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Use of multivariate statistical techniques to optimize the separation of 17 capsinoids by ultra performance liquid chromatography using different columns

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ABSTRACT

In this work a multivariate statistical tool (Derringer and Suich optimization) was proposed for the separation of seventeen capsinoids (natural and synthetic) using the UHPLC-DAD chromatography. Capsinoids were analyzed at 280 nm. The variables optimized were the mobile phase (water (0.1% acetic acid as solvent A) and acetonitrile (0.1% as solvent B)), gradient time and flow rate. Two columns with different length (50 and 100 mm) were used for the chromatographic separation. The two columns used properly separated the seventeen capsinoids, however the 100 mm column length showed a better chromatographic separation with a shorter run time and smaller peak widths. These results provided better values of limit of detection and quantification for the 100 mm column length. The better conditions of separation with the 100 mm column length were established with: initial mobile phase with 41.8% of solvent B; 3.96 min of linear gradient time to reach 100% of solvent B; flow rate of 0.679 mL min^{-1}. A validation of the method has been done with excellent values of repeatability (RSD < 1.92) and intermediate precision (RSD < 3.92). The developed method has been applied to real samples. Capsiate has been identified and quantified in some varieties of peppers.

1. Introduction

Chilli peppers are the spicy fruits from plants of the genus *Capsicum* (Solanaceae), which are native to Central and South America [1]. Peppers are used as spices to add aroma and flavor to foods and they are commercially very important [2]. The main characteristic of peppers is their pungency, which is caused by chemical compounds known as capsaicinoids [3–5]. Apart from capsaicinoids, this fruit is also a source of other nutraceutical compounds such as phenolic compounds, carotenoids, ascorbic acid and tocopherol [6]. However, the consumption of these functional compounds is limited by the pungent flavor caused by capsaicinoids [7].

Capsinoids are non-pungent compounds that are similar to capsaicinoids in terms of structure and biological activities and they were recently isolated from some sweet peppers. Capsinoids have been shown to have similar benefits to capsaicinoids, e.g. anti-inflammatory, analgesic [8], anti-microbial, anti-mutagenic, anti-tumor [9] and antioxidant properties [10], but they do not have a pungent flavor [11–14]. The structural difference between the capsaicinoids and capsinoids is the way in which the carbon chain is bound to the aromatic ring: by an amide moiety in capsaicinoids and by an ester moiety in capsinoids [7]. Capsinoids have proven to be beneficial for human health since they promote the body's metabolism and suppress body fat accumulation [15–18].

Quantification of the major and minor capsinoids in chilli peppers is problematical because the analysis of these compounds has not been studied previously. There are some reports on the synthesis of capsinoids and their biological activities have been studied [14,19–21]. Very few studies have been carried out on the separation of capsiate and dihydrocapsiate [7] and the chromatographic separation and quantification of minor compounds in chilli peppers have not been studied at all.

The main benefit of the optimization process is the reduction of the time and costs of the general process. The multivariate

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optimization allows seeing interactions factors between the optimized variables. This is not possible when the univariate optimization is used.

Since food samples studied often involve a large number of analytical peaks, many of which must be separated, the optimization process must take into consideration all the critical separation simultaneously. A set of experimental conditions that results in good separations for some peaks may not resolve other peaks that are overlapped. As such multi-criteria methods such as the one proposed by Derringer and Suich [22] are very convenient to use if accurate response surfaces have been determined from experimental results of a statistical design. This experimental strategy has been recently applied to the optimization of analytical systems in high performance liquid chromatography [23,24].

The objective of the work described here was to use multivariate statistic techniques to separate capsiate, dihydrocapsiate and another fifteen minority capsinoids by UHPLC-DAD.

2. Experimental

2.1. Reagents

2.1.1. Chromatographic reagents

Acetonitrile and acetic acid, both HPLC grade, were obtained from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q water deionization system (Millipore, Bedford, MA, USA). Capsinoid standards were synthesized according to the methodology described by Barbero et al. [25]. All solvents and the standard solution were filtered through a membrane system with a pore diameter of 0.2 μm. The standard solution was stored at −20 °C prior to analysis.

2.1.2. Reagents for the synthesis of capsinoids

Propionyl chloride (98%), butyryl chloride (98%), pentanoyl chloride (98%), hexanoyl chloride (99%), heptanoyl chloride (99%), octanoyl chloride (99%), nonanoyl chloride (96%), decanoyl chloride (98%), lauroyl chloride (98%), tridecanoic acid (98%), myristoyl chloride (97%), pentadecanoic acid (99%), palmitoyl chloride (98%), t-butyldimethyl silyl chloride (97%), and diisobutyl aluminum hydride (1 M in toluene) (DIBAL) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Acetic anhydride (98%), hydrochloric acid (37%), nitric acid (65%), sodium chloride (99.0–100.5%), ethanol (99.5%), sodium hydrogen carbonate (99.0–100.5%), sodium hydroxide (98.0–100.5%), *N*,*N*-dimethylmethanamide (DMF) (99%), dehydrated pyridine (99%) and tetrahydrofuran (THF) (99.5%) were purchased from Panreac Química S.A. (Castellar del Valle´s, Barcelona, Spain). Undecanoic acid (99%) and 8-methylnonanoic acid (97%) were purchased from Acros Organics (New Jersey, USA). *cis*-8-Methyl-6-nonenoic acid (97%) was purchased from Maybridge (Tintagel, Cornwall, UK). Ethyl acetate, chloroform, stabilized with ethanol, and hexane were purchased from Scharlau Chemie S.A. (Sentmenat, Barcelona, Spain). Thionyl chloride (99%) was purchased from Merck (Hohenbrunn, Germany). Sodium nitrite (99%) was purchased from Merck (Darmstadt, Germany).

2.2. Capsinoids identification

The capsinoids that were chemically synthesized were 4-hydroxy-3-methoxybenzyl ethanoate (O2C), 4-hydroxy-3-methoxybenzyl propanoate (O3C), 4-hydroxy-3-methoxybenzyl butanoate (O4C), 4-hydroxy-3-methoxybenzyl pentanoate (O5C), 4-hydroxy-3-methoxybenzyl hexanoate (O6C), 4-hydroxy-3-methoxybenzyl heptanoate (O7C), 4-hydroxy-3-methoxybenzyl octanoate (O8C), 4-hydroxy-3-methoxybenzyl nonanoate (O9C), 4-hydroxy-3-methoxybenzyl decanoate (O10C), 4-hydroxy-3-methoxybenzyl undecanoate (O11C), 4-hydroxy-3-methoxybenzyl dodecanoate (O12C), 4-hydroxy-3-methoxybenzyl tridecanoate (O13C), 4-hydroxy-3-methoxybenzyl tetradecanoate (O14C), 4-hydroxy-3-methoxybenzyl pentadecanoate (O15C), 4-hydroxy-3-methoxybenzyl hexadecanoate (O16C), 4-hydroxy-3-methoxybenzyl 8-methylnonanoate (Dihydrocapsiate, DHCTO) and 4-hydroxy-3-methoxybenzyl (*E*)-8-methyl-6-nonenoate (Capsiate, CTO).

The purity of each compound was determined by ^{1}H NMR and ^{13}C NMR analyses, and was found to be ≥ 98%. ^{1}H and ^{13}C spectra were recorded using $CDCl_3$ as the solvent, in a Varian INOVA spectrometer, at 399.952 and 100.577 MHz, respectively. The resonances of residual chloroform for ^{1}H and ^{13}C were set to δ_H 7.25 ppm and δ_C 77.00 ppm, respectively, and used as internal reference. UV–vis spectra were obtained using a Varian Cary 50 BIO spectrophotometer, with chloroform as the solvent.

To confirm the structure of the synthesized capsinoids, a chromatographic method using ultra-performance liquid chromatography (UHPLC) coupled to quadrupole-time-of-flight mass spectrometry (Q-ToF-MS) (Synapt G2, Waters Corp., Milford, MA, USA). has been developed. The injection volume was set to 3 μL. The chromatographic separation was performed on a reverse-phase C18 analytical column (Acquity UPLC BEH C18, Waters) of 2.1 mm × 100 mm and 1.7 μm particle size. Masslynx software, version 4.1, was used to control the equipment and for the acquisition and treatment of data.

For the identification of capsinoids, water (0.1% formic acid) and methanol (0.1% formic acid) as mobile phases at a flow rate of 0.5 mL min^{-1} was used. The elution gradient employed was as follows: 0 min, 45% B; 5 min, 100% B; 8.00 min, 100% B. Total run time was 12 min, including 4 min for re-equilibration. The determination of the analytes was carried out using an electrospray source operating in positive ionization mode under the following conditions: desolvation gas flow = 850 L h^{-1}, desolvation temperature = 500 °C, cone gas flow = 10 L h^{-1}, source temperature = 150 °C, capillary = 0.7 eV, cone voltage = 20 V and trap collision energy = 4 eV. Full-scan mode was used (m/z = 100–600). Capsinoids structures were confirmed by the results obtained by UHPLC-Q-ToF-MS.

2.3. Equipment

For the separation study a UHPLC (ACQUITY UPLC H-Class, Waters) system was used and this was equipped with an ACQUITY UPLC quaternary pump system, an ACQUITY UPLC auto sampler with temperature control adjusted to 15 °C, an ACQUITY UPLC Photodiode Array Detector and a column oven. Two columns with different lengths were used: Waters ACQUITY UPLC BEH C18 (2.1 mm I.D.; 1.7 μm particle size; 50 mm length) and Waters ACQUITY UPLC BEH C18 (2.1 mm I.D.; 1.7 μm particle size; 100 mm length). Two mobile phases were used: water (0.1% acetic acid) as solvent A and acetonitrile (0.1% acetic acid) as solvent B. For the analysis, capsinoids were measured at a wavelength of 280 nm and the column oven was set at 50 °C for the chromatographic separation.

2.4. Experimental design and data treatment

Four variables were optimized simultaneously: mobile phase, gradient time, flow rate and column length. The column was fixed at two levels (50 and 100 mm) and for each level a central composite design with the other variables was performed [23]. A central composite design with three variables was used for the separation study with the two columns. The first variable, 'initial percentage of acetonitrile', was varied from 0 to 50% for the two columns. The second variable, 'linear gradient time to 100% of acetonitrile', was varied from 3 to 10 min for the two columns. The

third variable, 'flow rate', was varied from 0.4 to 0.8 mL min^{-1} for the shortest column (50 mm column length) and from 0.4 to 0.7 mL min^{-1} for the longest column (100 mm column length). It was not possible to use a flow rate of 0.8 mL min^{-1} for the 100 mm column because this flow rate exceeds the pressure limit of the column. The response chosen to evaluate the best separation conditions was the resolution. Response values were calculated using the equation below:

$$R_S = \frac{2(t_2 - t_1)}{(w_2 + w_1)}$$

for which t_1 and t_2 are retention times and w_1 and w_2 are the corresponding widths of the bases of the pair of adjacent peaks. Each model was validated by analysis of variance (ANOVA $p < 0.05$) and the optimum conditions for the 17 capsinoids were determined by a response surface graph and the multi-criteria response technique of Derringer and Suich [13,23]. The objective of the chromatographic optimization was firstly to separate the 17 capsinoids in the lowest possible analysis time and secondly to identify which column had the best relation between the chromatographic separation and analysis time. All experiments were carried out randomly in triplicate in the central point.

2.5. Validation

Several parameters, including linearity, repeatability (intraday and interday), limit of quantification (LOQ) and limit of detection (LOD), were studied for method validation. The linearity was verified with an analytical curve consisting of seven points (in triplicate) for each compound. The intraday repeatability was calculated by the relative standard deviation of 10 injections of the solution containing the 17 capsinoids. The intermediate precision was calculated by the relative standard deviation of 10 injections of the same standard solution on 3 consecutive days (30 injections in total). The LOQ and LOD were estimated as 3 and 6 times the signal-to-noise ratio, respectively. A lack of fit test for each calibration curve was performed as recommended by Danzer and Currie [26].

2.6. Extraction procedure

The extracts from the pepper samples were obtained using an ultrasound-assisted extraction technique, according to our previously developed method for capsaicinoids [5]. Ultrasonic irradiation was carried out using a UP200S sonifier (200 W, 24 kHz) (Hielscher Ultrasonics, Teltow, Germany), with the sample immersed in a water bath coupled to a temperature controller (Frigiterm-10, J.P. Selecta, S.A., Barcelona, Spain). For the extraction of the capsinoids the following extraction parameters were used: extraction solvent: methanol; temperature: 50 °C; output amplitude of the nominal amplitude of the transducer: 100% (200 W); duty cycle: 0.5 s; solvent volume: 25 mL; extraction time: 10 min; amount of sample: 0.5 g. The extracts were filtered through a 0.22 μm nylon syringe filter (Membrane Solutions, Dallas, USA) prior to chromatographic analysis. All the extractions were carried out in triplicate.

3. Results and discussion

3.1. Statistical analysis

The resolutions were calculated for the three pairs of peaks that were most difficult to separate (all the other peaks were completely separated when these three pairs of peaks were separated sufficiently). The three pairs of peaks in question were O2C-O3C, O9C-CTO and O10C-DHCTO. Run time was added to the optimization, meaning that a total of four variables were optimized simultaneously. The responses for each set of experimental conditions for the 100 mm and 50 mm column are shown in Tables 1 and 2, respectively.

After the experiments each response was analyzed by analysis of variance (ANOVA $p < 0.05$) in order to evaluate the F values of the regression and the lack of fit. The ANOVA results and the comparison between the F values and their respective critical F values are shown in Table 3.

In the response 'analysis time' for the two columns of different lengths it can be seen that very high F values are obtained from the regression and lack of fit. The F value is the result of a division by the pure error and the MS (regression or lack of fit). For these responses the pure error was very small, which in turn led to a marked increase in the F values. In these cases there is a false positive and this mainly concerns the lack of fit. The false positive is also evident on comparing the 'predicted values' with the 'real values' for these models and these data, (Table 5) showing a good

Table 1
Central composite design and resolutions of the three critical pairs of peaks and run time for each experiment with the 100 mm column.

N° Experiment	Variables			O2C-O3C*	O9C-CTO*	O10C-DHCTO*	Run time**
	Initial ACN (%)	Gradient time (min)	Flow rate (mL min^{-1})				
1	10.1 (−1)	4.2 (−1)	0.46 (−1)	1.89	0.95	0.94	5.24
2	39.9 (1)	4.2 (−1)	0.46 (−1)	1.68	1.04	0.99	4.89
3	10.1 (−1)	8.58 (1)	0.46 (−1)	4.10	1.27	1.20	7.79
4	39.9 (1)	8.58 (1)	0.46 (−1)	2.37	1.28	1.30	7.05
5	10.1 (−1)	4.2 (−1)	0.64 (1)	2.87	1.03	1.02	4.54
6	39.9 (1)	4.2 (−1)	0.64 (1)	2.07	1.07	1.08	4.21
7	10.1 (−1)	8.58 (1)	0.64 (1)	4.11	1.14	1.40	7.01
8	39.9 (1)	8.58 (1)	0.64 (1)	2.57	1.15	1.50	6.21
9	25 (0)	6.5 (0)	0.55 (0)	3.49	1.11	0.93	5.88
10	25 (0)	6.5 (0)	0.55 (0)	3.62	1.11	1.02	5.88
11	25 (0)	6.5 (0)	0.55 (0)	3.58	1.14	1.07	5.88
12	0 (−1.68)	6.5 (0)	0.55 (0)	3.72	1.10	1.06	6.23
13	50 (1.68)	6.5 (0)	0.55 (0)	1.67	1.13	1.28	5.29
14	25 (0)	3 (−1.68)	0.55 (0)	1.78	1.01	0.91	3.87
15	25 (0)	10 (1.68)	0.55 (0)	4.84	1.06	1.49	7.77
16	25 (0)	6.5 (0)	0.4 (−1.68)	2.76	1.19	1.14	6.66
17	25 (0)	6.5 (0)	0.7 (1.68)	3.82	1.10	1.19	5.41

* Resolutions in each optimization condition.
** Minutes.

Table 2
Central composite design and resolutions of the three critical pairs of peaks and run time for each experiment with the 50 mm column.

N° Experiment	Variables			O2C-O3C*	O9C-CTO*	O10C-DHCTO*	Run time**
	Initial ACN (%)	Gradient time (min)	Flow rate (mL min^{-1})				
1	10.1 (−1)	4.42 (−1)	0.48 (−1)	2.13	0.88	0.78	4.45
2	39.9 (1)	4.42 (−1)	0.48 (−1)	1.28	0.87	0.95	4.07
3	10.1 (−1)	8.58 (1)	0.48 (−1)	4.01	0.85	1.03	6.74
4	39.9 (1)	8.58 (1)	0.48 (−1)	3.95	0.86	1.11	6.74
5	10.1 (−1)	4.42 (−1)	0.72 (1)	3.11	0.90	0.91	3.84
6	39.9 (1)	4.42 (−1)	0.72 (1)	1.68	0.91	1.20	3.44
7	10.1 (−1)	8.58 (1)	0.72 (1)	4.67	1.01	1.11	6.00
8	39.9 (1)	8.58 (1)	0.72 (1)	1.67	1.01	1.17	5.08
9	25 (0)	6.5 (0)	0.6 (0)	2.28	0.86	0.98	4.96
10	25 (0)	6.5 (0)	0.6 (0)	2.34	0.94	1.09	4.96
11	25 (0)	6.5 (0)	0.6 (0)	2.36	0.92	1.07	4.96
12	0 (−1.68)	6.5 (0)	0.6 (0)	3.77	0.99	1.04	5.38
13	50 (1.68)	6.5 (0)	0.6 (0)	1.13	0.95	1.17	4.26
14	25 (0)	3 (−1.68)	0.6 (0)	1.74	0.87	0.86	3.16
15	25 (0)	10 (1.68)	0.6 (0)	2.96	0.98	1.19	6.58
16	25 (0)	6.5 (0)	0.4 (−1.68)	1.38	0.86	0.97	5.74
17	25 (0)	6.5 (0)	0.8 (1.68)	2.72	0.99	1.08	4.52

* Resolutions in each optimization condition.
** Minutes.

Table 3
Summary of ANOVA with the significance of regression and lack of fit.

Column	Response	Regression		Lack of fit	
		MS_R/MS_r*	*F* 95%***	MS_{lof}/MS_{PE}**	*F* 95%***
100 mm Column	O2C-O3C Resolution	4.42 (3.3)	9.28	41.48 (5.2)	19.3
	O9C-CTO Resolution	4.82 (3.3)	9.28	15.37 (11.2)	19.4
	O10C-DHCTO Resolution	7.11 (3.3)	9.28	0.47 (5.2)	19.3
	Analysis Time Resolution	168.53 (3.3)	9.28	234.55 (5.2)	19.3
50 mm Column	O2C-O3C Resolution	8.87 (3.3)	9.28	318.69 (11.2)	19.4
	O9C-CTO Resolution	7.5 (3.3)	9.28	0.77 (11.2)	19.4
	O10C-DHCTO Resolution	15.01 (3.3)	9.28	1.11 (11.2)	19.4
	Analysis Time Resolution	128.16 (3.3)	9.28	1.7 E^{+5} (11.2)	19.4

* MS_R/MS_r, mean square of regression/mean square of residual (grade of freedom).
** MS_{Lof}/MS_{Pe}, mean square lack of fit/mean square pure error (grade of freedom).
*** F95%, *F* value at 95% of confidence for the same grade of freedom.

predictive power. Thus, the models described above were considered to be validated for use in the optimization process.

The resolution response for the pair of peaks O2C-O3C on using the 50 mm column gave rise to *F* values for the regression that were lower than the critical values. This finding led to some concern regarding the use of this response in the optimization. However, on considering the pure error value (false positive) and comparing the 'predicted values' and 'real values', it can be seen that this model has an acceptable predictive power and therefore this model was also chosen for the optimization.

For the response of the two pairs of peaks O2C-O3C and O9C-CTO, the *F* values obtained for the resolution with the 100 mm column were lower than the critical *F* value. The lack of fit values for the resolution response of the pair of peaks O9C-CTO had a non-significant *F* value, but for the O2C-O3C resolution the *F* value was twice the *F* critical value. As a consequence, the 'predicted values' were compared with the 'real values', and in both cases the predicted and real values were close, thus demonstrating the good predictive power of these models. In a previous study [13] an *F* value for the lack of fit was obtained that was four times higher than the critical *F* value and in this case, the model was considered to be valid because the 'predicted values' and 'real values' were very close. In this work the model showed a good predictive power in the optimization. The model for the responses O2C-O3C and O9C-CTO resolutions was used in the optimization.

The resolution of the pair of peaks O10C-DHCTO with the 100 mm column and the resolution of the pairs of peaks O9C-CTO and O10C-DHCTO with the 50 mm column were calculated by statistical regression analysis of the *F* values obtained near to the critical *F* values, and the lack of fit of *F* values were lower than the critical *F* values. These models are therefore valid for the optimization. Based on the results described above, all of the models were used in the optimization. The significant coefficients for each model are given in Table 4.

3.2. Determination of the best conditions

3.2.1. 100 mm Column length

The different conditions used for the optimization with the 100 mm column are shown in Table 1. The resolution of the pairs of peaks O2C-O3C3, O9C-CTO and O10C-DHCTO ranged from 1.67 to 4.84, 0.95 to 1.28 and 0.91 to 1.49, respectively. The optimal conditions for each response were defined after considering the chromatograms and resolutions for each set of conditions in the optimization. The pair of peaks O2C-O3C, were completely separated with a resolution of 1.67. However, at a resolution higher than 2.5 the analysis time was longer, so for this response it was desirable to obtain a resolution between 1.67 and 2.5. For the pairs of peaks O9C-CTO and O10C-DHCTO, a resolution less than 1.1 was observed. In these cases there is partial co-elution between these

Table 4
Significant coefficients and standard error for each analyzed response.

Response	Significant coefficient ± standard error									
	Int.	A	B	C	A^2	B^2	C^2	AB	AC	BC
O2C-O3C Resolution*	3.58 ± 0.21	−0.57 ± 0.09	0.72 ± 0.09	0.25 ± 0.09	−0.39 ± 0.11	–	–	–	–	–
O9C-CTO Resolution*	1.12 ± 0.03	–	0.06 ± 0.01	–	–	–	–	–	–	–
O10C-DHCTO Resolution*	1.01 ± 0.03	0.05 ± 0.015	0.17 ± 0.015	0.05 ± 0.015	0.05 ± 0.017	0.06 ± 0.017	0.05 ± 0.017	–	–	–
RunTime*	5.88 ± 7.4E^{-3}	−0.28 ± 3.5E^{-3}	1.15 ± 3.5E^{-3}	−0.37 ± 3.5E^{-3}	−0.044 ± 3.9E^{-3}	−0.023 ± 3.9E^{-3}	0.054 ± 3.5E^{-3}	−0.11 ± 4.6E^{-3}	–	−0.28 ± 4.6E^{-3}
O2C-O3C Resolution**	2.54 ± 0.16	−0.72 ± 0.18	0.6 ± 0.18	–	–	–	–	–	–	–
O9C-CTO Resolution**	0.92h ± 9.2E^{-3}	–	0.024 ± 0.01	0.042 ± 0.01	–	–	–	–	–	–
O10C-DHCTO Resolution**	1.04 ± 0.015	0.059 ± 0.017	0.083 ± 0.017	0.052 ± 0.017	–	–	–	–	–	–
RunTime **	4.99 ± 0.054	−0.26 ± 0.06	1.06 ± 0.6	−0.42 ± 0.06	–	–	–	–	–	–

A: Initial percentage of acetonitrile; B: Gradient time; C: Flow rate.
* 100 mm column.
** 50 mm column.

Table 5
Comparison of predicted and real values for the optimal conditions. Capsinoids resolution in the spiked sample of Bell pepper.

Response	100 mm Column		50 mm Column		Spiked sample
	Predicted values	Real values	Predicted values	Real values	Real values
O2C-O3C Resolution	1.77	1.81	1.95	1.52	1.75
O9C-CTO Resolution	1.12	1.09	1	0.97	1.02
OC10-DHCTO Resolution	1.17	1.05	1.27	1.15	1.06
Time analysis	3.84	3.85	4.47	4.25	3.84

pairs of peaks. The highest resolution values observed for these pairs of peaks were 1.28 and 1.49 respectively. The desirable resolution values were defined in the range from 1.1 to 1.28 and from 1.1 to 1.49 for the pairs of peaks O9C-CTO and O10C-DHCTO respectively. The response 'run time' ranged from 3.87 to 7.79 min. The minimum time in this range was the most desirable.

3.2.2. 50 mm Column length

On using the 50 mm column the pairs of peaks O2C-O3C, O9C-CTO and O10C-DHCTO were once again the most difficult to separate (Table 2). During the optimization of the experiments the resolution of the pairs of peaks O2C-O3C, O9C-CTO, O10C-DHCTO were in ranges 1.13 to 4.65, 0.85 to 1.01 and 0.78 to 1.2, respectively. Analysis of the chromatograms for each set of separation conditions showed that the pair of peaks O2C-O3C showed a satisfactory separation with a resolution greater than 1.13. However, with resolution values higher than 2, the separation required a longer analysis time. As a result, for this pair of peaks it was established that a resolution in the range from 1.13 to 2 was desirable. For the pairs of peaks O9C-CTO and O10C-DHCTO resolution values below 1.1 did not give rise to an efficient separation, so for these two pairs of peaks, a resolution value in the range 1.1 to 1.5 were defined as a desirable condition. For the response 'run time, the minimum possible value was desirable.

3.3. Optimal point and desirability

The simultaneous optimization for the 50 mm and 100 mm columns was carried out using the Design Expert 6.0.10 (Minneapolis, USA) software. In this software it is possible to choose an importance value (1 to 5) for each response. For this optimization all the responses were determined using the same importance value of 3.

An experimental region was found for the 100 mm column that met all the specifications of the separation. This theoretical condition had a chromatographic solvent run starting with 41.8% of solvent B (acetonitrile, 0.1% acetic acid), a gradient time of 3.96 min until 100% of solvent B was reached and a flow rate of 0.679 mL min^{-1}. For the 50 mm column the theoretical region of greatest desirability had a chromatographic solvent run starting with 50% of solvent B, a gradient time of 7.72 min to 100% of solvent B and a flow rate of 0.8 mL min^{-1}. These theoretical conditions were analyzed and the theoretical results were compared with the experimental results. The data are shown in Table 5.

It can be seen from the results in Table 5 that the predicted values calculated by the theoretical models for optimization are similar to the experimental values. This finding indicates that the considerations outlined in Section 3.1 were appropriate to explain the variation in results and to make predictions. The experimental

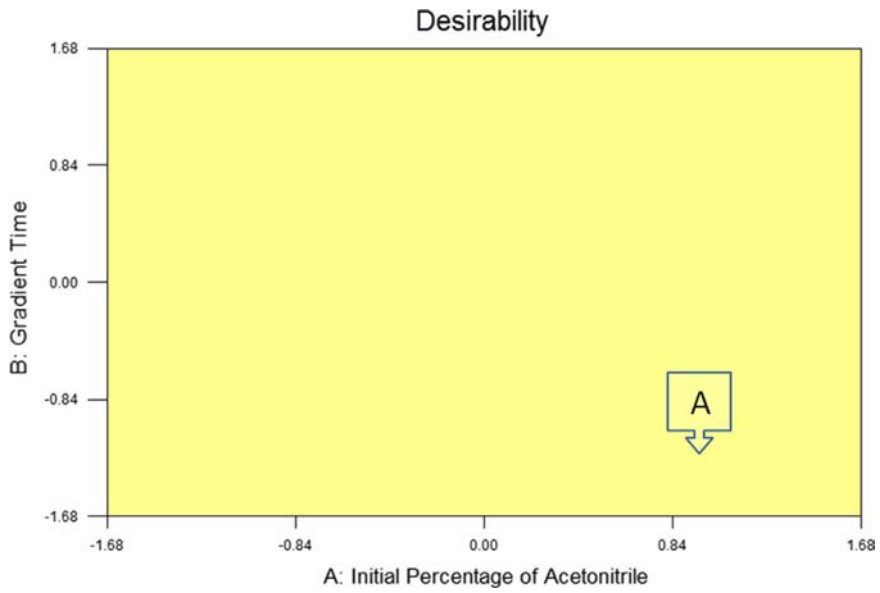

Fig. 1. Optimal experimental point for the three variables optimized. 1—Gradient time; 2—Initial acetonitrile percentage; 3—Flow rate (fixed at 0.679 mL min^{-1}).

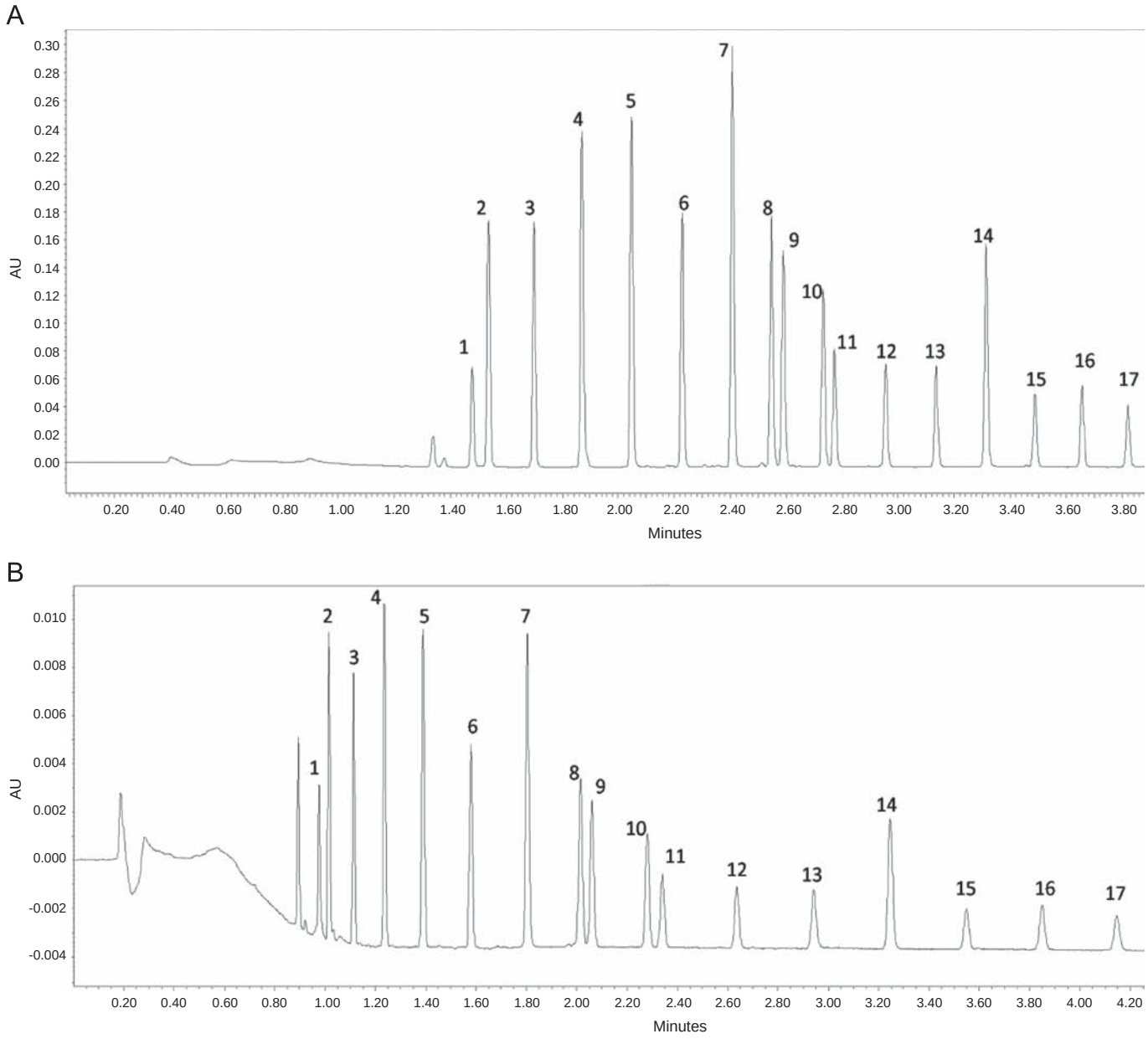

Fig. 2. Chromatograms obtained with the 100 mm column (A) and 50 mm column (B). 1-O2C, 2-O3C, 3-O4C, 4-O5C, 5-O6C, 6-O7C, 7-O8C 8-CTO, 9-O9C, 10-DHCTO, 11-O10C, 12-O11C, 13-O12C, 14-O13C, 15-O14C, 16-O15C, 17-O16C.

Table 6
Validation parameters for the method developed for the 100 mm column.

Compound	Linearity (μg mL^{-1})	R^2	p-Value (lack of fit test)	Repeatibility ($n=10$)	Intermediate precision ($n=3$)	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
CTO	3.8–38	0.999	0.6149	1.08	2.17	0.1	0.201
DHCTO	2.68–26.8	0.999	0.7177	1.01	2.34	0.102	0.204
O2C	0.68–6.8	0.999	0.7238	0.91	2.06	0.040	0.080
O3C	2.96–29.6	0.999	0.6876	1.10	2.64	0.097	0.197
O4C	2.68–26.8	0.999	0.8100	1.03	2.64	0.069	0.138
O5C	4.28–42.8	0.999	0.5003	1.92	3.92	0.084	0.168
O6C	4.08–40.8	0.999	0.4722	1.08	2.60	0.077	0.154
O7C	3.36–33.6	0.999	0.1931	1.03	2.47	0.080	0.175
O8C	5.16–51.6	0.999	0.3801	1.01	2.03	0.081	0.161
O9C	3.76–37.6	0.999	0.5967	0.97	2.25	0.117	0.234
O10C	1.76–17.76	0.999	0.6730	1.27	2.59	0.101	0.203
O11C	1.64–16.4	0.999	0.5390	1.44	3.01	0.108	0.216
O12C	1.76–17.6	0.999	0.4751	1.48	3.09	0.118	0.236
O13C	3.6–36	0.999	0.5981	1.21	2.43	0.109	0.217
O14C	1.68–16.8	0.999	0.1989	1.17	2.39	0.051	0.101
O15C	2.24–22.4	0.999	0.4863	0.98	2.22	0.214	0.427
O16C	2.6–26	0.999	0.0926	1.14	2.74	0.286	0.572

region, containing the point with a desirability value of 1 (maximum desirability) for the 100 mm column is shown in Fig. 1. The desirability can fall in the range from 0 to 1, where 0 does not meet the desired conditions and 1 meets all the desired conditions.

It can be seen in Fig. 2 that, after the optimization, the best separation conditions for the 100 mm column give a better separation than the best conditions for the 50 mm column. It is possible to separate all the compounds with the two columns after the optimization but the separation for the pair of peak O2C-O3C was better for the 100 mm column. The separations for the pairs of peaks O9C-CTO and O10C-DHCTO are equivalent in both cases. Comparison of the separation and the run time for the other pairs of peaks showed that the 100 mm column gave a better performance, so a further validation with the 100 mm column was carried out.

3.4. Validation

The values of repeatability and intermediate precision were expressed as relative standard deviation (RSD) for the peak area. For all the compounds analyzed, the RSD values were less than 1.92 and 3.92 for the repeatability and intermediate precision, respectively. The RSD for the retention time was also calculated. All the compounds had values less than 0.04. The 'p' values for the lack of fit for each linear regression were calculated by ANOVA and the resulting values were not significant. The limit of detection (LOD) and limit of quantification (LOQ) ranged from 0.051 to 0.286 and from 0.080 to 0.572 μg mL^{-1}, respectively. The validation parameters are shown in Table 6.

3.5. Application to real samples

Since no one has found peppers which have all these capsinoids, 1 mL of a mixture of the 17 capsinoids dissolved in methanol (8.42–10.16 μg/mL) was added to 1 ml of Bell Pepper (*Capsicum annuum* L.) extract. The optimized method was used to separate the capsinoids in this sample, obtaining resolution results equivalent to those obtained with the capsinoids dissolved in methanol (Table 5). On the other hand, capsinoids in different varieties of peppers were quantified by the method developed. 11 varieties of peppers (sweet and spicy ones) have been estudied. The milled peppers were extracted in triplicate. Based on the results, it can be seen that the only capsinoid that is found in some of these varieties of peppers is capsiate. The results of this quantification are shown in Table 7. The variety of pepper that has more quantity of capsiate is Cumari do Pará. The capsiate is presented in both sweet (Biquinho) and spicy (Naga Jolokia, Malagueta, etc.) varieties of peppers. All the studied species of peppers (*Capsicum chinense*, *Capsicum frutescens* and *Capsicum annuum*) have some variety containing capsiate.

Table 7
Quantification of capsinoids (capsiate) in real samples.

Variety of pepper	Amount of capsiate (μg g^{-1} FW*)
Cumarí do Pará (*Capsicum chinense*)	175.31 ± 14.62
Biquinho (*Capsicum chinense*)	119.74 ± 6.62
Naga Jolokia (*Capsicum chinense*)	98.24 ± 8.84
Bode (*Capsicum chinense*)	94.52 ± 2.43
Malagueta (*Capsicum frutescens*)	94.14 ± 4.71
Murupí (*Capsicum chinense*)	83.86 ± 3.76
Jalapeño (*Capsicum annuum*)	77.15 ± 5.24
Baiana (*Capsicum chinense*)	n.d.**
Bell (*Capsicum annuum*)	n.d.**
Italian (*Capsicum annuum*)	n.d.**
Padrón (*Capsicum annuum*)	n.d.**

* Fresf weight.
** Non detected.

4. Conclusions

Central composite design, response surface analysis and the Derringer and Suich multi-criteria method were used to optimize the chromatographic separation of 17 capsinoids with different chain length providing maximum resolution between peaks and shorter runtime. Two different columns have been used for the study: Waters ACQUITY UPLC BEH C18 (2.1 mm mm I.D.; 1.7 μm particle size; 50 mm length) and Waters ACQUITY UPLC BEH C18 (2.1 mm I.D.; 1.7 μm particle size; 100 mm length). Both columns were able to separate all the capsinoids after the optimization, but comparing the separation and the run time for all the peaks it can be seen that the 100 mm column gave a better performance, so a further validation with the 100 mm mm column was carried out.

A validation of the method has been done with excellent values of repeatability and intermediate precision. The developed method has been applied to real samples. Capsiate has been identified and quantified in some varieties of peppers.

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