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Original Article

Capsaicin determination from pain patch for the calculation of Scoville heat units by gas chromatography-mass spectrometry

İbrahim Daniş[1,2], Durişehvar Özer Ünal[1,2]

[1]İstanbul University, Faculty of Pharmacy, Department of Analytical Chemistry, İstanbul, Turkey
[2]İstanbul University, Drug Research Center, İstanbul, Turkey

ORCID IDs of the authors: İ.D. 0000-0003-4646-4129; D.Ö.Ü. 0000-0003-0754-1240

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ABSTRACT

Background and Aims: A sensitive, accurate and precise method has been developed for the determination of Capsaicin from pain patches by Gas chromatography Mass Spectrometry (GC-MS). Capsaicin has irritant effects in high concentrations, so these effects can be minimized by knowing the amount present in pain patches for the efficacy and safety of patches.
Methods: Capsaicin was extracted by using liquid-liquid extraction from patches. The Gas Chromatographic separation was performed by using 5% diphenyl 95% dimethylpolysiloxane column with high a purity 2 mL/min flow rate helium gas. The separation was made with a gradient oven temperature program. The oven temperature started at 250°C and was increased to 275°C at 10°C.min^{-1} ramp rate and held at 275°C for 2.5 min. The injection port was adjusted at 300°C and a split injection mode was used. The analysis was carried out in a split mode of 5:1. MS ionization potential was determined at 70 eV.
Results: The calibration curve was found to be linear in the range 5 - 50 µg/mL. The limits of detection and quantification for capsaicin was found to be 3.46 µg/mL and 5 µg/mL, respectively. The method developed was validated and successfully applied to the patch analysis.
Conclusion: This method is simple, reproducible, and can be used safely for the routine analysis of Capsaicin without derivatization. This study has the potential how to calculate the Scoville Heat Units (SHU) of pain patches that contain Capsaicin. The amount of Capsaicin in the pain patch, its irritant effects, and its efficacy and safety appear to be low when evaluated by the SHU.

Keywords: Capsicum, Capsaicin, pain patch, gas chromatography-mass spectrometry, Scoville

INTRODUCTION

Capsaicin is an alkaloid, derived from hot chilli pepper plants. It is an active component of the plants belonging to the Capsicum (pepper) genus. Capsaicin ($C_{18}H_{27}NO_3$), E-N-(4-hydroxy-3-methoxybenzyl)–8 –methylnon–6 enamide) has analgesic and antioxidant properties (Figure 1) (Lu, Ho, & Huang, 2017). Certain capsicum preparations have been used for the treatment of postherpetic neuralgia pain in recent years that are however, a strong irritant to skin and mucous membranes. Topical Capsaicin therapy may be a benefit in providing pain relief. Capsaicin patches are applied to the most painful areas of the skin.

Capsaicin and other members of the group of Capsaicinoids produce a large number of physiological and pharmacological effects such as effects on the gastrointestinal tract, the cardiovascular, and the respiratory system, as well as the sensory and thermoregulation systems. These effects result principally from the specific action of Capsaicinoids on primary afferent neurons of the C-fiber

Address for Correspondence:
İbrahim DANİŞ, e-mail: ibrahimdanis@outlook.com

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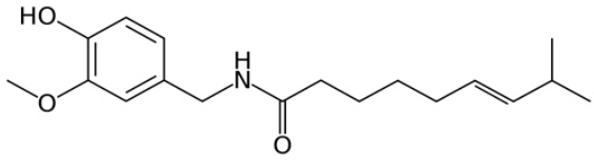


Figure 1. Chemical structure of Capsaicin.

type. This provides the rationale for their use to treat some peripheral painful states, such as rheumatoid arthritis (Surh & Lee, 1995). In addition, Capsaicinoids are powerful irritants, causing burning and pain at low concentrations on the skin and mucous membranes. Given orally, they induce an increase of salivation and gastric secretion, a rapid change of sensation, warm to intolerable burning, and gastrointestinal disorders depending on the dose (Govindarajan & Sathyanarayana, 1991).

Capsaicin was first isolated by John Clough Thresh in 1876 (Tresh, 1876) and the structure determined by E.K Nelson, and was first synthesized by E Spath and S.F Darling. Besides the analgesic properties of Capsaicin, in recent years, law enforcement has been using Capsaicin based pepper sprays against lawbreakers (Benzon2013).

Capsaicin helps to relieve chronic soft tissue pain, back pain, and neuropathic pain. Topical Capsaicin products are commonly used for pain relief. They have been available in various formulations such as lotions, creams, gels, or patches in low concentrations of Capsaicin. They have been in clinical use for many years to relieve pain, however, the effectiveness of Capsaicin in pain relief also has some adverse side effects, like allergies and irritation at specific concentrations causing burning and pain on the skin and mucous membranes. So the concentration of Capsaicin in topical formulations is important in avoiding side effects. A topical application of gel and cream contains 2.5% and 8% concentration of Capsaicin (Christo & Cauley, 2009). Because of the irritant and burning effect, the concentration of Capsaicin is reduced in strengths of 0.025% and 0.075% (Moon 2017). Capsaicin patches are used to treat patients with postherpetic neuralgia or neuropathy (especially HIV associated) and non-diabetic adults. The FDA and EU approved the use of the Capsaicin 8% patch in 2009 (Baranidharan et al., 2013; Anand et al., 2011; Laklouk et al., 2016). The amount of Capsaicin in the patches were important to the efficacy, safety, and tolerability of the patches (Anand et al., 2011; Laklouk et al., 2016). In this study, the methods were developed and validated for the determination of Capsaicin from patch formulations.

In the literature, various chromatographic methods were reported for the analysis of Capsaicinoids from natural products including High-Performance Liquid Chromatography (HPLC) with the detection of flourimetric (Daood et al., 2015), ultraviolet (Ciulu-Costinescu et al., 2015; Kuzma et al., 2015; Ashwini et al., 2015; Barbero et al.,2016) and mass spectrometric analysis (Barbero et al., 2016). A GC-MS determination of Capsaicin was also used for its analysis from pepper (You et al., 2013; Bononi et al., 2012; Pena-Alvarez et al., 2012; Peña-Alvarez et al., 2009; Ha et al., 2008). The Ultra-Fast Liquid Chromatographic method was developed by Usman et al. For analyzing multiple samples in a short time, the total run time was about 12 min (Usman et al., 2014). As a result of a full literature review, Capsaicin and DihydroCapsaicin (DHC) determination from pharmaceutical preparations by liquid chromatographic (LC) method was found only in the topical cream formulation. Sample preparation involves liquid-liquid extraction prior to LC analysis (Kaale et al., 2002).

This study aimed to develop and validate a sensitive and straightforward GC-MS method, then to analyze the Capsaicin level in patches to evaluate the irritant and burning effect. This is the first time in the literature that the SHU for the patch are determined and calculated. The chromatographic peak area of DihydroCapsaicin is used to calculate the Scoville Heat Units from the formula (Usman et al., 2014). A GC-MS method has been developed and validated for the calculation of SHU, which are important for the quantitation of Capsaicin from the pain patch and the control of the effectiveness and irritation properties of the patches.

MATERIAL AND METHODS

Chemicals and reagents

Methanol MS grade was purchased from Merck (Darmstadt, Germany). Capsaicin was supplied from Medigen (Medigen Pharma, Turkey). The Capsicum Oleoresin patch (53 mg Capsicum Oleoresin / 4.6 mg Capsaicin, 17 × 12 cm^2) was used for analysis. The Capsicum Oleoresin patch was purchased from the pharmacy.

Preparation of standard solutions

The standard stock solution of Capsaicin was prepared by dissolving with MeOH to obtain a final concentration of 1 $mg.mL^{-1}$. Capsaicin calibration curve solutions (5, 10, 20, 40, 50 µg/mL) and quality control samples (5; 25; 50 µg/mL) were prepared from stock solution by diluting with MeOH. All standard solutions were kept at +4°C.

Extraction procedure from patches

A 51.47 cm^2 patch containing 4.6 mg of Capsaicin was cut into four equal parts, each was placed in 50 mL falcon tubes, and each portion of the patch was extracted separately with 50 mL of MeOH (final concentration: 23 µg/mL). Samples were extracted with a rotary shaker for 5 hours. The alcoholic extract was taken by filtration and injected directly into the GC / MS system. The patches were stored at 4°C until assayed for volatile components such as menthol and camphor contained to keep the formulation stable.

Instrumentation and conditions

An Agilent 7890B Gas Chromatographic system equipped with a split or splitless injector and a 5977A MSD (Mass Spectrometer Detector) was used for the determination (Darmstad, Germany). HP-5MS (30 m X 0.25 mm) 0.25 µm film thickness (Agilent Technologies) analytical column was used in the separation process. The separation was made with a gradient oven temperature program. The oven temperature started at 250°C and was increased to 275°C at 10°C.min^{-1} ramp rate and held at 275°C for 2.5 min. The injection port was adjusted at 300°C and a split injection mode was used. The analysis was carried out in a split mode of 5:1 and the MS ionization potential was determined at 70 eV. The ion source and GC-MS transfer line temperature was selected as 300°C. The Scan mode spectra

of compounds are needed in mass spectrometric analysis. When examining the spectrum to determine candidate SIM ions to be used in the analysis, the compound-specific m/z ion or ions must be selected. SIM ions are important in a mass spectrometric analysis for precision. An analysis performed in a scanning mode in the range of 40-350 m/z and a value of m/z: 137, which is specific to the molecule and has the highest ion abundance, was selected for Capsaicin quantitation.

RESULTS

Development and optimization of the GC-MS method

Total ion chromatograms (TIC's) were obtained by using a standard solution of Capsaicin. When using an MS detector in scan mode, quantitation is usually done by monitoring a response for a specific ion in an analyte's mass spectrum. In many cases, this ion, termed the "quantitation ion", is the most abundant in the spectrum. Other lesser abundant ions may also be monitored to aid in proper identification of the analyte. These are often termed "qualifier" ions, and are not used in quantification of the peak. It is common practice to monitor 3 ions per compound. One ion signal is used to quantitate, and the others are used for qualitative information. The *m/z*: 137 ion was chosen for the quantification of Capsaicin (Figure 2). DihydroCapsaicin also has the same m/z: 137 fragment ion.

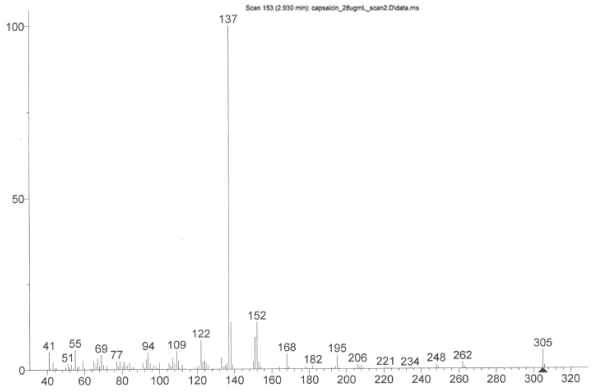


Figure 2. Mass spectrum of Capsaicin Standard.

Fragmentation of Capsaicin to m/z 137 is shown in Figure 3. In the method, developed retention time of Capsaicin and DihydroCapsaicin 2.9 and 3.0 min, respectively.

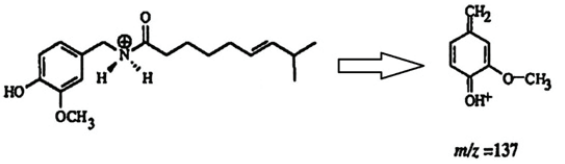


Figure 3. Fragmentation of m/z: 137.

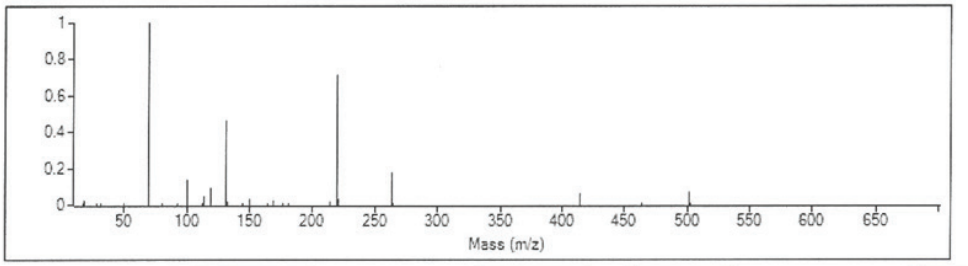
Strength and robustness studies aim to examine the effect of potential sources of variation in the response of the method. According to the ICH guidelines, the effect of flow rate and oven temperature in GC analyses is examined for the robustness of the method. When analyses were performed between flow rate ± 0.05 mL.min^{-1} and temperature ± 0.5°C, it was observed that it had no effect on the peak shape and area and retention time. Therefore the method developed is robust and rugged.

System suitability and tuning mass spectrometer

Tuning and calibration is performed to ensure that the mass spectrometer is working correctly, or that mass assignment and relative abundance of spectral signals resemble a previously determined standard. The tuning process will check that spectrometer contamination or degraded electronic components have not changed the assigned calibration of the mass axis. The MS system is tuned with a perfluorotributylamine (PFTBA) which is a known mass spectrum. The Autotune uses three ions from the PFTBA spectrum for tuning m/z: 69, 219 and 502. Before the analysis, systems were checked by tuning. Autotune provides information about the mass spectrometer; their sensitivities and responses change with time and usage. During tuning, the relative and absolute abundances of fragments of a known tuning compound are established, and the mass assignment, resolution, and spectral peak width generated by the mass analyzer are also adjusted and set. (Table 1). All the tuning parameters were found in acceptable limits (Figure 4).

Table 1. Autotune acceptance limits.

m/z	Relative abundance %	Isotop m/z	Isotop Ratio %
69.0	100	70.0	0.5-1.6
219.0	>35	220.0	3.2-5.4
502.0	>1	503.0	7.9-12.3



Target m/z	Actual m/z	Abund	Rel Abund	Iso m/z	Iso Abund	Iso Ratio
69.00	69.00	399,296	100.0%	70.00	4,503	1.1%
219.00	219.00	284,224	71.2%	220.00	12,325	4.3%
502.00	502.00	27,520	6.9%	503.00	2,847	10.3%

Figure 4. Autotune Results.

Linearity/Limit of quantification

A set of 5 calibration standards were prepared and analyzed in duplicate in three days. The calibration curve was constructed by plotting the area against the concentration at Capsaicin using linear regression analysis. The linearity of the method was demonstrated by the calibration equation and correlation coefficient (Table 2). The linearity of the method was found satisfactory $r^2 \geq 0.990 \pm 0.002$. The detection limit and quantification limit of the method are determined according to the signal / noise ratio. The LOD value was found to be 3.46 µg/mLand LOQ as 5 µg/mL (signal-noise>10).

Table 2. The Linearity data of the method (n=6).

Parameters	
Calibration Equation	y= 56158x -141036
Correlation Coefficient (r^2)	0.9973
Linear range (µg.mL^{-1})	5-50
LOQ (µg.mL^{-1})	5
LOD (µg.mL^{-1})	3.46

Selectivity

The selectivity of the method was performed by preparing the analyte and solvent that was used. It was observed that the signal was represented only by the analyte and chromatogram showed a very fine peak of analyte. There were no considerable changes in the area under curve or retention time evidently indicated the selectivity of the proposed method (Figure 5). There is no carry over seen during analysis.

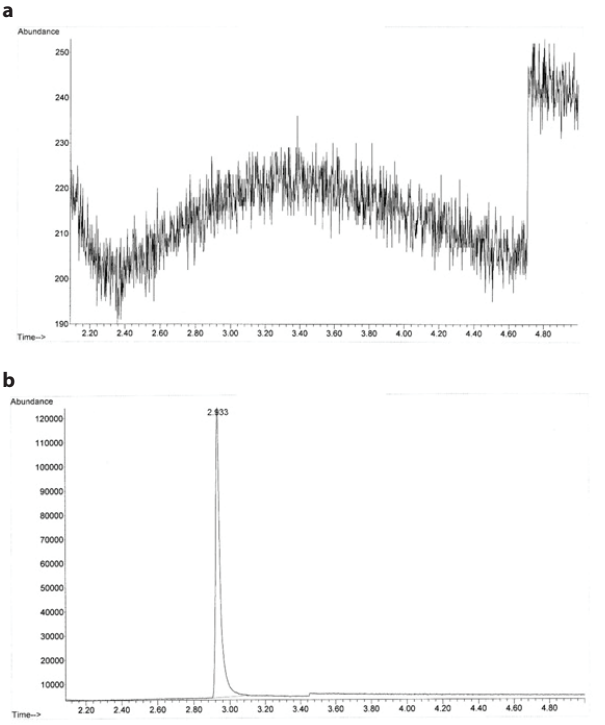


Figure 5. Blank sample chromatogram (a) and 5 µg.mL^{-1} Capsaicin chromatogram (b).

Accuracy and precision

The accuracy and precision was demonstrated by preparing low, high and medium concentrations samples according to calibration samples. The precision and accuracy of intra-day were settled by an analysis of six replicates of 3 concentrations including low, medium and high concentrations of quality control samples. Inter-day precision and accuracy were examined by the analysis of these quality control samples on three separate batches. The precision of the method was shown as the percentage of the coefficient of variation and the accuracy of the method was shown in terms of relative errors. The intra-day and inter-day accuracy as indicated by the standard deviation (SD) ranged from 0.1914 to 3.8383 (Table 3, 4). Intra-day

Table 3. Intra-day Accuracy and precision results.

Sample	Concentration (µg.mL^{-1})	Mean	Mean%	Standard Deviation (SD)	Relative Standard Deviation RSD	n
QC1	5	5.5369	110.7385	0.2877	5.1963	12
QC2	25	23.3753	93.5012	1.7569	7.5160	12
QC3	50	50.2949	100.5898	2.7037	5.3756	12

Table 4. Inter-day Accuracy and precision results (3 days for every concentration).

Sample	Concentration (µg.mL^{-1})	Mean	Mean%	Standard Deviation (SD)	Relative Standard Deviation RSD	n
QC1	5	5.1526 5.5547 5.4790	103.0526 111.0934 109.5801	0.5310 0.1914 0.3191	10.3048 3.4458 5.8237	18
QC2	25	23.6233 22.8733 24.1378	94.4933 91.4932 96.5513	2.3606 1.8400 2.0638	9.9925 8.0445 8.5501	18
QC3	50	49.3284 50.3633 50.3638	98.6567 100.7226 100.7276	3.4207 3.8383 3.6894	6.9345 7.6215 7.3256	18

and inter-day precision expressed by relative standard deviation (RSD) ranged from 3.4458 to 10.3048. The method developed was found to be accurate and precise.

Patch analysis

Capsaicin topical is used for the temporary relief of muscle or joint pain. Capsaicin can cause a burning sensation wherever it is applied. The method developed was applied for the determination of Capsaicin content in the pain patches which contain Capsaicine to avoid sensation. The patches were prepared as mentioned before in an extraction procedure from patch. The patch extracts were analyzed. Capsaicin and dihydroCapsaicin were separated by the developed method (Figure 6).

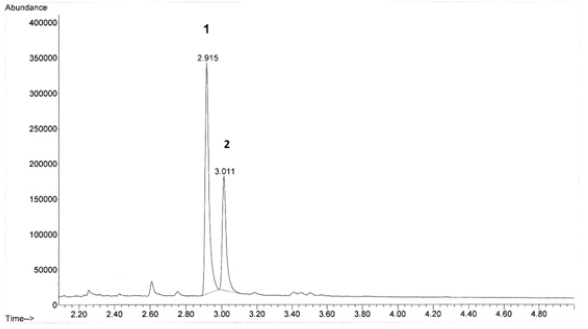


Figure 6. Chromatogram of Capsaicin and DihydroCapsaicin from the patch.

Calculation of Scoville heat units of patch

SHU was used to calculate the heat level of samples such as pepper. The units are calculated in parts per million of heat (ppmH) based on sample weigth (Usman et al., 2014). SHU is calculated by multiplying ppmH by a factor of 15. ppmH formula was converted to calculate the patch heat unit. The calculation of SHU of a Capsaicin pain patch is the first original study.

ppmH = [(peak area of Capsaicin in patch + 0.82) × (peak area of DHC in patch)] (Standart Capsaicin ppm in mL solution) / (total peak area of Standart Capsaicin) × (g Capsaicin in patch)

Capsaicin content is expressed in grams of Capsaicin per cm^2 of patch. For the conversion of the Scoville Heat Unit, the Capsaicin content in the patch is multiplied by a coefficient corresponding to the heat value for pure Capsaicin and calculated from the formula using the amounts of dyhydroCapsaicin. The Capsaicin Patch Scoville Heat Unit was calculated as 323.9 per cm^2 according to the equation given above.

DISCUSSION

In this study, the amount of Capsaicin from the pain patch was made by a gas chromatography-mass spectrometry and its irritating effects were determined by the SHU value. There are several methods to determine Capsaicin in the literature review. With the developed method, the retention times of Capsaicin and DihydroCapsaicin are 2.9 and 3.0 minutes, respectively. Compared to the methods found in the literature, it is one of the advantages of the method as it has a shorter analysis time compared to the UPLC method and other chromatographic methods. (Barbero et al., 2016). For compounds affected by temperature and light, it would be appropriate to reduce the analysis time to complete the analysis to reduce the risk of degradation of the compound. Capsaicin solution stability test results showed that protection from light at +4 ^oC increases the stability of the solution. (Kopec et al., 2002). This method can be applied to samples that take a long time to analyze and also in the case of a need for re-analysis for any reason. This investigation can also be used to determine the level of Capsaicin for Quality Control and the stability of a pharmaceutical preparation containing Capsaicin.

The calculated SHU of the Capsaicin patch was calculated as 323.9 per 1 cm^2 patch. Pure Capsaicin was rated between 15 and 16000000 SHU accordingly the SHU value of the Capsaicin patch calculated on the patch was found to be quite low. The amount of Capsaicin in the pain patch, its irritant effects, and its efficacy and safety appear to be low when evaluated by the SHU. Topical Capsaicin patch treatment can be beneficial in relieving pain without side effects.

CONCLUSION

The literature survey revealed that no Chromatographic determination of Capsaicin from pain patches is reported. This method was successfully applied to the analysis of Capsaicin from patches. The procedure is also accurate and precise, so recommended for routine quality control analysis. This study has the potential to be able to calculate the SHU of pain patches that contain Capsaicin.

Peer-review: Externally peer-reviewed.

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Conflict of Interest: The authors have no conflict of interest to declare.

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