



Analysis of Capsaicinoids in Chilli Sauce with Ultra Fast Liquid Chromatography

Ultra Hızlı Sıvı Kromatografisi ile Biber Sosunda Kapsaisinoidlerin Analizi

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ABSTRACT

Objective: In current study, quantification of the capsaicinoids in chilli sauces based on a sensitive ultra fast liquid chromatography method and derivatization with dansyl chloride (DNS-Cl) was described. Capsaicinoids are biosynthesized as secondary metabolites by chilli sauces. The major components of capsaicinoids are capsaicin (CPS) and dihydrocapsaicin (DCPS).

Methods: Phenol groups within the CPS and DCPS are suitable for derivatization reaction using DNS-Cl (chemically named as 5-(dimethylamino) naphthalene-1-sulfonyl chloride) at pH 10 with 0.5 M sodium bicarbonate which leads formation of a derivative highly fluorescent properties that can be measured at 520 nm following excitation at 360 nm wavelength. Separation of the compounds was conducted on a chromatographic system having a mobile phase formed by a combination of acetic acid (0.5 M, pH 7.0 with NaOH) solution and acetonitrile under solvent programming on a consistent flow rate of 0.4 mL. min⁻¹ using a C18 column.

Results: Method validation was evaluated as per the regulations described in International Conference on Harmonization Guidelines. The calibration graph for CPS and DCPS was linear between 0.2 and 200 µg mL⁻¹.

Conclusion: The proposed analytical procedure represents a simple, time and cost effective method with a suitable selectivity regarding quantification of capsaicinoids in chilli sauces.

Keywords: Capsaicin, chilli sauces, dansyl chloride, dihydrocapsaicin, UFLC, validation

ÖZ

Amaç: Mevcut çalışmada, hassas bir ultra hızlı sıvı kromatografisi yöntemine ve dansil klorür (DNS-Cl) ile türevlendirmeye dayalı olarak acı biber soslarındaki kapsaisinoidlerin miktar tayini tanımlanmaktadır. Kapsaisinoidler, biber sosları tarafından sekonder (ikincil) metabolitler olarak biyosentezlenir. Kapsaisinoidlerin ana bileşenleri kapsaisin (CPS) ve dihidrokapsaisindir (DCPS).

Yöntemler: CPS ve DCPS içindeki fenol grupları, pH 10'da DNS-Cl (kimyasal olarak 5-(dimetilamino) naftalen-1-sülfonil klorür olarak adlandırılır) kullanılarak 0,5 M sodyum bikarbonat ile türevlendirme reaksiyonu için uygundur, bu da yüksek floresan özelliklere sahip bir türevin oluşumuna yol açar ve 360 nm dalga boyunda uyarılmayı takiben 520 nm'de ölçülebilir. Bileşiklerin ayrılması, asetik asit (0,5 M, NaOH ile pH 7,0) çözeltisi ve asetonitrilin bir kombinasyonu ile oluşturulan bir mobil faza sahip kromatografik bir sistem üzerinde C18 kolonu kullanılarak, 0,4 mL dk⁻¹ akış hızında solvent programlaması altında tutarlı bir şekilde gerçekleştirildi.

Bulgular: Metot validasyonu, Uluslararası Harmonizasyon Topluluğu Konferansı'nda açıklanan düzenlemelere göre değerlendirildi. CPS ve DCPS için kalibrasyon grafiği 0,2 ve 200 µg mL⁻¹ arasında doğrusaldı.

Sonuç: Önerilen analitik prosedür, biber soslarında kapsaisinoidlerin miktar tayini ile ilgili olarak uygun bir seçiciliğe sahip, basit, zaman ve maliyet açısından etkin bir yöntemi temsil etmektedir.

Anahtar Sözcükler: Kapsaisin, biber sosları, dansil klorür, dihidrokapsaisin, UFLC, doğrulama

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Introduction

Chilli peppers are commonly preferred food additives due to high spicy flavor allowing preparation of tasty foods (1). Commercial food processing for chilli pepper became critical as there were a number of chilli pepper subtypes and high level of demand for chilli pepper. Capsaicinoids, as hydrophobic alkaloids, are the major component in chilli pepper (2,3). The majority, as 90%, of capsaicinoids are represented as capsaicin (trans-8-methyl-N-vanillyl-6nonenamide) (CPS) and dihydrocapsaicin (8-methyl-N-vanillyl-nonanamide) (DCPS) (4) (Figure 1). The recent update in regulations governing food industry suggests the recommended daily average and highest intake of CPS as 0.77 mg day⁻¹ and 2.64 mg day⁻¹, respectively, limiting CPS content of industrial food as 5 µg g⁻¹ (5). According to literature, capsaicinoids can be used in the therapy due to their following therapeutic properties such as being analgesic (6) and anti-inflammatory (7), and having gastroprotective properties against the gastrointestinal adverse effects of drugs (8,9), high level antioxidant effects (10,11) and anti-tumoral (12,13) properties.

Various analytical procedures were defined in literature for quantification of capsaicinoids as follows; high performance liquid chromatography (HPLC) (14-16), HPLC coupled with mass spectrometry (HPLC-MS) (17) gas chromatography coupled with MS (GC-MS) (18) and capillary electrophoretic methods (19). Sensitivity provided by HPLC method using ultraviolet detection is not adequate. Separation quality is better with LC-MS and GC-MS methods with high sensitivity however highly costed equipment and qualified operator are required. In this study, capsaicinoids were analyzed with UFLC method based on fluorescence detection through derivatization of CPS and DCPS with dansyl chloride (DNS-Cl). Detection based on fluorescence measurement enabled adequate sensitivity for the analytical procedure. Weberl was the first researcher using DNS-Cl, (5-(dimethylamino) naphthalene-1-sulfonyl chloride) for the formation of fluorescent derivatives of albumin

(20). DNS-Cl was used as a fluorescent derivatization agent in determination of several pharmaceutical active ingredients such as primary amines, secondary amines, imidazoles and phenols in their chemical structure (21-25). As the authors of the proposed study, we also used derivatization procedures carried out with DNS-Cl for some pharmaceutical analysis (26-28). In all these analysis, we trialed various conditions to gain the most effective derivatives. The pH values should be in alkaline range and temperatures of the mediums were supposed to be at about 40-60 °C with requiring short reaction durations.

In current study, DNS-Cl was selected as an efficient fluorescent labelling reagent for quantification of CPS in chilli hot sauces. The proposed method based on derivatization with DNS-Cl, has the advantages of being simple with faster sample preparation, having sensitivity and selectivity resulting from fluorescence spectra, use of widely available equipment. In addition to the advantages of the derivatization procedure, UFLC also provided advantageous separation procedure such as shorter chromatographic process, reduced mobile phase consumption, facility to study with trace amounts of sample and more sensitive assays than conventional HPLC applications. As per the results, currently developed analytical UFLC procedure was suitable for quantification of capsaicinoids in chilli sauce.

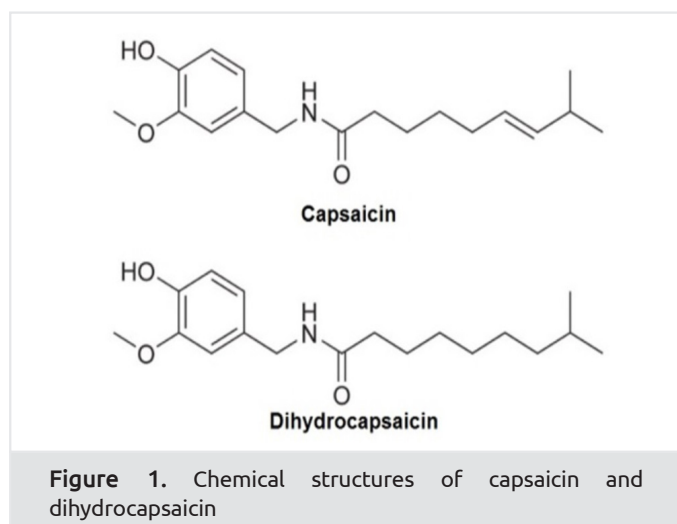
Method

Reagents and Solutions

The CPS and DCPS standards and DNS-Cl were procured through Sigma (St. Louis, MO). All reagents and chemical substances were of analytical-reagent grade. CPS and DCPS were solvated in methanol at 1 mg mL⁻¹ concentration as the stock solution which was the basis for preparation of working solutions through dilutions from this stock solution. Concentration of freshly prepared DNS-Cl solution in acetonitrile was 2.0 mg mL⁻¹ (0.02%). The sodium bicarbonate was dissolved in water at 0.5 M concentration and pH was adjusted with addition of 0.5 M sodium hydroxide to pH 10 by a pH meter. The prepared solution was stored in the fridge and available for use for approximately seven days.

Apparatus

Shimadzu LC 20A UFLC (Shimadzu, Kyoto, Japan) was used for chromatographic separations. Elements of the system were LC 20AB Binary pump, CTO-10As column oven. Data gathered from chromatographic system was analyzed by system software of LC Solution. Excitation wavelength of 360 nm and an emission wavelength of 520 nm was specifically set by the fluorescence detector. Inertsustain C18 column (4.0x100 mm, 3 µm) procured from GL Sciences, Tokyo-JAPAN was used for chromatographic separation. A mobile phase was consisted of the combination of acetonitrile (Mobile phase A) and solution of acetic acid at 0.5 M concentration at pH 7.0 (adjusted using



NaOH) (Mobile phase B) under gradient elution with a constant flow rate of 0.4 mL min⁻¹. The column temperature was kept constant at 25 °C.

General Procedure

The aliquots taken from the standard solution involving CPS and DCPS corresponding to the concentration interval of 0.02–200.0 µg mL⁻¹ were taken into a series of test tubes and a 250 µL bicarbonate solution at pH 10 and 750 µL of DNS-Cl solution were added to every test tube. The reaction solution mixture was kept at 40 °C during 10 min. They were left to cool at ambient temperature in air and then the formed drug fluorescent derivative was extracted using 5 mL of dichloromethane during 1.0 min. The extract was subjected to evaporation under nitrogen gas at 40 °C. The 0.5 mL of the mobile phase was used for dissolving of remaining residue. A 20 µL aliquots of the resulting solution was used for quantification by UFLC.

Sample Preparation

Three samples of daily-used chilli hot sauces (one red pepper-based, one jalapeno pepper-based and one cayenne pepper-based sauce) were procured from local market in Turkey. First step covered the transfer of 10 mL of hot sauce into a volumetric flask of 50 mL volume having absolute ethanol of 25 mL. Secondly, the resulting mixture was kept in an ultrasonic bath for sonification during 1 h. Following second step, the mixture was stirred during 2 h via a magnetic stirrer. Centrifugation of the resulting mixtures was performed at 10,000 rpm during 10 min followed by addition of absolute ethanol to complete the total volume to 50 mL. A vacuum rotatory evaporator was used for evaporation of the ethanol phase at 40 °C. The residue was dissolved with addition of 1.0 mL methanol solution and vortexed. Upon completion of solvation, a 750 µL of DNS-Cl solution and a 250 µL of bicarbonate solution at pH 10 were added to every

tube. The reaction mixture was kept at 40 °C during 10 min. They were then left to cool down at ambient temperature in air. Extraction of the resulting derivative was performed with 5 mL of dichloromethane during 1.0 min. A 0.5 mL of the mobile phase was used for solvation of the remaining residue. A 20 µL aliquots of the resulting solution was analyzed by UFLC for determination of capsaicinoids.

Results

Chromatographic Conditions

A C18 column (4.0x100 mm, 3 µm) was used for chromatographic separation under gradient elution and the column temperature was kept at 25 °C during the analysis. A mobile phase formed by the mixture of acetonitrile (Mobile phase A) and acetic acid solution at 0.5 M concentration (pH adjusted to 7.0 with NaOH) (Mobile phase B) was used with 0.4 mL min⁻¹ flow rate. The optimum chromatographic separation was performed by using acetic acid solution at 0.5 M concentration and with adjusting pH to 7 by NaOH additions with acetonitrile. Fluorimetric detection was carried out at emission wavelength of 520 nm following excitation at 360 nm excitation wavelength. Table 1 represents the gradient elution program. Figure 2 represents the typical chromatograms.

Optimization of Derivatization Reaction Parameters

The CPS and DCPS were derivatized with DNS-Cl to yield derivatives with fluorescence properties. According to the results of investigation for identification of optimum reaction conditions, a 750 µL of DNS-Cl in acetonitrile solution was found to be sufficient to achieve the highly effective derivatives. The effect of various pH values on fluorescent intensity were investigated in pH mediums changing between 9 and 11, using bicarbonate solution and borate buffer. As evidenced in literature, DNS-Cl has a tendency to yield reaction under

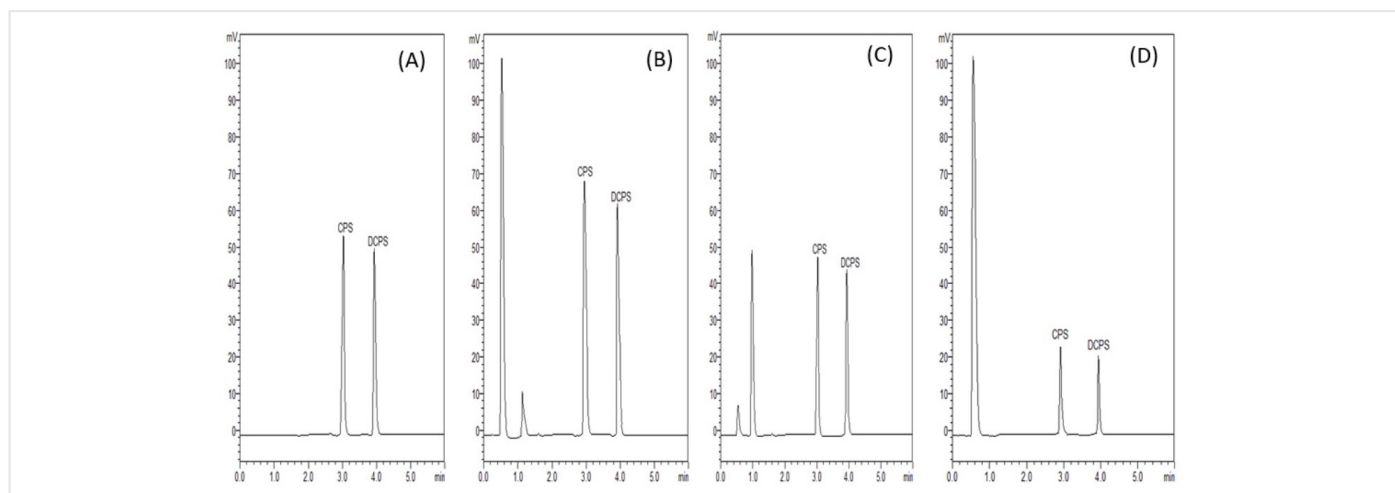


Figure 2. Representative chromatograms of (A) standard chromatogram of CPS (100 µg mL⁻¹) and DCPS (100 µg mL⁻¹), (B) red pepper-based sauce sample (C) jalapeno pepper-based sauce sample and (D) cayenne pepper-based sauce sample
CPS: Capsaicin, DCPS: Dihydrocapsaicin

alkaline conditions. The bicarbonate solution of pH 10 with a volume of 250 μL enabled the maximum fluorescence intensity for this derivatization reaction.

The effects of temperature and various durations on the intensity of fluorescence resulting from the formed derivatives were also examined between 40-60 $^{\circ}\text{C}$. The optimum fluorescence measurements were achieved and remained stable in 40 $^{\circ}\text{C}$ water bath during 10 min. The effect of different solvents on fluorescence intensity was investigated using benzene, diethyl ether, toluene, ethyl acetate, chloroform, dichloromethane. It was observed that dichloromethane, as a solvent, allowed the highest level of fluorescence intensity. The derivatives synthesized according to the pre-defined conditions, were stable during at least 2 h. Table 2 lists optimum values for the derivatization parameters.

Method Validation

Method validation was evaluated in line with ICH Guidelines (29).

Linearity

Linearity test was performed with solution involving CPS and DCPS prepared at six different concentration values covering the range of 0.2 to 200.0 $\mu\text{g mL}^{-1}$. The calibration curve was established by the plotting the substance peak area against the concentration. The correlation coefficients, y-intercept and slope values of the calibration curves were computed and presented (Table 2). Calculation of the detection limit (LOD) and the quantitation limit (LOQ) was performed according to the pre-

defined equation: $\text{LOD or LOQ} = \kappa\text{SDa}/b$. Standard deviation of the intercept is represented as SDa and the slope as b . Constant κ is assigned as 3 for calculation of LOD and as 10 for calculation of LOQ. Data for LOD and LOQ are represented in Table 3.

Precision

The precision investigation was performed during five consecutive days by quantitation of CPS and DCPS (each $n=5$). The within the day and between the days precisions (relative standard deviations) were $<2\%$, indicating good level of precision for the method. The results of the precision are presented in Table 4.

Accuracy

The accuracy of the proposed method was proved using the standard addition technique. Calculation of the percentage recovery regarding the added standard in each assay sample was performed according to the following equation:

$$\text{Percentage recovery \%}, \% = [(C_t - C_u)/C_a] \times 100$$

C_t standing for the total concentration of the analyte measured, C_u representing the concentration of the unknown analyte amount actually existing in the sauce, and C_a expressing the known concentration of the pure analyte which is deliberately added to the sauce. The findings are presented in Table 3. The percentage recovery values for the measured were quantitatively 88.08-93.64% for CPS, 84.15-94.78% for dihydrocapsaicin, leading to a high level of accuracy of the method. The findings of the recovery study have been presented in Table 5.

Robustness

The method was found to be robust based on the observed changes in the flow rate of the mobile phase ($\pm 0.1 \text{ mL min}^{-1}$) and the determined organic phase composition ($\pm 2\%$). During the analyses, the mobile phase pH (7.0 ± 0.5) and column oven temperature ($25 \pm 5 \text{ }^{\circ}\text{C}$) were measured and noted. The study demonstrated that minor variations in the variable parameters of the method did not have significant impact on the results, proving the robustness of the currently proposed method.

The chemical stability of the stock solutions, which were composed of the study compounds in mobile phase mixture, was assessed after storing the solutions at room temperature for 48 hours. All of the studied compounds were found out to be stable in the mobile phase for 48 hours at room temperature and in the refrigerator (at 4 $^{\circ}\text{C}$). The stability studies yielded no further peaks in the chromatograms.

Table 1. Gradient elution program

Time (min)	%A	%B
0	80	20
2	50	50
5	60	40
8	60	40
10	80	20

min: Minute

Table 2. Optimum values for the derivatization parameters

Reagent concentration	pH	Temperature	Duration	Extraction solvent
0.02%	10	40 $^{\circ}\text{C}$	10 min	CH_2Cl_2

min: Minute

Table 3. Calibration and sensitivity data of capsaicinoids

Capsaicinoids	Linear range ($\mu\text{g mL}^{-1}$)	Regression equation	R^2	LOD ($\mu\text{g mL}^{-1}$)	LOQ ($\mu\text{g mL}^{-1}$)
Capsaicin	0.2-200	$y=417.5x + 4795$	0.9961	0.0029	0.0099
Dihydrocapsaicin	0.2-200	$y=374x + 4193$	0.9952	0.0040	0.0133

LOD: Detection limit, LOQ: Quantitation limit

Determination of CPS and DCPS in Chilli Sauces

Application of the described methods to samples and the procedure for extraction were detailed at Determination of CPS and DCPS in Chilli sauces. The CPS and DCPS concentration values in the examined pepper samples and the pungency level defined as Scoville Heat Units (SHU) are presented in Table 5 (1 ppm CPS measured being roughly equivalent to 16 SHU) (30). Classification as per SHU is as follows: non-pungent (0-700 SHU) - mildly pungent (700-3,000 SHU) - moderately pungent (3,000-25,000 SHU) - highly pungent (25,000-70,000 SHU) - very highly pungent (>80,000 SHU). The mean concentration of capsaicinoids in daily-used chilli hot sauce (one red pepper-based, one jalapeno pepper-based and one cayenne pepper-based sauce) was 288.08, 180.57 and 81.5 ppm, respectively. According to the findings of the current study, the pungency level of the examined samples could be ranked as follows, as presented in Table 6: red pepper-based (moderately pungent) > jalapeno pepper-based (mildly pungent) > cayenne pepper-based sauce.

Conclusion

Development and validation of a cost and time effective and selective method aiming the quantification and separation of CPS and DCPS in different kind of chilli sauces were successfully completed. The currently developed method had good repeatability and selectivity. The quantitative analysis of capsaicinoids in chilli sauces was performed satisfactorily with this method. UFLC method could be readily implemented in the analysis of chili sauce extracts. DNS-Cl derivatization improved the selectivity of capsaicinoids. Two capsaicinoids were well separated from each other within 4 min. Capsaicinoid composition of chilli sauce samples were accurately determined. The observed pungency level from the highest to the lowest as expressed in SHU could be ranked as follows; highest SHU level with red pepper-based, while jalapeno pepper-based, cayenne pepper-based sauce had lower SHU values. In conclusion, the proposed method is faster, more sensitive and cost effective than the previously reported methods for the determination of CPS and DCPS in food samples.

Table 4. Intra-day and Inter-day precision results of capsaicinoids

	Intra-day (n=5)			Inter-day* (n=5)		
	Added concentration ($\mu\text{g mL}^{-1}$)	Found concentration ($\mu\text{g mL}^{-1}$) \pm SD	RSD %**	Added concentration ($\mu\text{g mL}^{-1}$)	Found concentration ($\mu\text{g mL}^{-1}$) \pm SD	RSD %*
Capsaicin	0.2	0.201 \pm 0.0015	0.75	0.2	0.203 \pm 0.003	1.38
	100	100.25 \pm 0.890	0.89	100	100.34 \pm 1.240	1.24
	200	201.32 \pm 1.270	0.63	200	202.45 \pm 1.700	0.84
Dihydrocapsaicin	0.2	0.203 \pm 0.0018	0.89	0.2	0.204 \pm 0.0024	1.18
	100	100.18 \pm 0.900	0.90	100	100.48 \pm 0.984	0.98
	200	201.26 \pm 1.251	0.75	200	203.02 \pm 1.865	0.92

*Five consecutive day
**RSD: Relative standard deviation

Table 5. Accuracy results of capsaicinoids

Capsaicin*			Dihydrocapsaicin*		
Spiked amount ($\mu\text{g mL}^{-1}$)	Recovery	RSD %*	Spiked amount ($\mu\text{g mL}^{-1}$)	Recovery	RSD %*
0.2	88.08	1.25	0.2	84.15	0.45
100	93.31	1.53	100	94.78	0.23
200	93.64	1.41	200	94.28	0.26

*Sample amount 10.0 mL; Concentration of capsaicin and dihydrocapsaicin in the initial sample was 150.04 and 120.17 $\mu\text{g/mL}$, respectively

Table 6. Concentrations of capsaicin, dihydrocapsaicin and Scoville heat units (SHU) in analyzed samples

Pepper type	Capsaicin (ppm)*	Dihydrocapsaicin (ppm)*	Total capsaicinoids (ppm)	Scoville heat units (SHU)	Pungency level
Red pepper-based sauce	152.21 \pm 1.017	135.87 \pm 1.1287	288.08	4609	moderately pungent
Jalapeno pepper-based sauce	95.36 \pm 0.9541	85.21 \pm 0.8365	180.57	2889	mildly pungent
Cayenne pepper-based sauce	41.36 \pm 0.2547	40.14 \pm 0.3698	81.5	1304	mildly pungent

*n=5

Ethics

Ethics Committee Approval: This article does not contain any studies with human participants or animal performed by any of the authors.

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: B.C., C.Ö., A.Ö., Design: B.C., C.Ö., A.Ö., Data Collection or Processing: B.C., C.Ö., A.Ö., Analysis or Interpretation: B.C., C.Ö., A.Ö., Literature Search: B.C., C.Ö., A.Ö., Writing: B.C., C.Ö., A.Ö.

Conflict of Interest: No conflict of interest was declared by the authors.

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