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Determination of capsaicinoids in topical cream by liquid–liquid extraction and liquid chromatography

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Abstract

A reversed-phase liquid chromatography (LC) method has been developed, optimised and validated for the separation and quantitation of capsaicin (CP) and dihydrocapsaicin (DHCP) in a topical cream formulation. Sample preparation involves liquid–liquid extraction prior to LC analysis. The method uses a Hypersil C_{18} BDS, 5 μm, 250 × 4.6 mm I.D. column maintained at 35 °C. The mobile phase comprises methanol, water, acetonitrile (ACN) and acetic acid (47:42:10:1, v/v/v/v) at a flow rate of 1.0 ml/min. Robustness was evaluated by performing a central composite face-centred design (CCF) experiment. The method shows good selectivity, linearity, sensitivity and repeatability. The conditions allow the separation and quantitation of CP and DHCP without interference from the other substances contained in the cream.

Keywords: Capsaicin; Dihydrocapsaicin; Liquid–liquid extraction; Liquid chromatography; Thermocream®

1. Introduction

Capsaicin (CP) and dihydrocapsaicin (DHCP) are the principal alkaloids derived from capsicum fruit extracts of different of *Capsicum* species [1–3]. The chemical structures of capsaicinoids are shown in Fig. 1. Topically applied CP creams are helpful for a range of conditions, including nerve pain in diabetes (diabetic neuropathy), cancer pain, fibromyalgia, and rheumatoid arthritis pain [4–7].

Chromatographic methods have been reported for analytical separation and quantitation of naturally occurring capsaicinoids by gas chromatography [8–11], thin layer chromatography [12–15] and high performance liquid chromatography (LC) [16–19]. In 2001, Reilly et al. described LC–MS whereby CP, DHCP and nonivamide were monitored in self-defence pepper spray weapons [20].

A simple and fast micellar electrokinetic capillary chromatography method for determination of CP and DHCP from fruit extracts of various *Capsicum* species was described by Laskaridou-

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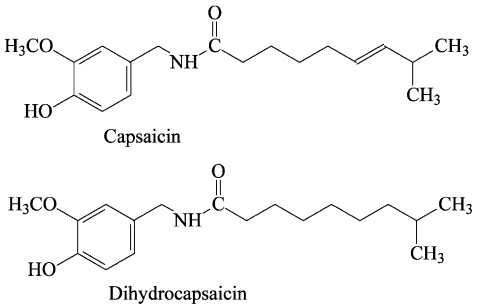

Fig. 1. Chemical structures of capsaicinoids.

Monnerville [21]. Perkins et al. recently reported a liquid chromatographic method with enzyme immunoassay detection for determination of capsaicinoids in salsa [22]. The United States Pharmacopeia (USP) prescribes an LC method for determination of CP, DHCP and other capsaicinoids in bulk products and in capsicum oleoresin [23].

None of these methods focuses on the determination of capsaicinoids in topical cream formulations. The aim of our study is to develop, optimise and validate a simple, fast and sensitive LC–UV method able to determine the total capsaicinoids in Thermocream®.

2. Experimental

2.1. Chemicals, samples and standards

Methanol HPLC grade was from Fisher chemicals (Loughborough, UK), acetonitrile (ACN) HPLC grade was from Acros Organics (Geel, Belgium), acetic acid was from Merck (Darmstadt, Germany). Double distilled water was used to prepare solutions. CP 97% and DHCP 90% were from Sigma-Aldrich Chemie (Steinheim, Germany). Pharmacobel Stérop (Brussels, Belgium) made available blank Thermocream base and Thermocream®. Composition: menthol 57.5 mg, methyl salicylate 57.5 mg, capsicum oleoresin 0.5% 7.2 mg, emulsifiers, purified water add to 1 g. A sample of 2.1 g for analysis corresponds to 76 μg (36 ppm) of capsaicinoids (CP+DHCP) and this concentration is assigned the 100% value. Normally, the ratio of CP/DHCP in capsicum oleoresin is about 3:1.

2.2. Chromatographic system

The isocratic LC system consisted of a Spectra Physics P4000 LC pump, a Spectra Series AS3000 autosampler, a Thermo Quest/Linear, UV–VIS 200 detector set at 280 nm (TSP, San Jose, CA, USA) and an HP 3396 series III integrator (Hewlett–Packard, Avondale, PA, USA). A Hypersil C_{18}, BDS 5 μm, 250 × 4.6 mm, column was used (Thermo Quest, Runcorn, Cheshire, UK). The flow rate was maintained at 1.0 ml/min. A 100 μl loop was used. The column was maintained at 35 °C by means of a water bath heated by Julabo EM thermostat (Julabo, Seelbach, Germany). The mobile phase composition was: methanol–water–acetonitrile–acetic acid (47:42:10:1, v/v/v/v).

2.3. Preparation of standards

Standard stock solutions: CP 0.076 mg/ml and DHCP 0.076 mg/ml in methanol–water (80:20). These concentration values are corrected for the standard content reported in Section 2.1. Aliquots of 0.5, 1.0, 1.5 ml of this solution were diluted to 50.0 ml with methanol–water (80:20) to prepare the standard solutions corresponding to 50, 100, and 150%, respectively. The 1.0 ml aliquot gives a concentration of 1.52 μg/ml.

2.4. Preparation of sample

Into a 100 ml conical flask, 2.1 g of the cream was weighed and 20.0 ml of methanol–water (80:20) and 20.0 ml of hexane were added. The mixture was thoroughly mixed with the help of magnetic stirring for 10 min to make a uniform emulsion. The emulsion was centrifuged at 2880 g for 20 min and 15.0 ml of the lower aqueous layer was pipetted into a 50.0 ml volumetric flask. The extraction procedure was repeated twice by adding each time 15.0 ml of methanol–water (80:20) and withdrawing the same volume. This makes a total volume of 45 ml, which was brought to 50.0 ml using the same diluent. This solution (100 μl) was

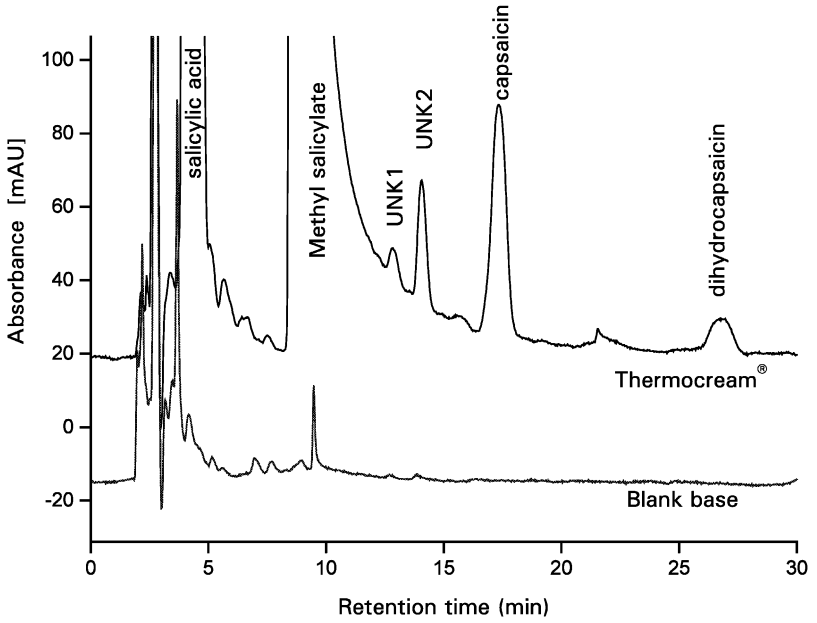

Fig. 2. Overlaid chromatograms of a Thermocream® (upper) and blank cream base (lower). Chromatographic conditions as described in the Section 2.

directly injected. The concentration of CP+DHCP corresponding to 100% is 1.52 μg/ml.

3. Results and discussion

3.1. Chromatography

Fig. 2 shows a typical chromatogram. Separation of CP and DHCP from all other components was achieved in a reasonable analysis time. The water–methanol–acetic acid system prescribed in the USP monograph for determination of capsaicinoids in capsicum oleoresin was first considered [23]. The use of methanol alone as organic modifier resulted in the co-elution of CP with two unknowns in the shoulder of the methylsalicylate peak. This problem was solved by combining methanol with ACN, which improves the resolution of CP from the adjacent unknown peaks to baseline separation. The consideration of ACN came in the light of the monograph for CP, which uses 40% ACN in water/phosphoric acid prescribed by USP [23]. The specificity of this method proved to be good, no peak coming from the matrix or other active ingredients interfered with the quantitation of peaks of interest. See Fig. 2.

3.2. Robustness

Due to the complex interaction of the different factors influencing the resolution among the components, the aid of appropriate computer software was employed in the design of robustness experiments and data analysis. MODDE 4.0 (Umetri, Umeå, Sweden) software was used for this purpose. A central composite face-centred design (CCF) permits the response to be modelled by fitting a second order polynomial Eq. (i), with a total of $N = 2^k + 2k + c$ experimental combinations. The first term is related to full factorial design, the second to the star points, which enables to estimate the degree of curvature and the third to the centre points. From the repetition of the centre points the experimental variance at the centre of the domain can be estimated. To determine the relationship between the controlled response variables characterising the separation process and the resolution obtained, a second order polynomial

Table 1
Factorial analysis values corresponding to low, central and high levels of separation parameters under investigation

Variables	Experimental range investigated		
	Low	Central	High
ACN (%)	8	10	12
Temperature (°C)	30	35	40
Acetic acid (%)	0.9	1.0	1.1
Flow rate (ml/min)	0.9	1.0	1.1

Number of experiments, $N = 2^k + 2k + 3 = 27$; k, number of variables.

Eq. (i) can be used to fit the data. Four controlled variables, i.e. amount of ACN = 1, acetic acid (AcOH) = 2, Temperature = 3 and Flow rate = 4 were investigated and are represented in this equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{12} X_1 X_2 + \ldots + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + E \quad \text{(i)}$$

Where β is regression coefficient, E is overall experimental error, X is variables and Y is response. The coefficient β_1 describes the linear quantitative effect of the variable in this model. The cross product, β_{12} will measure the interaction effect between the variables and the square term $\beta_{11} X_1^2$ will describe non-linear effect on response. The 95% confidence limits are expressed by using error bars. A regression coefficient smaller than the error bar interval shows that the variation of the response caused by changing the variable is insignificant. The low (−1), central (0) and high (1) values of the parameters used in the study are listed in Table 1. CP and unknown peak 2 (UNK2) shown in Fig. 2 was chosen as critical peak pair. In correlation with the four factors investigated, the experimental design required 27 randomised runs. A duplicate set of experiments was performed. Fixing the amount of methanol to 47% (v/v) and varying the amount of ACN, enabled investigation of the influence of the ratio of methanol–acetonitrile in this system. The regression coefficient plots in Fig. 3 and the response surface plots in Fig. 4 were generated. All parameter effects exhibited a negative influence on the resolution between CP and UNK2. The influence is significant only for ACN. The influences of the parameter interactions were non-significant. Increasing the concentration of ACN decreases resolution while changing temperature and amount of acetic acid do not affect the resolution significantly (Fig. 4). The

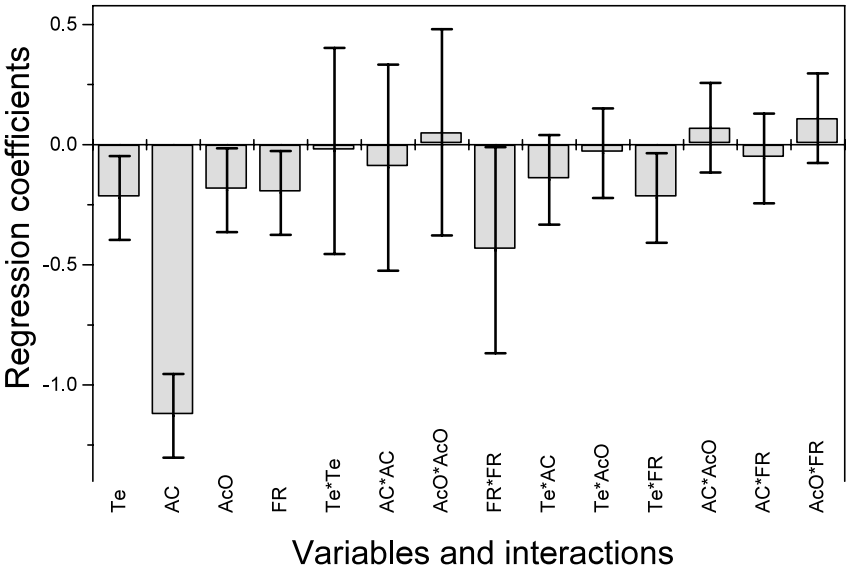

Fig. 3. Regression coefficients as a function of influential variables and interactions. Te, temperature; AC, acetonitrile; AcO, acetic acid; and FR, flow rate.

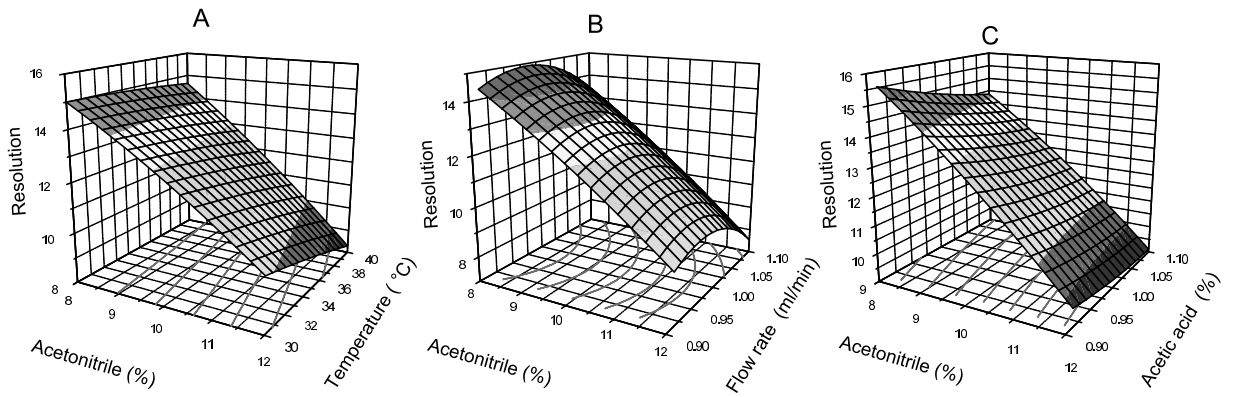

Fig. 4. Response surface plots showing the influence of amount of ACN, acetic acid, temperature and flow rate on the resolution of CP from UNK2.

Table 2
Recoveries at three concentration levels

Level	Extraction number Ψ	Recovery (%)
50%	I	94.8 (1.5)
	II	95.7 (0.9)
	III	95.9 (0.9)
Mean		95.5 (0.6)
100%	I	97.4 (0.9)
	II	96.3 (1.8)
	III	97.2 (1.4)
Mean		96.9 (0.6)
150%	I	95.8 (0.4)
	II	98.1 (3.8)
	III	97.7 (1.7)
Mean		97.2 (1.3)

Ψ number of analyses = 4, R.S.D. values are mentioned in parentheses.

effect of changing the flow rate displayed an optimal point at 1 ml/min (Fig. 4B). Nevertheless, throughout this study, the resolution of the critical pair was always above eight. It can be stated that the method robustness is sufficient to allow determination of capsaicinoids from cream formulation without any interference from other ingredients or blank.

3.3. Recovery

The percent recovery was determined at three CP concentration levels, i.e. 50, 100 and 150% relative to 1.52 μg/ml CP standard. This was achieved by spiking 0.5, 1.0 and 1.5 ml of 0.076 mg/ml into 2.1 g of the blank cream base followed by liquid–liquid extraction as described in Section 2.4. The percent recovery was expressed relative to 1.52 μg/ml CP standard. This concentration corresponds to the total concentration of capsaicinoids expected from 2.1 g of the cream sample. For each concentration three extractions were performed and each was analysed four times. In all determinations recoveries of more than 95% were achieved with R.S.D. values between 0.6 and 1.3% ($n = 4$). See Table 2.

3.4. Quantitative aspects

The limit of quantitation (LOQ) at signal-to-noise ratio (S/N) = 10 is for CP 13 ng/ml (9%), (R.S.D. 7.4% $n = 6$) and for DHCP 15 ng/ml (10%), (R.S.D. 7.5%, $n = 6$). The limit of detection (LOD) at S/N = 3 is 3.4 ng/ml (2.2%) for CP and is 4.9 ng/ml (3.2%) for DHCP relative to 1.52 μg/ml CP and 1.52 μg/ml DHCP, respectively. For a Thermocream® sample extract prepared as described in Section 2.4, the intraday and interday R.S.D. values were 0.9% ($n = 6$) and 3% ($n = 18$, 6 days), respectively, for CP and 12% ($n = 6$) and 14% ($n = 18$, 6 days), respectively, for DHCP.

The calibration curves show a linear relationship in the range investigated. CP, range 10–150%, $y = 7447x + 30\,527$, $r^2 = 0.9938$ and $S_{y,x} = 37\,772$ and DHCP, range 10–50%, $y = 7897x + 4085$, $r^2 = 0.9999$, $S_{y,x} = 3998$, y is peak area measurement,

Table 3
Determination of total capsaicinoids in Thermocream®

	Extraction number Ψ	CP	DHCP	CP+DHCP
Sample I	I	73.7 (0.8)	16.0 (6.4)	90.3 (1.6)
	II	74.4 (1.4)	17.4 (9.2)	91.9 (1.9)
	III	76.4 (0.2)	18.0 (8.5)	94.5 (1.4)
Sample II	I	73.6 (0.9)	17.5 (1.7)	90.8 (0.7)
	II	75.4 (1.3)	17.6 (3.9)	93.1 (1.6)
	III	74.4 (0.6)	17.9 (7.2)	92.3 (1.9)
Sample III	I	73.4 (0.9)	16.4 (4.2)	89.9 (1.2)
	II	74.3 (1.7)	17.9 (5.8)	92.2 (2.1)
	III	75.1 (1.5)	17.6 (10.0)	93.1 (2.0)
Sample IV	I	74.3 (1.5)	17.3 (3.3)	91.6 (1.7)
	II	76.0 (0.9)	19.4 (4.5)	95.5 (1.5)
	III	76.0 (0.9)	17.9 (11.5)	94.0 (3.2)
Mean		74.7	17.6	92.3
S.D.		1.0	0.8	1.6
R.S.D.% ($n = 12$)		1.3	4.7	1.8

Ψ Number of analyses = 4, R.S.D. values are mentioned in parentheses.

x is the percent concentration, r is the coefficient of correlation, $S_{y,x}$ is standard error of y estimate, three injections per concentration.

3.5. Analysis of Thermocream® sample

Four tubes of Thermocream® were randomly selected and analysed by using the described method. Table 3 summarises the percent content and the respective R.S.D. values. Twelve samples (three from each tube) were extracted and each extract was analysed four times. The mean of each group of analyses is reported in Table 3. The mean for the total capsaicinoids is 92.4% with R.S.D. value of 1.8%, $n = 12$.

4. Conclusion

A simple, robust and selective method for the determination of capsaicinoids in topical cream has been developed and validated. This method shows good sensitivity, selectivity, repeatability and linearity. It was applied satisfactorily to the quantitative analysis of capsaicinoids in Thermocream®.

References

[1] R. Gamse, A. Wax, R. Zigmond, S. Leeman, Neuroscience 6 (1981) 437–441.
[2] F. Heresch, J. Jurenitsch, Chromatographia 12 (1979) 647–650.
[3] A. Gbolade, O. Omobuwajo, O. Soremekun, J. Pharm. Biomed. Anal. 15 (1997) 545–548.
[4] B. Lynn, Pain 41 (1990) 61–69.
[5] R. Tandan, G. Lewis, P. Knisinski, G. Badger, T. Files, Diabetes Care 15 (1992) 8–14.
[6] W.Y. Zhang, A.L. Po, Eur. J. Clin. Pharmacol. 46 (1994) 517–522.
[7] H. McQuay, D. Carroll, A. Jadad, P. Wiffen, A. Moore, Br. Med. J. 311 (1995) 1047–1052.
[8] Y. Masada, K. Hashimito, T. Inoue, M. Suzuki, J. Food Sci. 36 (1971) 858–862.
[9] P. Todd, M. Bensinger, T. Biftu, J. Food Sci. 42 (1977) 660–667.
[10] K. Iwai, T. Suzuki, H. Fujiwake, S. Oka, J. Chromatogr. 172 (1979) 303–311.
[11] J. Jurenitsch, R. Leinmuller, J. Chromatogr. 189 (1980) 389–397.
[12] S. Kosuge, M. Furuta, Agr. Biol. Chem. 34 (1970) 248–256.
[13] P. Todd, M. Bensinger, T. Biftu, J. Chromatogr. Sci. 13 (1975) 577–579.
[14] T. Suzuki, T. Kawada, K. Iwai, J. Chromatogr. 198 (1980) 217–223.
[15] D. Pankar, N. Magar, J. Chromatogr. 144 (1977) 149–152.
[16] O. Sticher, F. Soldati, R. Joshi, J. Chromatogr. 166 (1978) 221–231.
[17] J. Jurenitsch, E. Bingler, H. Becker, W. Kubelka, Planta Med. 36 (1979) 54–60.

[18] F. Heresch, J. Jurenitsch, Chromatographia 12 (1979) 647–650.
[19] J. Jurenitsch, I. Kampelmühler, J. Chromatogr. 193 (1980) 101–110.
[20] C. Reilly, D. Crouch, G. Yost, A. Fatah, J. Chromatogr. A 912 (2001) 259–267.
[21] A. Laskaridou-Monnerville, J. Chromatogr. A 838 (1999) 293–302.
[22] B. Perkins, R. Bushway, K. Guthrie, T. Fan, B. Stewart, A. Prince, M. Williams, J. AOAC Int. 85 (2002) 82–85.
[23] United States Pharmacopeia, 25th ed., United States Pharmacopeia Convention, Rockville, MD, 2002, pp.296–297.