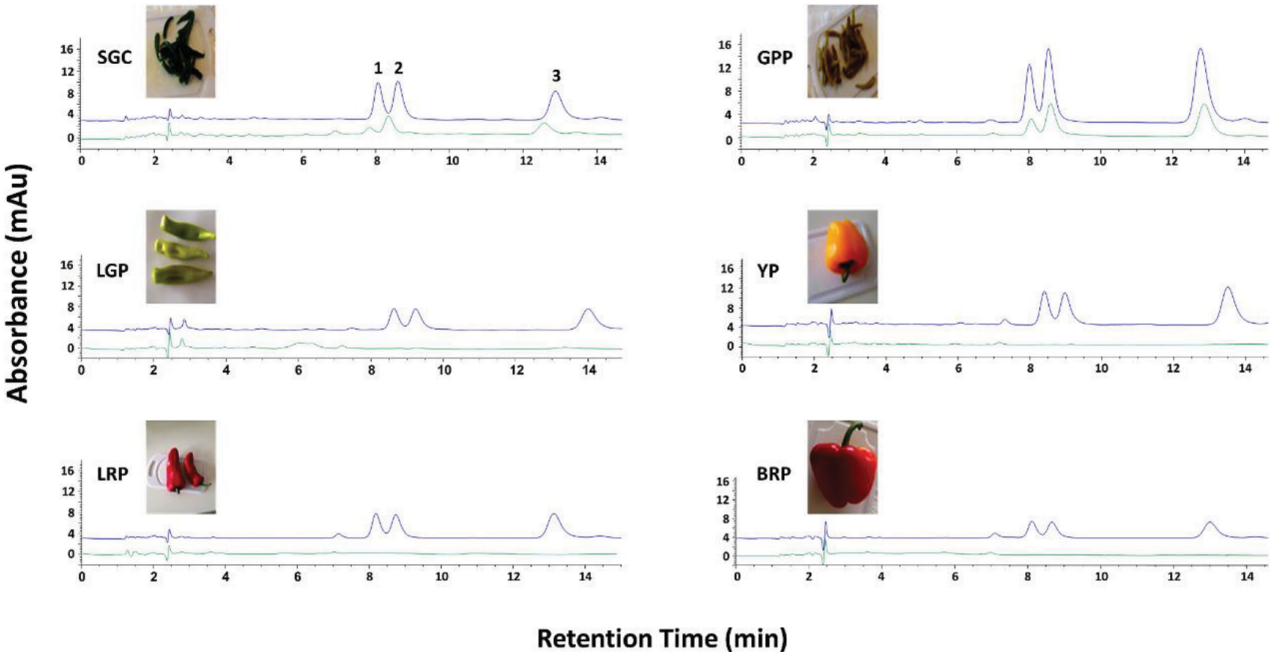

Figure 3. Representative chromatograms of pepper samples extracted and analyzed under optimum DLLME-HPLC-DAD conditions. Top chromatogram: sample spiked at 5.0 µg mL^{-1} of each analyte; bottom chromatogram: unspiked sample. Peaks: 1, NDHC; 2, CAP; and 3, DHC. SGC: small green chili; GPP: green pepper pickle; LGP: light green pepper; YP: yellow pepper; LRP: long red pepper; BRP: big red pepper.

3.2. Optimization of back-extraction conditions

Chloroform, obtained from DLLME was incompatible with reversed-phase HPLC. Hence, back-extraction of the analytes into an aqueous solution facilitated injection of the BES into the instrument. Impact of the BES volume on extraction efficiency was assessed starting from 100 to 400 µL. Extraction performance improved with an increase of up to 300 µL BES volume (Figure 2g), beyond which it started to decrease due to dilution of the analytes in excess volume of the BES. Thus, 300 µL was considered as an optimum BES volume. The effect of back-extraction time was evaluated within the range of 0 s to 60 s. Equilibrium was achieved within 15 s only due to microemulsion formation (Figure 2h). Hence, the optimum back-extraction time was taken as 15 s.

3.3. Analytical performance

Both external aqueous and standard-addition calibration graphs were plotted as a function of peak area versus concentration of the three capsaicinoids under optimized conditions to test the analytical performance. External aqueous calibrations were constructed by dissolving appropriate amounts of the analytes in the BES at the concentration range of 25–150 µg mL^{-1}, whereas, standard-addition calibrations were obtained by spiking the pepper samples, prior to SLE, with the standards at concentrations within the range of 2.5–15 µg mL^{-1} and applying DLLME followed by the back-extraction step. A good linearity was achieved as reflected by coefficients of determination (R^2) in the range of 0.9962 to 0.9999 (Table 1). Intra- and interday precision, expressed as percentage relative standard deviation (%RSD), was found to be less than 6.1 and 7.5%, respectively, indicating good repeatability. Limits of detection (LOD), ranged between 0.1 and 0.3 µg mL^{-1} (or 5.0 and 15.0 µg g^{-1}). Limits of quantitation (LOQ) ranged from 0.3 to 1.0 µg mL^{-1} (or 15.0 and 50.0 µg g^{-1}). Linear dynamic range (LDR) extended from the LOQ to 15.0 µg mL^{-1} (0.75 mg g^{-1}). The method's EF is a measure of how sensitive this method is. The higher the EF, the more sensitive it is. EFs ranged from 3.3 to 14.7, which are significant considering the high complexity of the food samples analyzed. EFs ranged between 3.3 and 14.7 times. The proposed method's figures merit of are summarized in Table 1.

3.4. Recovery studies and matrix effect

Applicability, recovery and matrix effect were evaluated by analyzing six pepper samples for their capsaicinoid content. Typical chromatograms, shown in Figure 3, show the absence of extraneous peaks from the samples at the retention times of the three capsaicinoids, confirming high selectivity of the method. Percentage relative recoveries (%RR) obtained fro

Table 2. Percentage relative recoveries of capsaicinoids from capsicum samples.

Pepper sample	Added (μg mL^{-1})	Found (μg mL^{-1})			%RR[a]		
		NDHC	CAP	DHC	NDHC	CAP	DHC
SGC	-	< LOQ	1.1 (±0.1)[b] (55 μg g^{-1})	< LOQ	-	-	-
	2.5	2.3 (±0.2)	2.6 (±0.1)	2.5 (±0.1)	92	104	100
	5.0	5.3 (±0.3)	5.3 (±0.3)	5.2 (±0.3)	106	106	104
GPP	-	3.2 (±0.2) (160 μg g-^{1})	4.8 (±0.1) (240 μg g^{-1})	4.4 (±0.2) (220 μg g^{-1})	-	-	-
	2.5	2.6 (±0.1)	2.6 (±0.1)	2.7 (±0.2)	104	104	108
	5.0	5.3 (±0.3)	5.2 (±0.2)	5.3 (±0.3)	106	104	106
LGP	-	< LOD	< LOD	< LOD	-	-	-
	2.5	2.4 (±0.1)	2.3 (±0.2)	2.7 (±0.2)	96	92	108
	5.0	5.1 (±0.1)	5.1 (±0.1)	4.9 (±0.1)	102	102	98
YP	-	< LOD	< LOD	< LOD	-	-	-
	2.5	2.3 (±0.2)	2.6 (±0.1)	2.4 (±0.1)	92	104	96
	5.0	5.1 (±0.1)	5.2 (±0.2)	4.9 (±0.1)	102	104	98
LRP	-	< LOD	< LOD	< LOD	-	-	-
	2.5	2.5 (±0.1)	2.4 (±0.1)	2.4 (±0.2)	100	96	96
	5.0	4.8 (±0.2)	4.8 (±0.3)	4.7 (±0.3)	96	96	94
BRP	-	< LOD	< LOD	< LOD	-	-	-
	2.5	2.6 (±0.1)	2.5 (±0.1)	2.4 (±0.1)	104	100	96
	5.0	4.8 (±0.2)	5.1 (±0.1)	5.0 (±0.1)	96	102	100

a. Percentage relative recovery, a value obtained considering extraction yields from standard-addition calibrations.
b. SD, n -= 3

Table 3. Comparison of DLLME-HPLC-DAD with other methods for the extraction and determination of capsaicinoids in capsicum samples.

Analyte[a]	Extraction method/ technique[b]	Extraction time (min)	Vorg.c (mL)	LOD[d] (μg mL^{-1})	LOQ[e] (μg mL^{-1})	R^{2f}	%RSD[g]	Ref.
CAP, β- carotene	SEP-HPLC	120	58	-	-	>0.9987	-	(34)
NDHC, CAP, DHC, HDHC	SFE-US-HPLC	60	10	4.10–9.76	13.67–32.55	>0.9997	-	(32)
NDHC, CAP, DHC, PAVA	PHWE-LC-APCI-MS	30	0	-	5 ng mL-1	>0.9998	-	(31)
NDHC, CAP, DHC, IDHC, HDHC	PLE-LC-MS	12	50	0.036–0.112	0.121–0.375	>0.9998	<7	(33)
NDHC, CAP, DHC	DLLME-HPLC-DAD	0.75	1.9	0.1–0.3	0.3–1.0	>0.9962	<7.5	This study

a. PAVA: nonivamide; IDHC: isodihydrocapsaicin; HDHC: homodihydrocapsaicin.
b. SEP-HPLC: stage extraction process-high performance liquid chromatography; SFE-US-HPLC: supercritical fluid extraction-ultrasound-high performance liquid chromatography; PHWE-LC-APCI-MS: pressurized hot-water extraction-liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry; PLE-LC-MS: pressurized liquid extraction-liquid chromatography-mass spectrometry.
c. Total volume of organic extraction solvent used.
d. Limit of detection (μg mL^{-1}).
e. Limit of quantitation (μg mL^{-1}).
f. Coefficient of determination.
g. Percentage relative standard deviation, n = 3.

spiked samples at two concentration levels (i.e. 2.5 and 5.0 μg mL^{-1}) were calculated to be in the range of 92.0–108.0 (Table 2). Possible matrix effect was assessed from the slopes of the calibration graphs for the three analytes and the samples (Table 1) and the p-value calculated using a single-factor analysis of variance (ANOVA). The difference was statistically significant ($P < 0.05$), suggesting the presence of matrix effect due to the wide variety of the studied samples. The standard-addition method was therefore appropriate to suppress this effect.

3.5. Comparison with other methods

DLLME-HPLC-DAD was contrasted with other extraction methods used to analyze capsicum samples in terms of parameters like extraction time, consumption of organic solvent used per sample, LOD, LOQ, R^2 and %RSD. The shortest extraction time (i.e. 0.75 min) and the least volume of organic solvent (i.e. 1.9 mL) are the key benefits of this approach over the others (Table 3), with the exception of the pressurized hot-water extraction method (PHWE) suggested by Bajer et al. [31], where water was used as the extraction solvent. Nevertheless, PHWE required special instrumentation, incurring extra capital cost. Supercritical fluid extraction (SFE) proposed by Santos et al. [32] used carbon dioxide as a 'green' extraction solvent, however, the final extract had to be conditioned with 10 mL of methanol before injection into HPLC. The lower LODs and LOQs reported by Bajer et al. [31] and Barbero et al. [33] compared to this study were largely due to the higher sensitivity of the MS detector used in their method. MS detectors are more sensitive than UV, but they are more complicated and expensive.

4. Conclusion

DLLME, combined with a back-extraction step, was proposed prior to HPLC-DAD for the extraction of the three major capsaicinoids from different cultivars of pepper (*Capsicum annuum* L.) samples. Several advantages of this method as compared to other existing extraction methods include cost-effectiveness, rapidness, high extraction efficiency, good selectivity and sample clean up as well as minimum consumption of organic solvents. Despite the complexity of the studied matrix, high recoveries, good linearity, reproducibility and interference-free chromatograms were attained. These advantages of DLLME-HPLC-DAD encourages its use for the quantitation of capsaicinoids in pepper samples in routine food analysis and quality control laboratories. The ability to scale-up DLLME for the isolation of capsaicinoids with high purity suggests that this method be applied for the isolation of various intermediate- to low-polarity compounds from natural products, which reduces the extraction time and organic solvent consumption, satisfying the criteria for green chemistry.

Acknowledgments

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Supplementary material

1. MS and NMR spectra analysis of CAP

The positive ion ES-MS spectrum of CAP (CA-114) exhibited a quasi-molecular ion peak at $m/z = 306.5$ $[M+H]^+$ and 328.5 $[M+Na]^+$, indicating a molecular formula of $C_{18}H_{27}NO_3$ (calculated molecular weight $= 305.4$ g mol^{-1}) as shown in Figure S1.

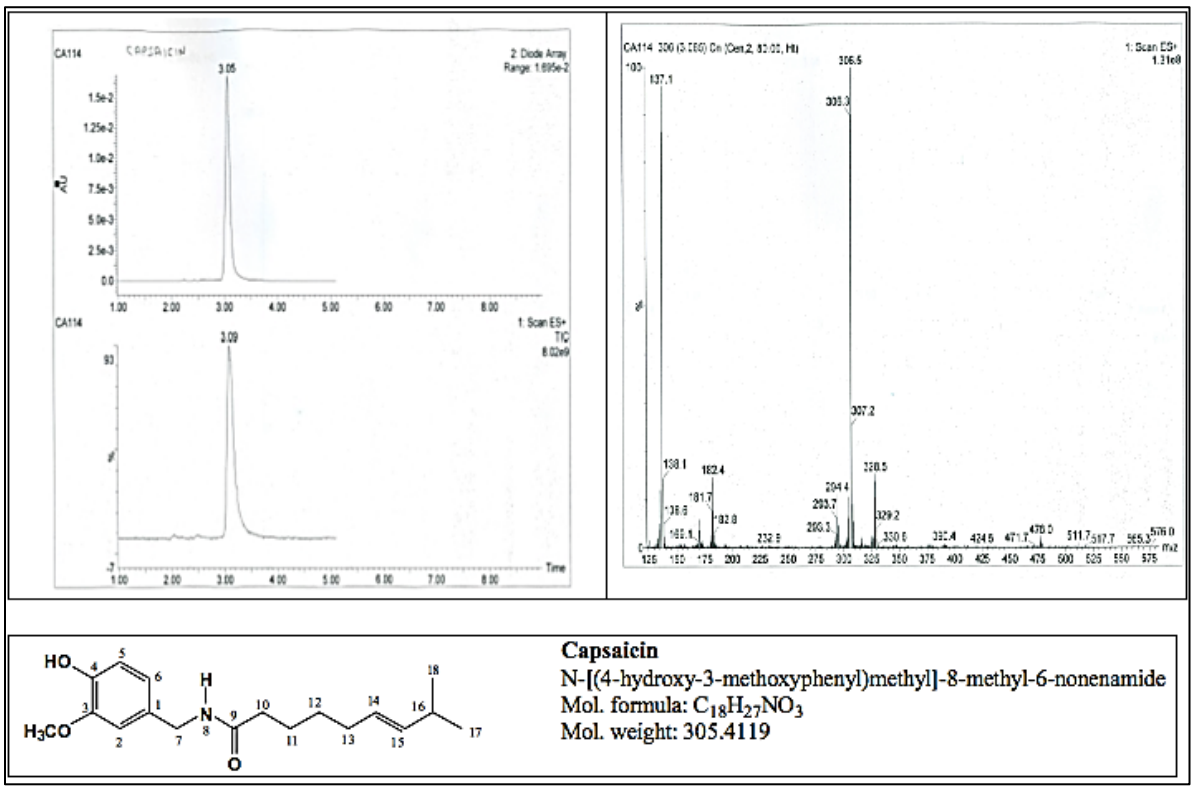


Figure S1. MS spectra of CAP.

The ^{1}H-NMR spectrum of CAP showed the signals between 0.80 ppm and 7.0 ppm (Figure S2). In the low field of the spectrum (aromatic region), three protons were observed as an ABX system arising from the 1,3,4-trisubstituted phenyl unit of vanillylamine. These signals observed at δ 6.75 (dd, J 8.1 and 1.6 Hz), 6.80 (d, J 1.6 Hz) and δ 6.85 (d, J 8.1 Hz) were assigned as H-6, H-2 and H-5, respectively, of vanillylamine moiety of the structure together with an aromatic methoxy

signal at δ 3.85. Additional signals in this region were two olefinic protons at δ 5.30 (ddt, J = 15.4, 5.7 and 5.7 Hz) and δ 5.36 (dd, J = 15.4, 5.7 Hz) assigned to trans located H-14 and H-15 of the acyl unit. The signal observed as a broad singlet at δ 5.73 was assigned to the NH of amide functionality. The signal at 4.35 ppm (d, J = 5.6 Hz) with two proton intensity were assigned to the methylene protons of the vanilliylamine. The rest of the signals were observed at the high field of the spectrum arising from the aliphatic protons of the acyl unit.

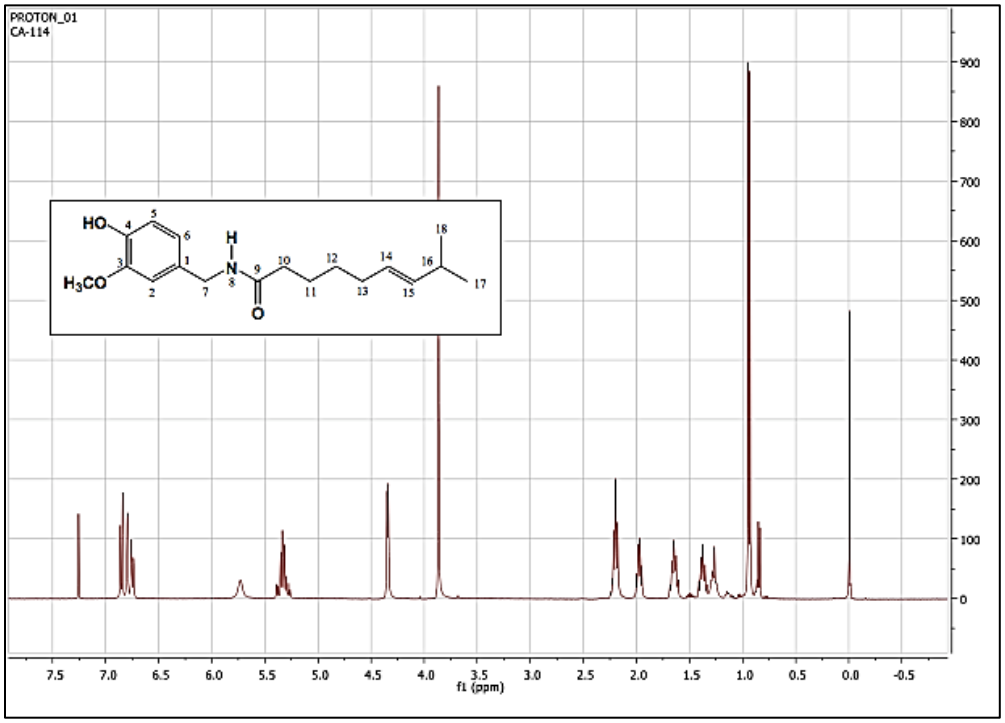


Figure S2. ^{1}H-NMR spectrum of CAP (400 MHz, $CDCl_3$).

Magnified versions of the ^{1}H-NMR spectra of CAP is given in Figure S3 and S4 at different δ range for clarity.

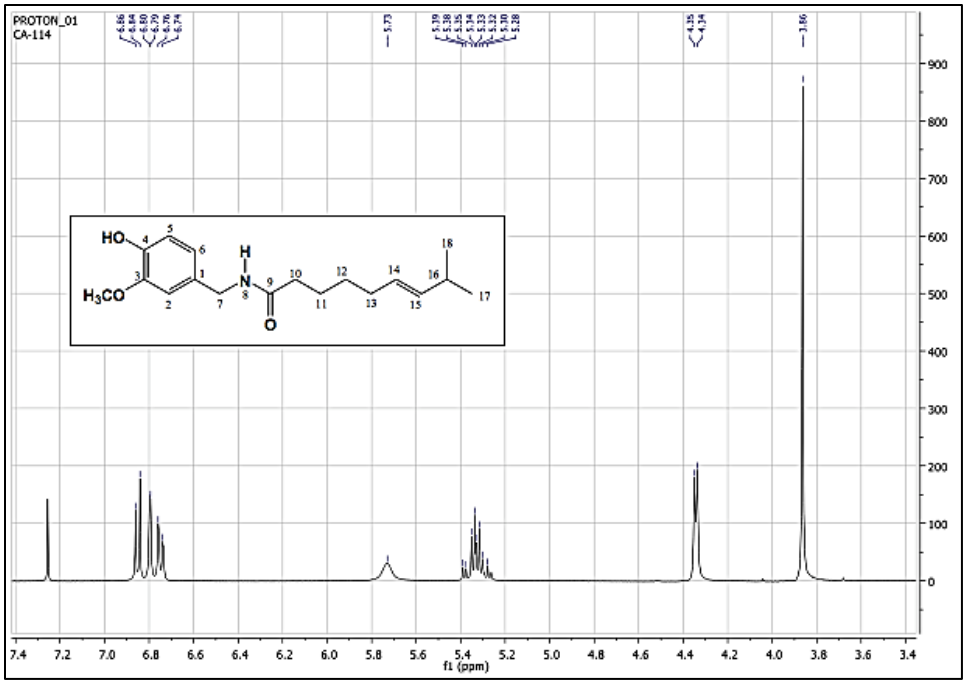


Figure S3. A magnified ^{1}H-NMR spectrum of CAP from 7.4 to 3.4 ppm.

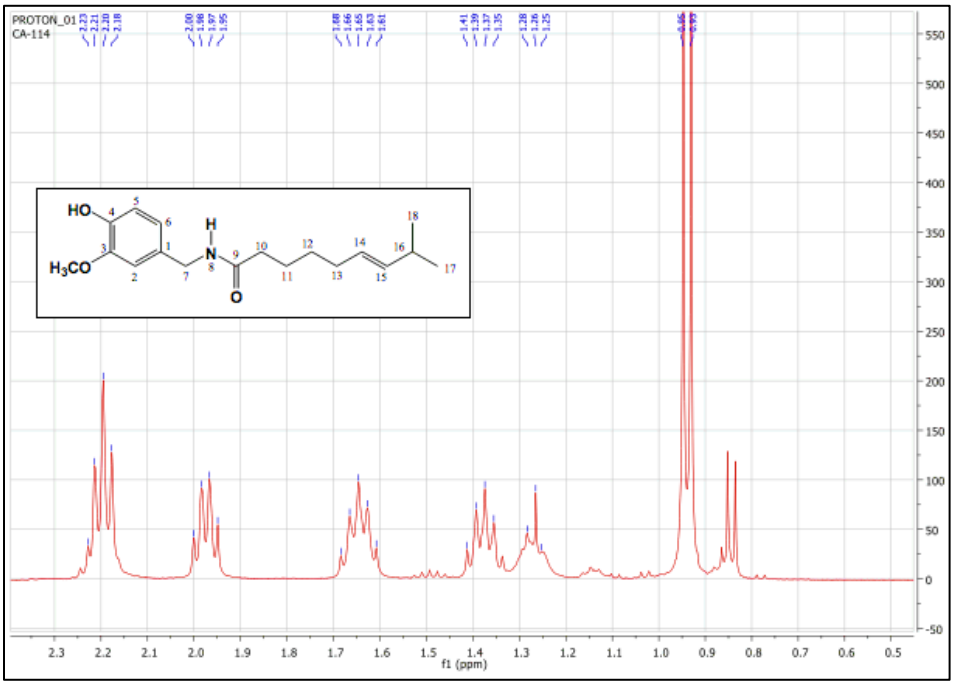


Figure S4. A magnified ^{1}H-NMR spectrum of CAP from 2.3 to 0.5 ppm.

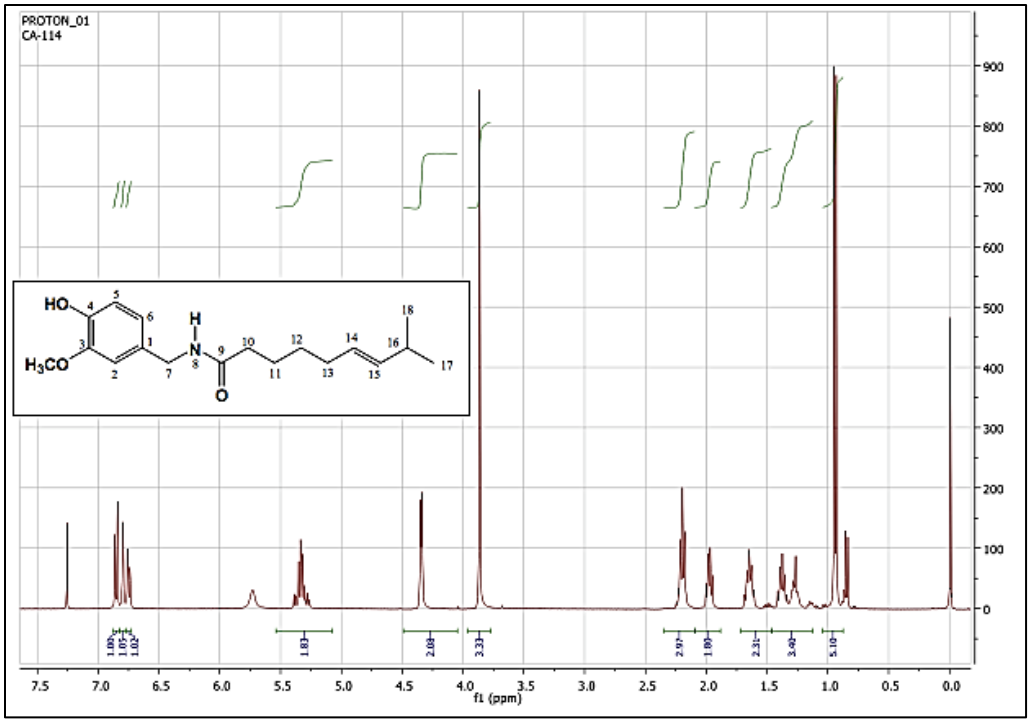


Figure S5. ^{1}H-NMR spectrum of CAP with integration.

In the correlation spectroscopy (COSY) spectrum (Figure S6), all protons were observed in three spin systems, of which the first was the aromatic protons of the vanilliylamine unit, while the second spin system consisted of the two protons, NH and H_2-7 of the same unit. The remaining protons were observed in the third spin system attributed to the acyl unit. For the interpretation of the protons of the acyl unit, the signals with six proton intensity assigned as the terminally located two secondary methyl resonances at 0.94 ppm (d, J = 6.7 Hz) were selected. With the help of COSY correlations, a methine signal was assigned to H-16 (δ 2.20). Further COSY correlation was between the H-16 and one of the two olefinic protons, H-15 (δ 5.36 dd, J = 15.4 and 5.7 Hz). H-15 showed further correlation with the second olefinic proton H-14 (δ 5.30 ddt, J = 15.4, 5.7 and 5.7 Hz). Apart from H-14, the rest of the third spin system was found to be consisting of four methylene protons of H_2-13, H_2-12, H_2-11, and H_2-10, respectively.

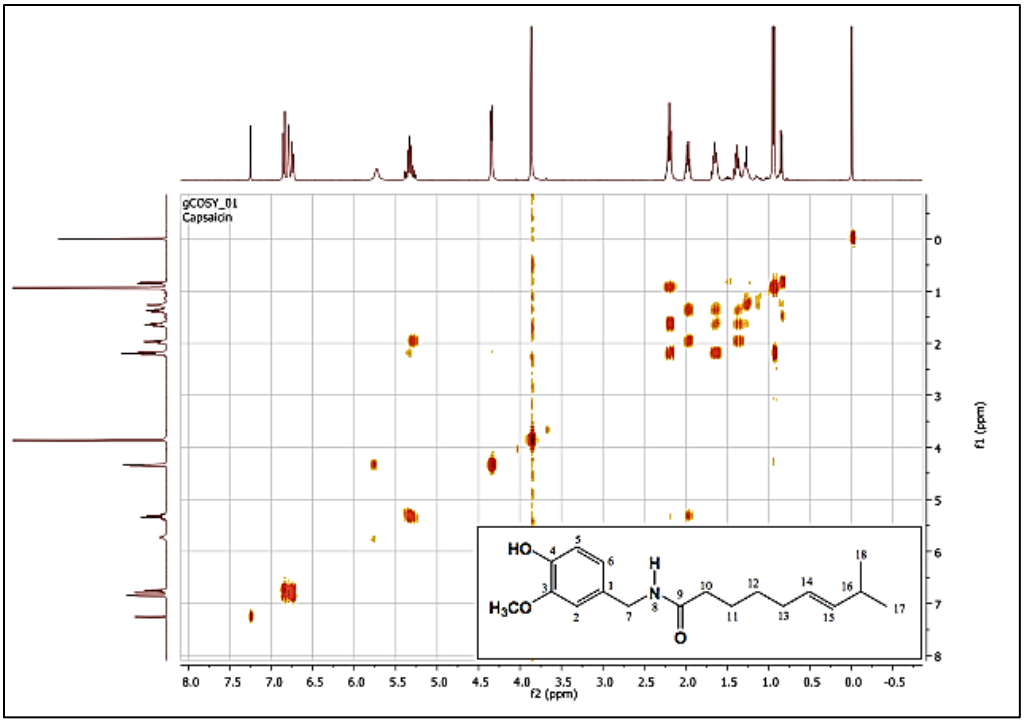


Figure S6. COSY (=^{1}H, ^{1}H-correlated spectrum) of CAP (CA-114).

The ^{13}C-NMR spectrum of CAP exhibited 18 carbon resonances as shown in Figure S7.

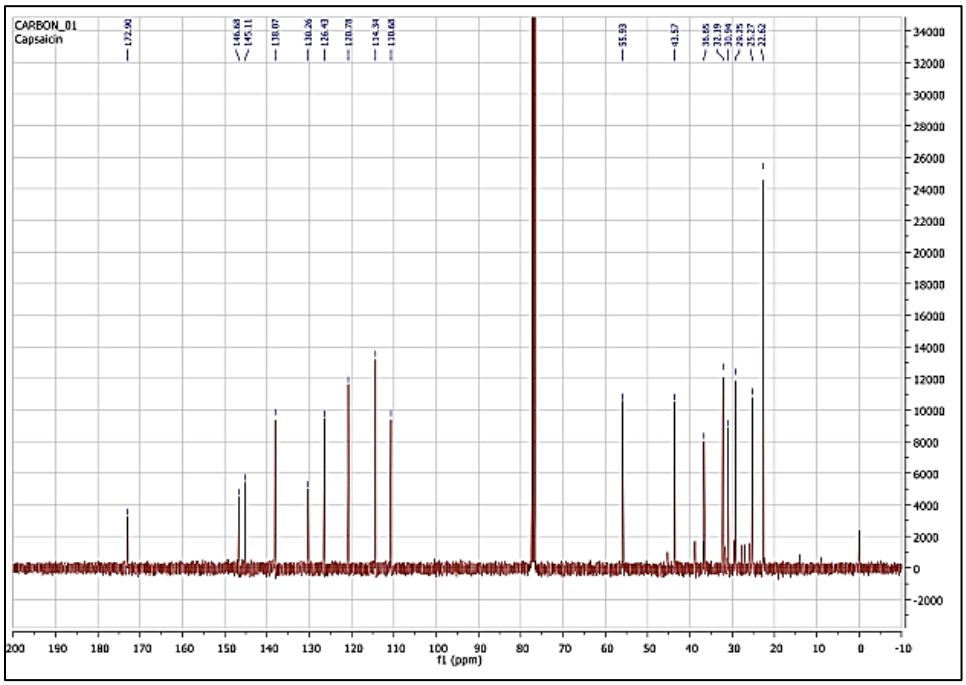


Figure S7. ^{13}C-NMR spectrum of CAP (CA-114) (100 MHz, $CDCl_3$).

A heteronuclear single quantum coherence spectroscopy (HSQC) experiment made clear the assignments of each carbon resonance involved in the three spin systems. By the help of this experiment, the carbon resonances of vanillylamine and the acyl unit were clearly assigned. The HSQC spectrum of CAP is given in Figure S8.

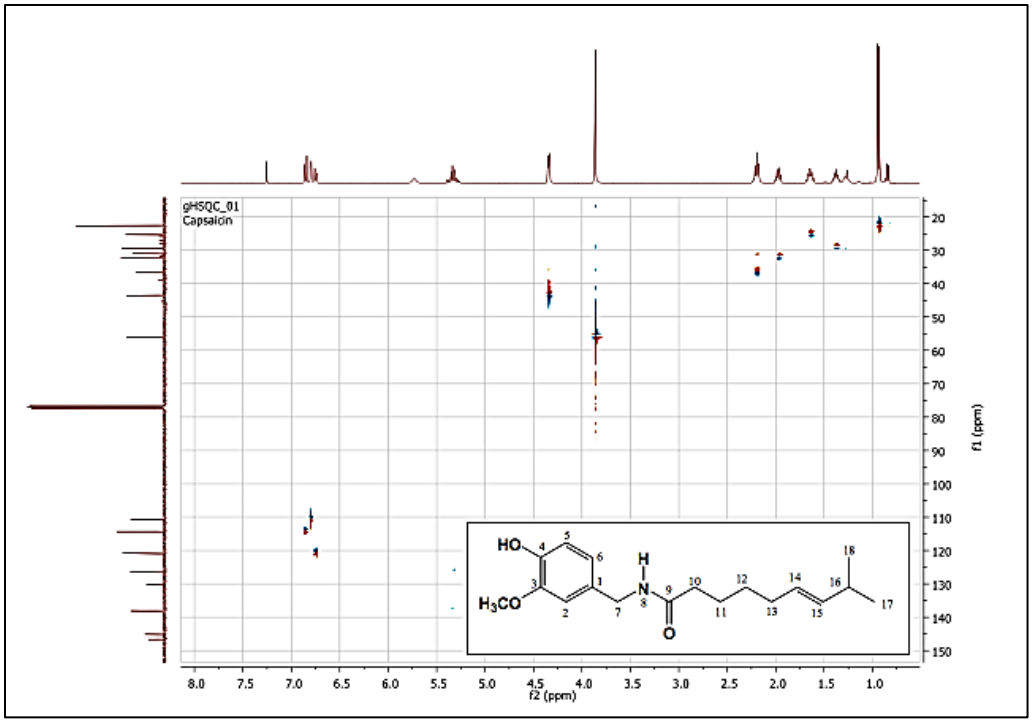


Figure S8. HSQC (=^{1}H, ^{13}C- short-range correlation spectrum of CAP) (CA-114).

A heteronuclear multiple bond correlation (HMBC) experiment made clear the intermolecular connectivity showing the ^{1}H and ^{13}C heteronuclear long-range correlations (Table S1). The significant HMBC correlations were observed between the C-3/ Ar-OCH_3, C-1/H_2-7, H-2 and H-6, C-7/ H-2 and H-6, C-9/H_2-7, C-16/H_3-17, H_3-18, H-14 and H-15 (Figure S9).

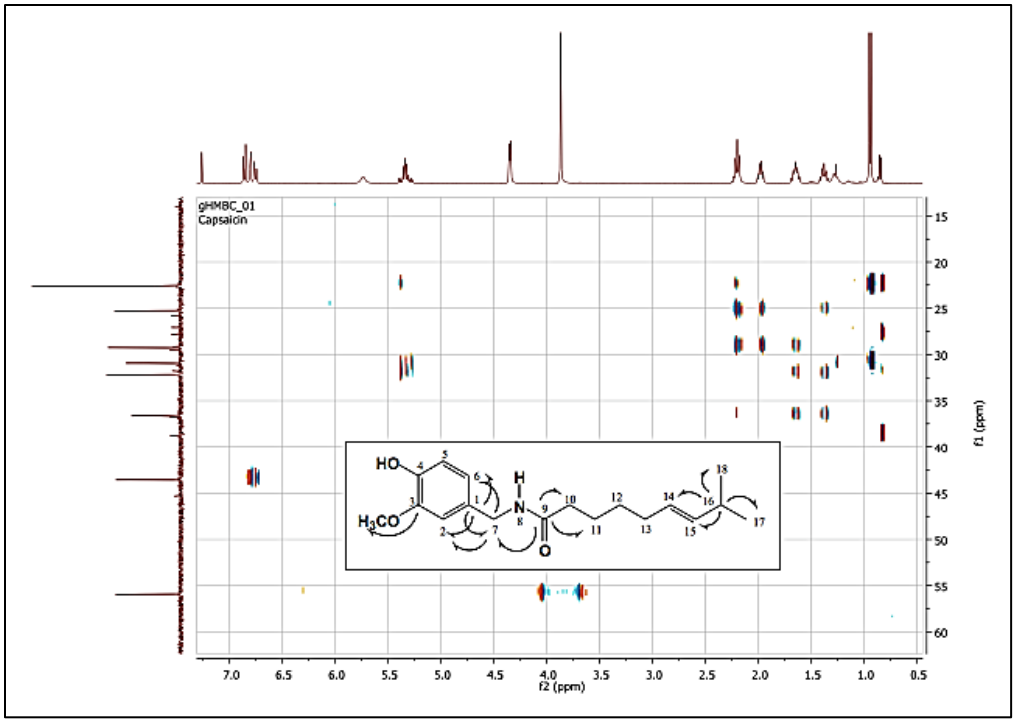
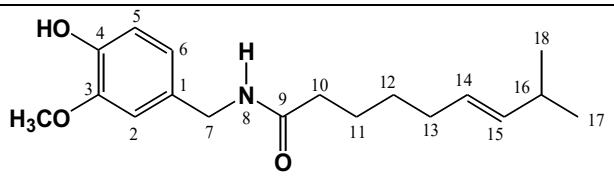


Figure S9. HMBC (=^{1}H, ^{13}C- long-range correlated spectrum) of CAP (CA-114).

The NMR spectral results for CAP are summarized in Table S1.

Table S1. ^{1}H and ^{13}C-NMR data of CAP and HMBC correlations (^{1}H: 400 MHz; ^{13}C: 100 MHz, $CDCl_3$).



CA-114: CAP				
C/H		δ_c ppm	δ_H ppm, *J* (Hz)	HMBC (from C to H)
1	C	130.26	-	H-2, H-5, H_2-7
2	CH	110.68	6.75 dd (8.1 / 1.6)	H-6, H_2-7
3	C	146.68	-	OMe, H-2, H-5
4	C	145.11	-	H-2, H-6
5	CH	114.34	6.85 d (8.1)	-
6	CH	120.78	6.80 d (1.6)	H-2, H-5, H_2-7
7	CH_2	43.57	4.35 d (5.6)	H-2, H-6
8	-	-	-	-
9	C	172.90	-	H_2-7, H_2-10, H_2-11
10	CH_2	36.65	2.20 dd "t" (7.8)	H_2-11, H_2-12
11	CH_2	25.27	1.65 q (7.8)	H_2-10, H_2-12
12	CH_2	29.25	1.37 q (7.8)	H_2-10, H_2-11, H_2-13
13	CH_2	32.19	1.98 q (7.8)	H-14, H-15, H_2-12, H_2-11
14	CH	126.43	5.30 ddt (15.4 / 5.7 / 5.7)	H-16, H_2-12
15	CH	138.07	5.36 dd (15.4 / 5.7)	H_3-17, H_3-18, H-16, H_2-13
16	CH	30.94	2.20 dq†	H_3-17, H_3-18, H-14, H-15
17	CH_3	22.62	0.94 d (6.7)	H-15, H-16
18	CH_3	22.62	0.94 d (6.7)	H-15, H-16
OCH_3	CH_3	55.93	3.85 s	-
NH	-	-	5.73 br s	-

Based on these experiments, the full assignment of proton and carbon resonances of CAP was determined.

2. MS and NMR spectra analysis of NDHC

The positive-ion ES-MS spectrum of NDHC (CA-112) exhibited a molecular ion peak at $m/z = 294$ $[M+H]^+$, and 316.5 $[M+Na]^+$, indicating a molecular formula of $C_{17}H_{27}NO_3$ (calculated molecular weight $= 293\ g\ mol^{-1}$). The MS spectrum of NDHC is given in Figure S10.

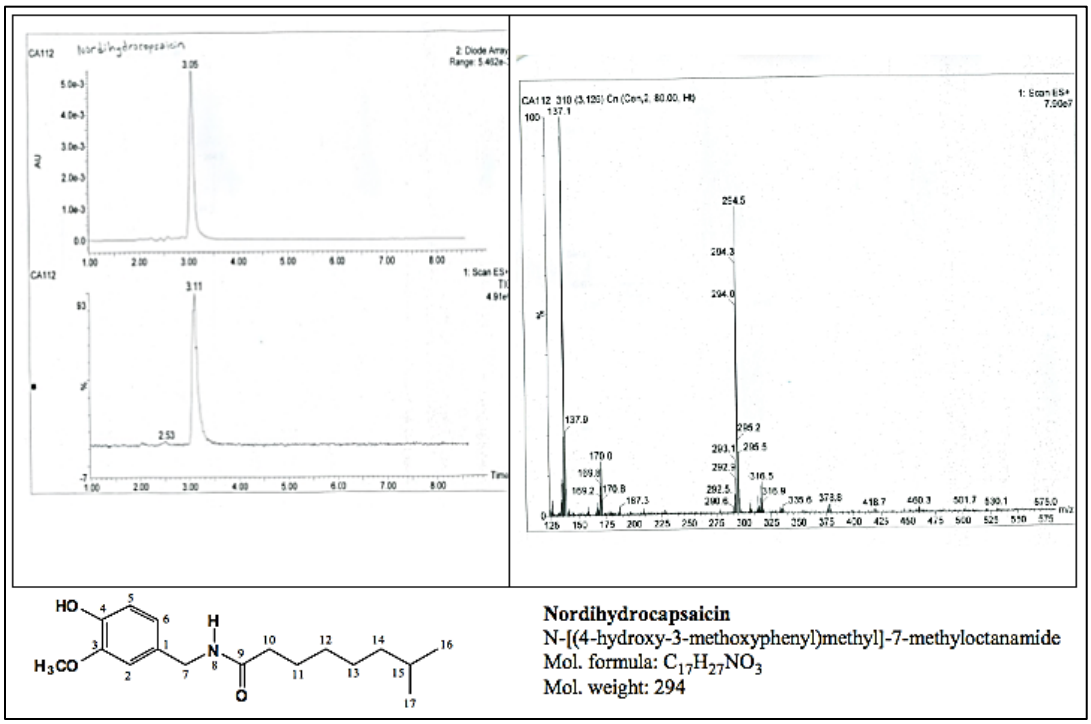


Figure S10. MS spectrum of NDHC.

The ^{1}H-NMR spectrum of NDHC was very similar to that of CAP. All chemical shifts associated with the proton in the vanillylamine portion of the molecule and the first two carbons along with the acyl chain are identical. The major difference is the length of the acyl chain, which is shorter by a single unit, and also the absence of an olefinic proton that was observed in CAP. The ^{1}H-NMR spectrum of NDHC is given in Figure S11, while the integrated version is given in Figure S12 for better details. A magnified ^{1}H-NMR spectrum of NDHC is given in Figure S13. The ^{1}H-NMR data for NDHC is summarized in Table S2.

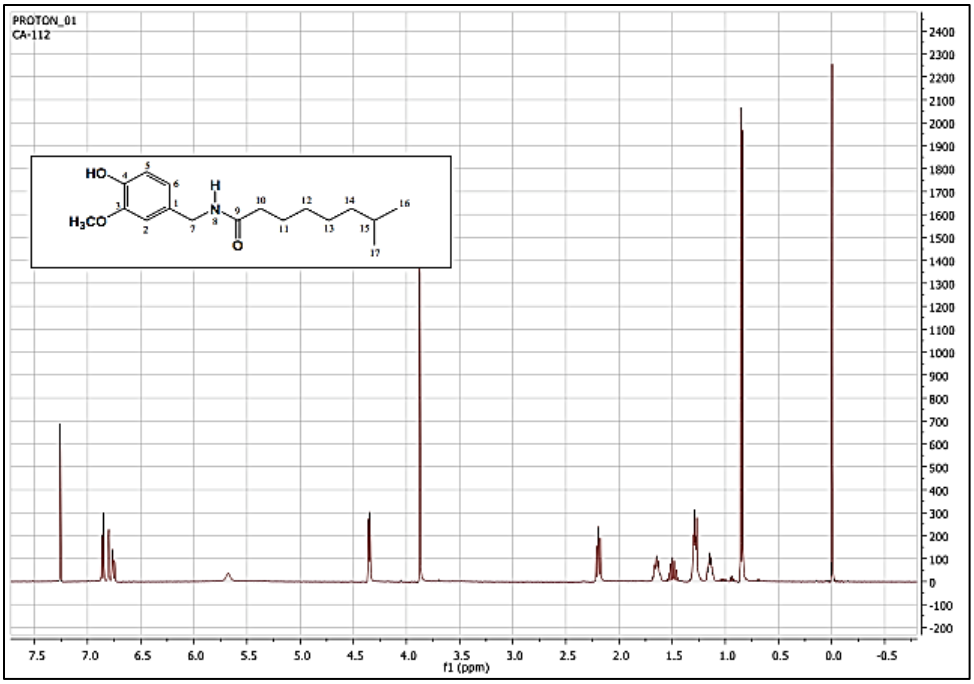


Figure S11. ^{1}H-NMR spectrum of NDHC (CA-112) (400 MHz, $CDCl_3$).

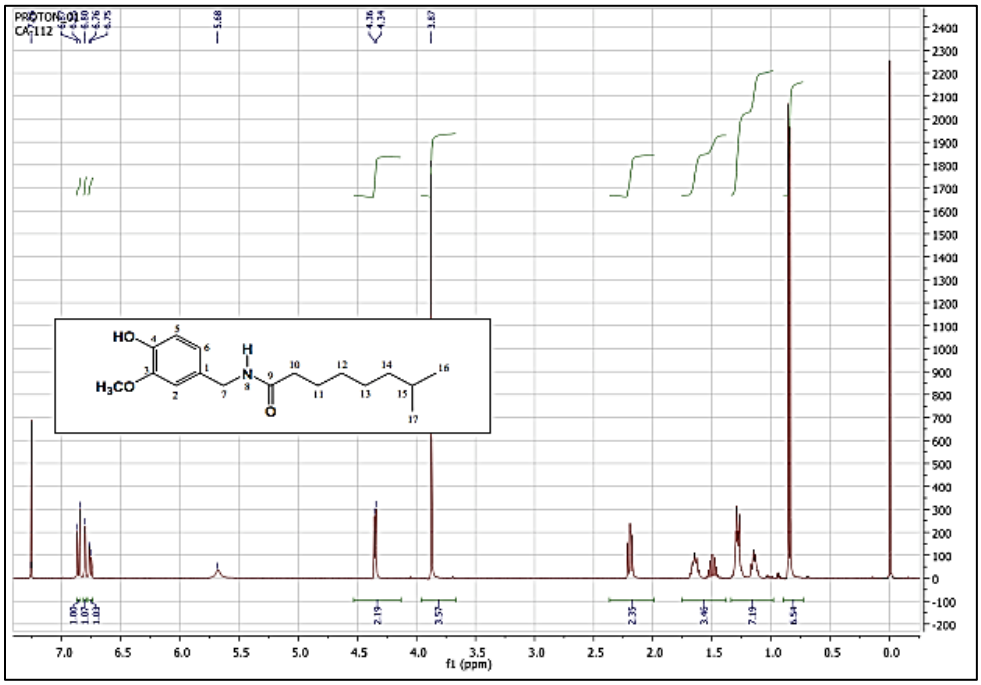


Figure S12. ^{1}H-NMR spectrum of NDHC with integration.

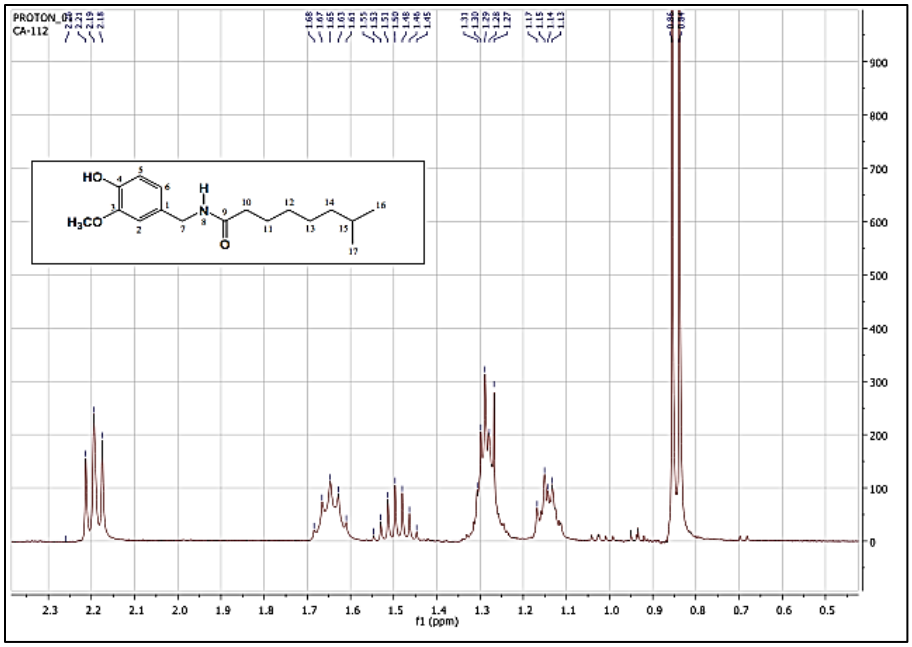


Figure S13. A magnified ^{1}H-NMR spectrum of NDHC.

The ^{13}C-NMR spectrum of NDHC exhibited 17 carbon resonances, also one unit shorter than that of CAP. The spectrum is given in Figure S14, while the data is summarized in Table S2.

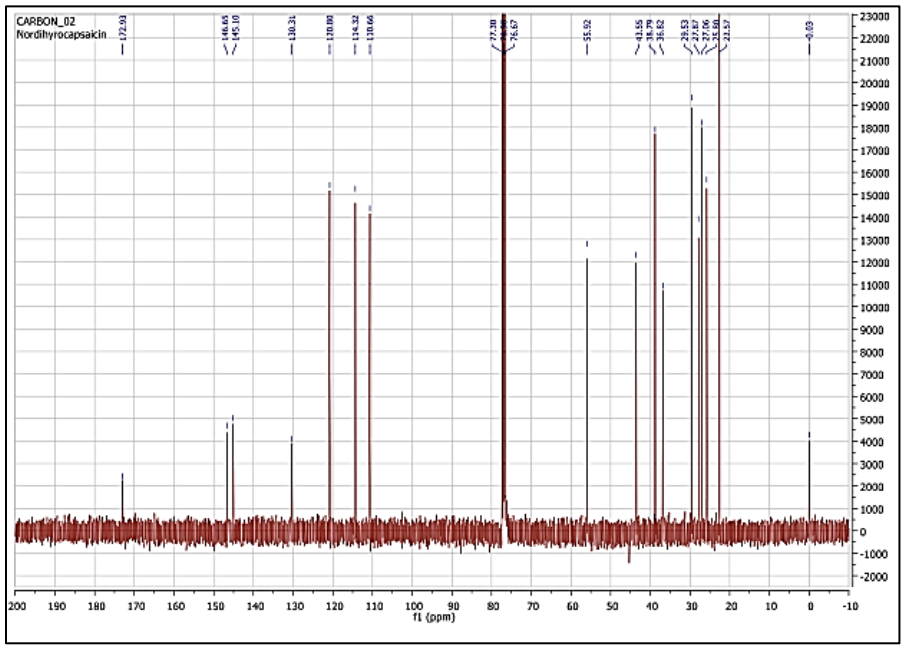
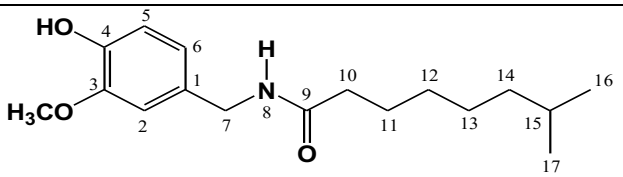


Figure S14. ^{13}C-NMR spectrum of NDHC (CA-112) (100 MHz, $CDCl_3$).

Table S2. ^{1}H- and ^{13}C-NMR data of NDHC (^{1}H: 400 MHz; ^{13}C: 100 MHz, $CDCl_3$).



	CA-112: NDHC		
C/H		δ_c ppm	δ_H ppm, *J* (Hz)
1	C	130.31	-
2	CH	110.66	6.76 dd (8.1 / 1.8)
3	C	145.65	-
4	C	145.10	-
5	CH	114.32	6.86 d (8.0)
6	CH	120.80	6.81 d (1.8)
7	CH_2	43.55	4.35 d (5.1)
8			
9	C	173.08	-
10	CH_2	36.82	2.19 t (7.4)
11	CH_2	25.80	1.65 q (7.4)
12	CH_2	29.53	1.29 †
13	CH_2	27.06	1.29 †
14	CH_2	38.79	1.14 m
15	CH_2	27.87	1.50 septet (6.7)
16	CH_3	22.57	0.85 d (6.7)
17	CH_3	22.57	0.85 d (6.7)
OCH_3	CH_3	55.92	3.87 s
NH	-	-	5.74 br s

3. MS and NMR spectra analysis of DHC

The positive ion ES-MS spectrum of DHC (CA-120) exhibited a quasi-molecular ion peak at $m/z = 308.4$ $[M+H]^+$ and 330.0 $[M+ Na]^+$, indicating a molecular formula of $C_{18}H_{19}NO_3$ (calculated molecular weight $= 307.4$ g mol^{-1}) as shown in Figure S15.

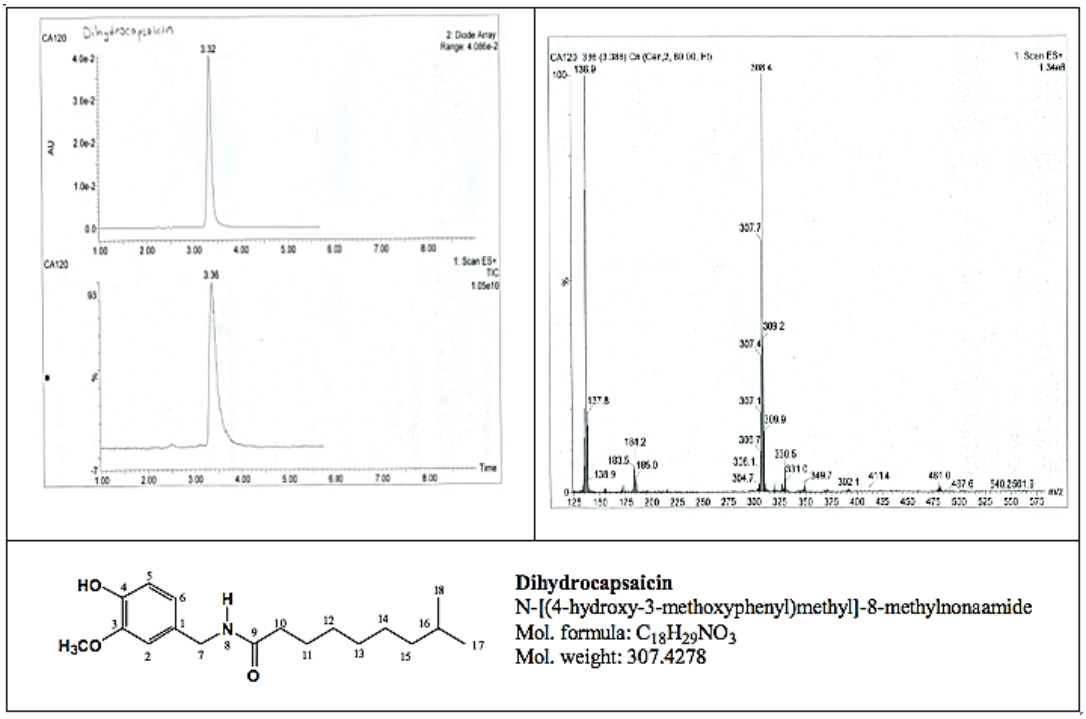


Figure S15. MS spectrum of DHC.

The ^{1}H-NMR spectrum of DHC is also very similar to that of CAP, also exhibiting similar chemical shifts associated with the protons in the vanillylamin portion of the molecule and the first two carbons along the acyl chain. The length of the acyl chain is also the same with that of CAP with the only difference being the absence of olefinic proton in the case of DHC. The ^{1}H-NMR spectrum of DHC is given in Figure S16, while the integrated version is given in Figure S17. A magnified ^{1}H-NMR spectrum of NDHC is given in Figure S18. ^{1}H-NMR data of DHC is summarized in Table S3.

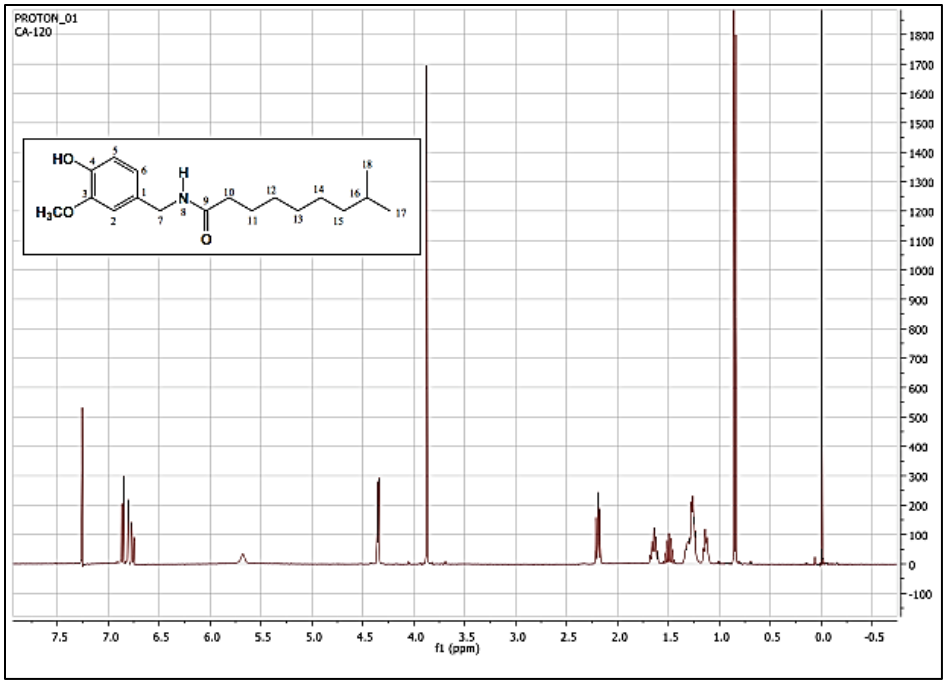


Figure S16. ^{1}H-NMR spectrum of DHC (CA-120) (400 MHz, $CDCl_3$).

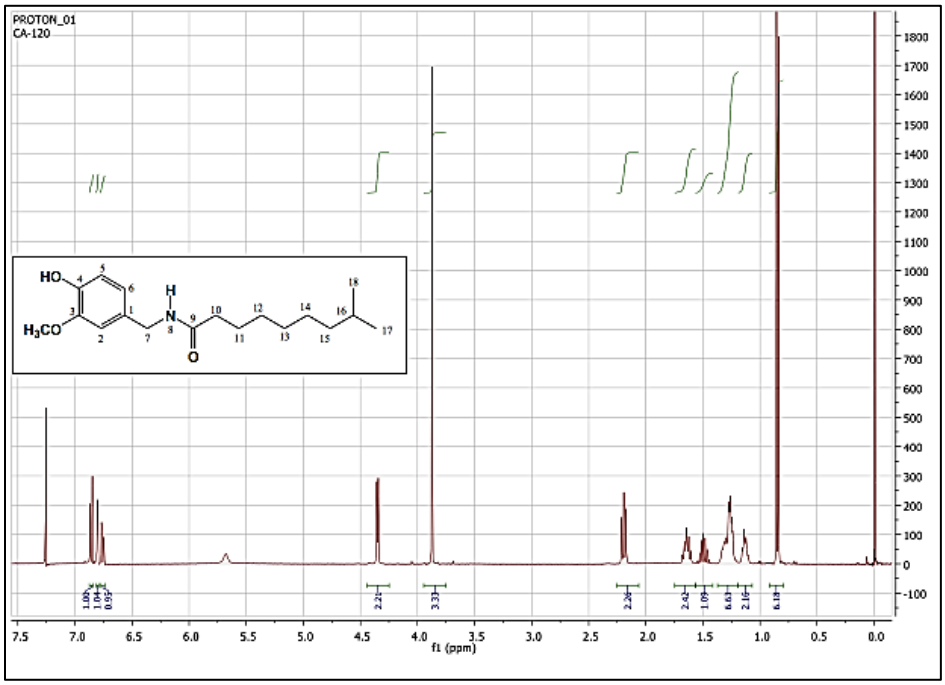


Figure S17. ^{1}H-NMR spectrum of DHC with integration.

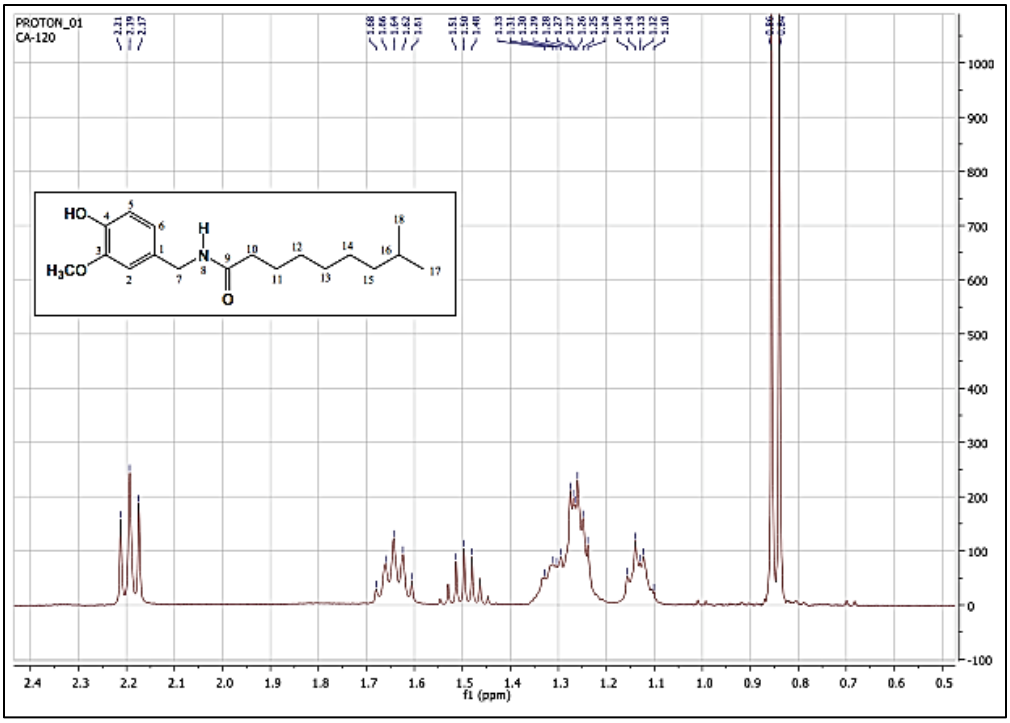


Figure S18. A magnified ^{1}H-NMR spectrum of DHC from 2.4 to 0.5 ppm.

The ^{13}C-NMR spectrum of DHC exhibited 18 carbon resonances as shown in Figure S19. The data is summarized in Table S3.

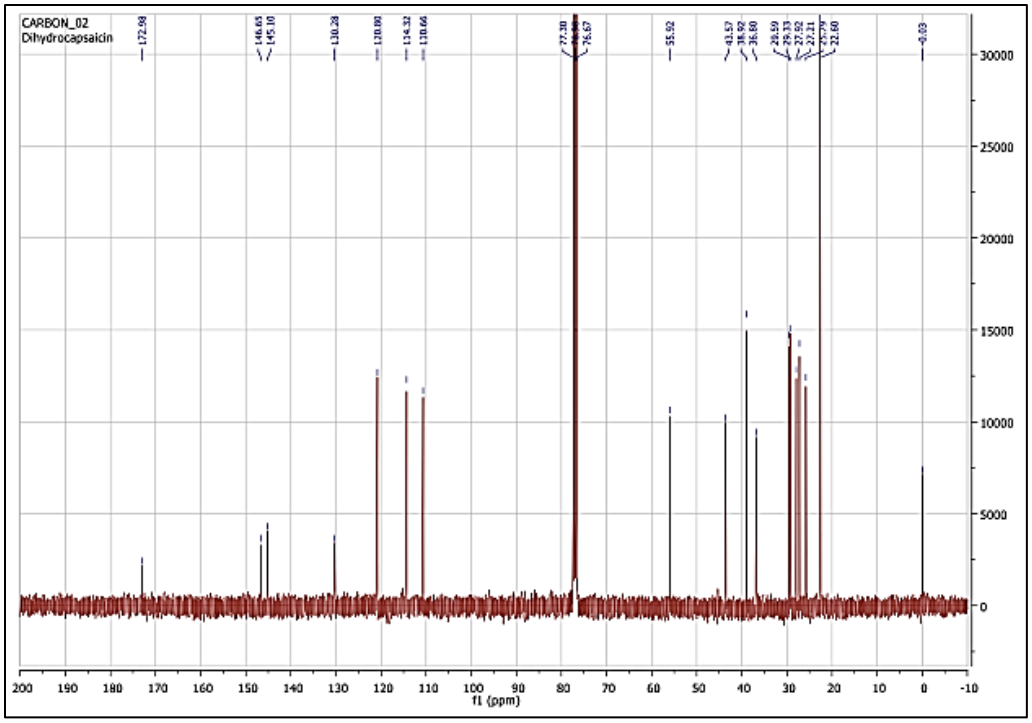
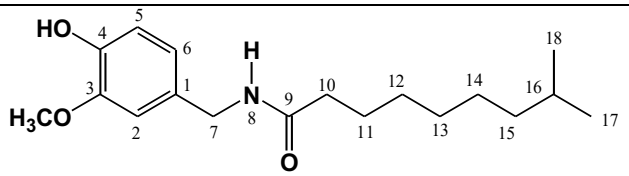


Figure S19. ^{13}C-NMR spectrum of DHC (CA-120) (100 MHz, $CDCl_3$).

Table S3. ^{1}H- and ^{13}C-NMR data of DHC (^{1}H: 400 MHz; ^{13}C: 100 MHz, $CDCl_3$).



CA-120: DHC			
C/H		δ_c ppm	δ_H ppm, *J* (Hz)
1	C	130.28	-
2	CH	110.66	6.76 dd (8.1 / 1.8)
3	C	145.65	-
4	C	145.10	-
5	CH	114.32	6.86 d (8.0)
6	CH	120.80	6.81 d (1.8)
7	CH_2	43.57	4.36 d (5.1)
8			
9	C	172.82	-
10	CH_2	36.80	2.19 t (7.8)
11	CH_2	25.79	1.64 q (7.8)
12	CH_2	29.33	1.31 †
13	CH_2	29.59	1.28 †
14	CH_2	27.21	1.26 †
15	CH_2	38.92	1.13 m
16	CH	27.92	1.50 septet (6.7)
17	CH_3	22.60	0.85 d (6.7)
18	CH_3	22.60	0.85 d (6.7)
OCH_3	CH_3	55.92	3.87 s
NH	-	-	5.74 br s