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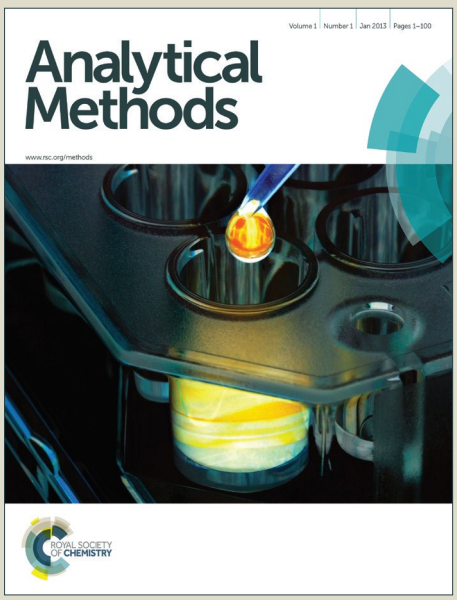

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Multivariate optimization by statistical methods of Ultra High Performance Liquid Chromatography conditions for 17 capsaicinoids separation

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Abstract

In this work a multivariate optimization by statistical methods (Derringer and Suich optimization) was proposed in order to find the optimum conditions of an Ultra High Performance Liquid Chromatograph with Diode Array Detection (UHPLCDAD) for the separation of seventeen capsaicinoids (natural and synthetic).. Capsaicinoids 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 fifteen of the seventeen capsaicinoids, but capsaicin (C) and N-(4-hydroxy-3-methoxybenzyl) nonanamide (N9C) could not be separated. However the 50 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 50 mm column length. The better conditions of separation with the 50 mm column length were established with: initial mobile phase with 0% of solvent B; 8.12 minutes of linear gradient time to reach 100% of solvent B; flow rate of 0.8 mL min^{-1}. A validation of the method has been done with good values of repeatability (RSD < 1.92) and intermediate precision (RSD < 3.92). The developed method has been applied to real food samples. Capsaicin and dihydrocapsaicin have been identified and quantified in all of the spicy foods analyzed.

1. Introduction

Chilli peppers are fruits of species from genus *Capsicum* (Solanaceae), and are used as a popular additive employed all over the world due to their aroma and color.[1] These properties make the peppers be a very important product in the food industry and in the cuisine. The main feature of chilli peppers is their pungent flavor, caused by a family of chemical compounds known as capsaicinoids.[2] Capsaicin (C) and dihydrocapsaicin (DHC) are the most abundant capsaicinoids and are responsible of approximately the 90% of the pungent flavor of peppers. In addition, less abundant capsaicinoids have also been detected, such as nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin, nornorcapsaicin, nornordihydrocapsaicin, nonivamide, among others.[3,4]

Additionally, capsaicinoids have several biological activities such as antimicrobial,[5] antioxidant,[6] anti-inflammatory,[7] analgesic,[8] anti-cancer[9,10] and stimulate the cardiovascular and respiratory system, increasing the metabolism (thermogenesis) and reducing the fat accumulation and body weight.[11,12]

Numerous methods have been applied in the determination of capsaicinoids in both peppers and biological fluids, and these include thin layer chromatography (TLC),[13] electrochemical sensors,[14] electrophoresis,[15] gas chromatography (GC)[16] and high performance liquid chromatography (HPLC).[17] By far the most commonly technique used for the identification and quantification of these compounds is RP-HPLC.[18]

There are many works of capsaicinoids analysis, but many of them analyze only the major capsaicinoids (capsaicin and dihydrocapsaicin),[19-21] however there are only a few studies focused in analyzing the minority capsaicinoids[22-24] and non-natural capsaicinoids, easy and cheap to synthetize, that could be used as synthetic additives.

The main benefit of the optimization process is the reduction of the time and cost of the general process. The multivariate optimization allows seeing interaction factors between the optimized variables. This is not possible when the univariate optimization is used. Since food samples studies often involve a large number of analytical peaks, where many of them must be separated, the optimization process must take into consideration all the critical separation parameters simultaneously. A set of experimental conditions that results in a good separation for some peaks may not resolve other peaks that are overlapped. In this situation, multi-criteria methods such as the one proposed by Derringer and Suich[25] 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.[26, 27]

This work proposes the use of multivariate statistic techniques in order to separate capsaicin, dihydrocapsaicin and other fifteen synthetic capsaicinoids with similar properties to natural capsaicinoids with different chain length by UHPLC-DAD.

2. Materials and Methods

2.1. Reagents

2.1.1. Chromatographic reagents

Acetonitrile, methanol and acetic acid (HPLC grade) were obtained from Merck (Darmstadt, Germany). Capsaicin (97%) and dihydrocapsaicin (90%) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Water was obtained from a Milli-Q water deionization system (Millipore, Bedford, MA, USA). Capsaicinoid standards were synthesized according to the methodology described by Barbero et al.[28] 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 capsaicinoids

4-Hydroxy-3-methoxy benzylamine hydrochloride (98%), 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%) and palmitoyl chloride (98%) were purchased from Sigma-

Aldrich Chemie (Steinheim, Germany). Acetic anhydride (98%), sodium hydrogen carbonate (99.0-100.5%), sodium hydroxide (98.0-100.5%), N,N-dimethylmethanamide (99%), dehydrated pyridine (99%) and tetrahydrofuran (99.5%) were purchased from Panreac Química S.A. (Castellar del Valle´s, Barcelona, Spain). Undecanoic acid (99%) was purchased from Acros Organics (New Jersey, USA). 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).

2.2. Capsaicinoids identification

The capsaicinoids that were chemically synthesized were *N*-(4-hydroxy-3-methoxybenzyl) ethanamide (N2C), *N*-(4-hydroxy-3-methoxybenzyl) propanamide (N3C), *N*-(4-hydroxy-3-methoxybenzyl) butanamide (N4C); *N*-(4-hydroxy-3-methoxybenzyl) pentananamide (N5C), *N*-(4-hydroxy-3-methoxybenzyl) hexanamide (N6C), *N*-(4-hydroxy-3-methoxybenzyl) methyl] heptanamide (N7C), *N*-(4-hydroxy-3-methoxybenzyl) octanamide (N8C), *N*-(4-hydroxy-3-methoxybenzyl) nonanamide (N9C-nonivamide), *N*-(4-hydroxy-3-methoxybenzyl) decanamide (N10C), *N*-(4-hydroxy-3-methoxybenzyl) undecanamide (N11C), *N*-(4-hydroxy-3-methoxybenzyl) dodecanamide (N12C), *N*-(4-hydroxy-3-methoxybenzyl) tridecanamide (N13C), *N*-(4-hydroxy-3-methoxybenzyl) tetradecanamide (N14C), *N*-(4-hydroxy-3-methoxybenzyl) pentadecanamide (N15C) and *N*-(4-hydroxy-3-methoxybenzyl) hexadecanamide (N16C).

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 δH 7.25 ppm and δC 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 capsaicinoids, 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 x 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 capsaicinoids, 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, 40% B; 6 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 ºC, cone gas flow=10 L h^{-1}, source temperature=150 ºC, capillary=0.7 eV, cone voltage=20 V and trap collision energy=4 eV. Full-scan mode was used (m/z=100–600). Capsaicinoids 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 ºC, 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, capsaicinoids were measured at a wavelength of 280 nm and the column oven was set at 50 ºC 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.[26] 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 minutes 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:

$$R_S = 2(t_2 - t_1) / (w_2 + w_1)$$

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 capsaicinoids were determined by a response surface graph and the multi-criteria response technique of Derringer and Suich.[26,29] The objective of the chromatographic optimization was firstly to separate the 17 capsaicinoids 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 capsaicinoids. The interday repeatability 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.[30]

2.6. Extraction procedure

The extracts from the spicy food samples were obtained using an ultrasound-assisted extraction technique, according to our previously developed method for natural capsaicinoids.[31] Ultrasonic irradiation was carried out using a UP200S sonifier (200W, 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 capsaicinoids, the following extraction parameters were used: extraction solvent: methanol; temperature: 50 ºC; output amplitude of the nominal amplitude of the transducer: 100% (200W); 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 two pairs of peaks that were most difficult to separate (all the other peaks were completely separated when these two pairs of peaks were separated sufficiently). The two pairs of peaks in question were N3C-N4C and N10C-DHC. Run time was added to the optimization, meaning that a total of three

variables were optimized simultaneously. The responses for each set of experimental conditions for the 100 mm and 50 mm column are shown in Table 1.

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 2.

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) and 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 3), showing a good 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 N3C-N4C 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.

The others responses show a significant values of F for the regression and not significant values of F for the lack of fit. As a consequence, the 'predicted values' were compared with the 'real values', and in both cases the predicted and real values were close, demonstrating the good predictive power of these models. In a previous study[29]

an *F* value for the lack of fit was obtained. This *F* value 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.

These models are therefore valid for the optimization. Based on the results described above, all of the models were used in the optimization. Table 4 shows the significant coefficients for each model.

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 N3C-N4C and N10C-DHC ranged from 0.00 to 1.25 and 0.92 to 1.64, 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 N3C-N4C was completely separated with a resolution of 1.10. However, at a resolution higher than 1.25 the analysis time was longer, so for this response it was desirable to obtain a resolution between 1.10 and 1.25. For the pair of peaks N10C-DHC a resolution less than 1.10 was observed. In these cases there is partial co-elution between these pairs of peaks. At a resolution higher than 1.50 the analysis time was longer, so the desirable resolution values were defined in the range 1.10 to 1.50 for this pair of peaks. The response 'run time' ranged from 3.37 to 6.72 minutes. The minimum time in this range was the most desirable.

3.2.2. 50 mm column length

Using the 50 mm column the pairs of peaks N3C-N4C and N10C-DHC were once again the most difficult to separate (Table 1). During the optimization of the experiments the resolution of the pairs of peaks N3C-N4C and N10C-DHC were in ranges 0.81 to 1.12 and 0.89 to 1.22, respectively. Analysis of the chromatograms for each set of separation conditions showed that the two pairs of peaks N3C-N4C and N10C-DHC have a satisfactory separation with a resolution greater than 1.10. As a result, for this pairs of peaks it was established that a resolution in the ranges from 1.10 to 1.12 and 1.10 to 1.22 respectively were desirable. For the response 'run time', the minimum possible value was desirable. The response 'run time' ranged from 2.86 to 5.73 minutes.

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 21.42% of solvent B (acetonitrile, 0.1% acetic acid), a gradient time of 7.89 minutes until 100% of solvent B was reached and a flow rate of 0.7 mL min^{-1}. For the 50 mm column the theoretical region of greatest desirability had a chromatographic solvent run starting with 0% of solvent B, a gradient time of 8.12 minutes to reach 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. These data are shown in Table 3.

It can be seen from the results in Table 3 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.

It can be seen in Fig. 1 that, after the optimization, the best separation conditions for the 100 mm column give the same separation than the best conditions for the 50 mm column. The separations for the pairs of peaks N3C-N4C and N10C-DHC are equivalent in both cases. Comparison of the separation and the run time for the other pairs of peaks showed that the 50 mm column gave a better performance, so a further validation with the 50 mm column was carried out. C and N9C could not be separated by any column.

3.4. Validation

The values of repeatability and reproducibility were expressed as relative standard deviation (RSD) for the peak area. For all the compounds analyzed, the RSD values were less than 1.99 and 4.30 for the repeatability and the reproducibility, respectively. The RSD for the retention time was also calculated. All the compounds had values of 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 0.005 to 0.076 and from 0.010 to 0.152 μg mL^{-1}, respectively. The validation parameters are shown in Table 5.

3.5. Application to real samples

The method developed with the 50 mm column length was used for the determination of the capsaicinoids (natural and non-natural) present in different spicy foods (sauces, ketchups and paprika). 12 spicy sauces, 4 spicy ketchups and 6 paprikas were analyzed. Capsaicin and dihydrocapsaicin were found in all the spicy foods analyzed. None of the non-natural capsaicinoids studied were found in these spicy foods. Capsaicin has been the major capsaicinoid in most of the foods studied (Table 6). Only a few spicy products had a higher concentration of dihydrocapsaicin (sauces 4 and 12 and ketchup 4). Spicy sauces studied showed great variability in the total content of capsaicinoids. The values of total capsaicinoids in sauces range between 19 μg g^{-1} (sauce 11) and 270 μg g^{-1} (sauce 4). Foods that showed a lower content of capsaicinoids were spicy ketchups (4 – 52 μg g^{-1}). Paprikas were the foods that showed a higher concentration of capsaicinoids (471 – 1242 μg g^{-1}). The results show that the developed method is valid for the analysis of food adulterations with non-natural capsaicinoids.

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 capsaicinoids with different chain length providing maximum resolution between peaks and shorter run time. Both columns were able to separate all the capsaicinoids, with the exception of capsaicin and N9C. Comparing the separation and the run time for all the peaks it can be seen that the 50 mm column gave a better performance, so a further validation with the 50 mm column was carried out.

The best theoretical condition for the 50 mm column length had a chromatographic solvent run starting with 0% of solvent B (acetonitrile, 0.1% acetic acid), a gradient time of 8.12 minutes until 100% of solvent B was reached and a flow rate of 0.8 mL min^{-1}. For all the compounds analyzed, the RSD values were less than 1.99 and 4.30 for the repeatability and the reproducibility, respectively. The limit of detection (LOD) and limit of quantification (LOQ) for all de capsaicinoids studied ranged from 0.005 to 0.076 and from 0.010 to 0.152 μg mL^{-1}, respectively. The results show that the developed method is valid for the analysis of food adulterations with non-natural capsaicinoids.

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Figure caption

Figure 1 Chromatograms obtained with the 100 mm column (A) and 50 mm column (B). 1-N2C; 2-N3C; 3-N4C; 4-N5C; 5-N6C; 6-N7C; 7-N8C; 8-C; 9-N9C; 10-DHC; 11-N10C; 12-N11C; 13-N12C; 14-N13C; 15-N14C; 16-N15C; 17-N16C

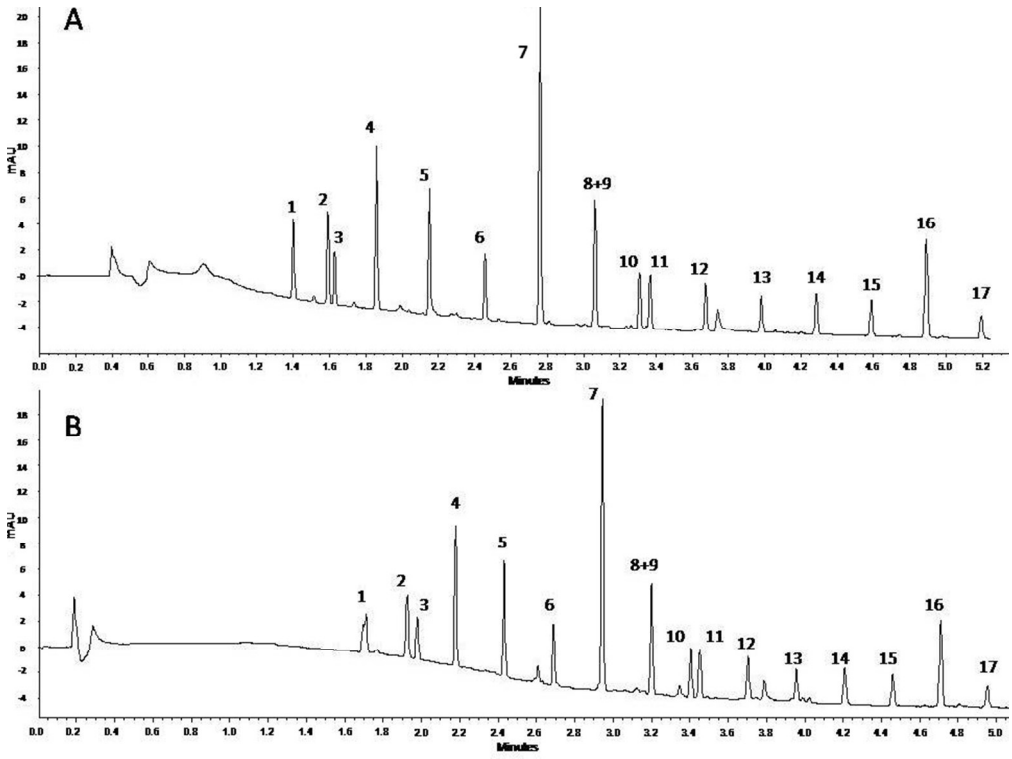

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Table 1 Central composite design and resolutions of the two critical pairs of peaks and run time for each experiment with the 100 and 50 mm column

N° Experiment	Column	Variables			N3C-N4C*	N10C-DHC*	Run Time**
		Initial ACN[a]	Gradient time[b]	Flow rate[c]			
1	**100 mm Column**	10.1 (-1)	4.2 (-1)	0.46 (-1)	0.77	1.03	4.59
2		39.9 (1)	4.2 (-1)	0.46 (-1)	0.00	1.10	4.18
3		10.1 (-1)	8.58 (1)	0.46 (-1)	1.22	0.95	6.72
4		39.9 (1)	8.58 (1)	0.46 (-1)	0.00	1.35	5.79
5		10.1 (-1)	4.2 (-1)	0.64 (1)	0.86	1.06	4.00
6		39.9 (1)	4.2 (-1)	0.64 (1)	0.49	1.11	3.57
7		10.1 (-1)	8.58 (1)	0.64 (1)	1.25	1.44	6.02
8		39.9 (1)	8.58 (1)	0.64 (1)	0.57	1.50	5.04
9		25 (0)	6.5 (0)	0.55 (0)	0.85	1.25	5.03
10		25 (0)	6.5 (0)	0.55 (0)	0.87	1.32	5.03
11		25 (0)	6.5 (0)	0.55 (0)	0.91	1.28	5.03
12		0 (-1.68)	6.5 (0)	0.55 (0)	1.09	1.25	5.48
13		50 (1.68)	6.5 (0)	0.55 (0)	0.00	1.25	4.28
14		25 (0)	3 (-1.68)	0.55 (0)	0.63	0.92	3.37
15		25 (0)	10 (1.68)	0.55 (0)	0.98	1.64	6.50
16		25 (0)	6.5 (0)	0.4 (-1.68)	0.78	1.26	5.74
17		25 (0)	6.5 (0)	0.7 (1.68)	0.95	1.38	4.59
		Initial ACN[a]	Gradient time[b]	Flow rate[c]	**N3C-N4C***	**N10C-DHC***	**Run Time****
1	**50 mm Column**	10.1 (-1)	4.2 (-1)	0.48 (-1)	1.04	0.97	3.91
2		39.9 (1)	4.2 (-1)	0.48 (-1)	0.81	0.89	3.42
3		10.1 (-1)	8.58 (1)	0.48 (-1)	1.11	1.08	5.74
4		39.9 (1)	8.58 (1)	0.48 (-1)	1.12	1.04	5.64
5		10.1 (-1)	4.2 (-1)	0.72 (1)	1.04	0.94	3.34
6		39.9 (1)	4.2 (-1)	0.72 (1)	0.81	0.99	2.87
7		10.1 (-1)	8.58 (1)	0.72 (1)	1.12	1.03	5.09
8		39.9 (1)	8.58 (1)	0.72 (1)	0.84	1.14	4.00
9		25 (0)	6.5 (0)	0.6 (0)	0.99	1.01	4.16
10		25 (0)	6.5 (0)	0.6 (0)	0.94	1.01	4.16
11		25 (0)	6.5 (0)	0.6 (0)	0.96	1.06	4.16
12		0 (-1.68)	6.5 (0)	0.6 (0)	1.12	1.00	4.23
13		50 (1.68)	6.5 (0)	0.6 (0)	0.74	0.96	3.32
14		25 (0)	3 (-1.68)	0.6 (0)	0.92	0.80	2.76
15		25 (0)	10 (1.68)	0.6 (0)	0.94	1.18	5.39

16	25 (0)	6.5 (0)	0.4 (-1.68)	1.06	0.94	4.85
17	25 (0)	6.5 (0)	0.8 (1.68)	0.94	1.22	3.78

[a] Percentage (%)
[b] Minutes
[c] mL min^{-1}
* Resolution
** Minutes

Table 2 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	NC3-NC4 Resolution	7.13 (3.3)	9.28	13.84 (11.2)	19.4
	NC10-DHC Resolution	10.02 (3.3)	9.28	13.03 (11.2)	19.4
	Analysis Time Resolution	59.54 (3.3)	9.28	554.57 (5.2)	19.3
50 mm Column	NC3-NC4 Resolution	12.87 (3.3)	9.28	7.29 (11.2)	19.4
	NC10-DHC Resolution	10.96 (3.3)	9.28	5.52 (11.2)	19.4
	Analysis Time Resolution	53.91 (3.3)	9.28	$2.8\ E^{+5}$ (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 degree of freedom

Table 3 Comparison of predicted and real values for the optimal conditions

Response	100 mm column		50 mm column	
	Predicted values	Real Values	Predicted Values	Real Values
N3C-N4C Resolution	1.10	0.99	1.11	1.06
NC10-DHC Resolution	1.40	1.26	1.16	1.06
Time analysis	5.22	5.26	4.68	4.70

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
N3C-N4C Resolution*	0.88 ±0.058	-0.36 ±0.027	0.11 ±0.027	0.11 ±0.027	-0.14 ±0.03	-	-	-0.096 ±0.035	0.12 ±0.035	-
N10C-DHC Resolution*	1.24 ±0.029	-	0.16 ±0.032	-	-	-	-	-	-	-
Run Time (min)*	5.03 ±0.013	-0.35 $\pm 6.2E^{-3}$	0.91 $\pm 6.2E^{-3}$	-0.33 $\pm 6.2E^{-3}$	-0.053 $\pm 6.8E^{-3}$	-0.034 $\pm 6.8E^{-3}$	0.047 $\pm 6.8E^{-3}$	-0.13 $\pm 8.1E^{-3}$	-	-0.031 $\pm 8.1E^{-3}$
N3C-N4C Resolution**	0.97 ±0.016	-0.10 ±0.018	-	-	-	-	-	-	-	-
N10C-DHC Resolution**	1.02 ±0.015	-	0.084 ±0.017	0.045 ±0.017	-	-	-	-	-	-
Run Time (min)**	4.17 ±0.068	-0.26 ±0.076	0.84 ±0.76	-0.39 0.076	-	-	-	-	-	-

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

Table 5 Validation parameters for the method developed for the 50 mm column

Compound	Linearity (μg mL^{-1})	R^2	p-Value (lack of fit test)	Repeatibility (n=10)	Reproducibility (n=30)	LOD* (μg mL^{-1})	LOQ** (μg mL^{-1})
C	0.76-7.6	0.999	0.8221	0.92	2.30	0.005	0.010
DHC	0.608-6.08	0.999	0.5292	1.09	2.49	0.010	0.020
N2C	0.576-5.76	0.999	0.8231	0.91	2.55	0.015	0.029
N3C	0.52-5.2	0.999	0.4917	1.40	3.22	0.013	0.026
N4C	0.632-6.32	0.998	0.7877	1.71	3.42	0.027	0.053
N5C	1.2-12.0	0.998	0.7445	1.04	2.91	0.017	0.037
N6C	0.96-9.6	0.998	0.7536	1.22	2.81	0.019	0.038
N7C	0.544-5.44	0.999	0.6865	0.99	2.77	0.020	0.040
N8C	3.7-37.0	0.999	0. 8317	1.01	2.32	0.029	0.058
N9C	3.76-37.6	0.999	0.8221	0.97	2.81	0.011	0.023
N10C	0.528-5.28	0.997	0.4280	1.92	4.03	0.016	0.031
N11C	0.616-6.16	0.999	0.7637	1.87	4.30	0.027	0.055
N12C	0.552-5.52	0.998	0.5351	1.08	3.02	0.030	0.060
N13C	0.536-5.36	0.999	0.7794	1.42	3.55	0.037	0.075
N14C	0.536-5.36	0.998	0.1529	1.39	2.92	0.036	0.074
N15C	0.624-6.24	0.999	0.5637	0.98	2.45	0.076	0.152
N16C	0.624-6.24	0.999	0.0706	1.00	2.10	0.067	0.134

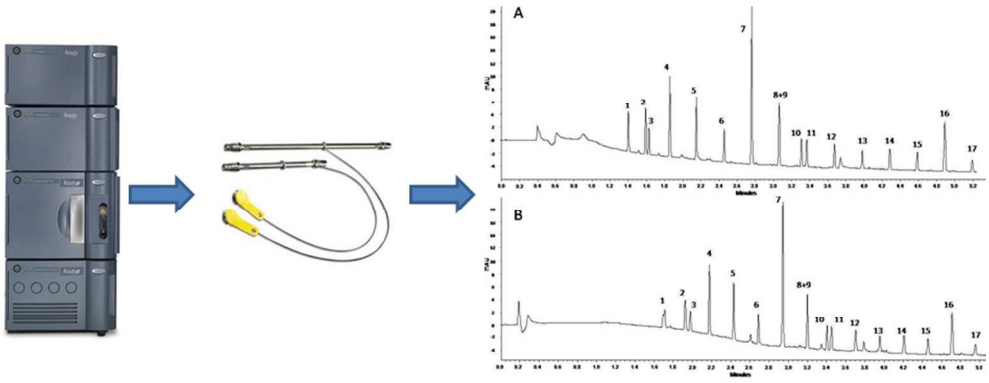
*Limit of Detection
**Limit of Quantification

Table 6 Quantification of capsaicinoids (capsaicin (C) and dihydrocapsaicin (DHC)) in foods

Type of food	C (µg/g food)	DHC (µg/g food)	Total Capsaicinoids (µg/g food)
Sauce 1	60.03 ± 1.38	33.07 ± 0.82	93.10 ± 2.20
Sauce 2	19.72 ± 0.45	7.65 ± 0.19	27.37 ± 0.64
Sauce 3	20.04 ± 0.46	9.83 ± 0.24	29.86 ± 0.71
Sauce 4	120.98 ± 2.78	148.54 ± 3.70	269.52 ± 6.48
Sauce 5	11.97 ± 0.28	7.52 ± 0.19	19.49 ± 0.46
Sauce 6	69.39 ± 1.60	36.10 ± 0.90	105.49 ± 2.59
Sauce 7	65.36 ± 1.50	40.24 ± 1.00	105.60 ± 2.50
Sauce 8	157.47 ± 3.62	64.94 ± 1.62	222.40 ± 5.24
Sauce 9	137.09 ± 3.15	73.50 ± 1.83	210.58 ± 4.98
Sauce 10	36.80 ± 0.85	22.98 ± 0.57	59.77 ± 1.42
Sauce 11	16.03 ± 0.37	6.74 ± 0.17	22.77 ± 0.54
Sauce 12	72.88 ± 1.68	107.69 ± 2.68	180.56 ± 4.36
Ketchup 1	30.01 ± 0.69	16.72 ± 0.42	46.73 ± 1.11
Ketchup 2	3.16 ± 0.07	1.24 ± 0.03	4.40 ± 0.10
Ketchup 3	5.40 ± 0.12	3.27 ± 0.08	8.67 ± 0.21
Ketchup 4	6.54 ± 0.15	45.73 ± 1.14	52.26 ± 1.29
Paprika 1	754.30 ± 17.35	487.49 ± 12.14	1241.78 ± 29.49
Paprika 2	479.12 ± 11.02	280.01 ± 6.97	759.14 ± 17.99
Paprika 3	302.75 ± 6.96	238.25 ± 5.93	541.01 ± 12.90
Paprika 4	355.97 ± 8.19	145.67 ± 3.63	501.63 ± 11.81
Paprika 5	374.41 ± 8.61	232.37 ± 5.79	606.78 ± 14.40
Paprika 6	286.23 ± 6.58	185.33 ± 4.61	471.56 ± 11.20

Textual abstract

Two new methods for the separation of capsaicin, dihydrocapsaicin and other fifteen synthetic capsaicinoids with similar properties to natural capsaicinoids with different chain length have been developed. The use of an experimental design for developing a chromatographic method for capsaicinoids has never been reported. With this work it has been shown that the use of multivariate statistical techniques is appropriate to develop quick and easy methods for the chromatographic separation of capsaicinoids using different columns.



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