Fast analysis of capsaicinoids in Naga Jolokia extracts (*Capsicum chinense*) by high-performance liquid chromatography using fused core columns

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ABSTRACT

A rapid high-performance liquid chromatography method with a C18 reverse-phase fused-core column has been developed for the determination and quantification of the main capsaicinoids (nornordihydrocapsaicin, nordihydrocapsaicin, capsaicin, dihydrocapsaicin, homocapsaicin and homodihydrocapsaicin) present in Naga Jolokia peppers. A fused-core Kinetex^{TM} C18 column (50 × 2.1 mm i.d.; 2.6 μm) was used for the analysis. The chromatographic separation was obtained with a gradient method in which the mobile phase was water (0.1% acetic acid) as solvent A and acetonitrile (0.1% acetic acid) as solvent B. The separation of all compounds was achieved in less than 3 minutes with a total analysis time (sample-to-sample) of 10 minutes. The robustness of the method was evaluated. The method showed excellent repeatability and intermediate precision expressed as coefficient of variance of less than 2%. The developed method was employed for the quantification of the major capsaicinoids present in different peppers and commercial products containing chilli peppers.

Keywords:

Capsaicinoids; *Capsicum chinense*; fused-core columns; HPLC; Naga Jolokia; peppers.

Chemical compounds studied in this article:

Nornordihydrocapsaicin (PubChem CID: 25200611); Nordihydrocapsaicin (PubChem CID: 168836); Capsaicin (PubChem CID: 1548943); Dihydrocapsaicin (PubChem CID: 107982); Homocapsaicin (PubChem CID: 71448975); Homodihydrocapsaicin (PubChem CID: 3084336).

1. Introduction

The chilli pepper is the fruit of a Solanaceous crop belonging to the genus *Capsicum*. It is native to South and Central America, although India is now the largest producer of chillies in the world (Barbero, Liazid, Azaroual, Palma, & Barroso, 2016). Chillies are sources of capsaicinoids, which are alkaloids that represent the plants secondary metabolites that are mainly involved in plant defence against herbivores and pathogens. Capsaicinoids are also responsible for the pepper's characteristic pungent flavour, which is attributed to the amide bond connecting a vanillyl ring and an acyl chain in the capsaicinoid structure (Barbero, Palma, & Barroso, 2006). The two predominant capsaicinoids in chillies are capsaicin (*trans*-8-methyl-*N*-vanillylnon-6-enamide) and dihydrocapsaicin (8-methyl-*N*-vanillylnonanamide), which typically make up 80–90% of the total capsaicinoids concentration (generally with a ratio from 1:1 to 2:1) (Al Othman, Ahmed, Habila, & Ghafar, 2011; Sganzerla, Coutinho, de Melo, & Godoy, 2014). There are more than 20 known capsaicinoids and the most abundant, apart from capsaicin and dihydrocapsaicin, are nornordihydrocapsaicin, nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin, amongst others (Constant, Cordell, West, & Johnson, 1995; Giuffrida et al., 2013).

In addition to their wide culinary use and appreciation of their colour, aroma, flavour and pungency, chillies have been studied for their biological properties. Studies demonstrate that at high doses (above 100 mg capsaicin per kg bodyweight) and for a prolonged exposure time, capsaicin can cause peptic ulcers and accelerate the development of gastrointestinal cancers (Bley, Boorman, Mohammad, McKenzie, & Babbar, 2012; Mózsik, Past, Abdel Salam, Kuzma, & Perjési, 2009; Surh & Sup Lee, 1995). Studies have shown chemopreventive and antineoplastic activity on the

gastrointestinal system such as gastric epithelium restitution, repair of gastric mucosa and increase of mucosal blood flow (Jones, Shabib, & Sherman, 1997; Luo, Peng, & Li, 2011). The capsaicinoids have been extensively studied for their analgesic activity, as antioxidants and in preventing obesity by enhancing energy expenditure of the body (Janssens, Hursel, Martens, & Westerterp-Plantenga, 2013; Peppin & Pappagallo, 2014). Furthermore, it was demonstrated that capsaicin has protective activity on the cardiac system by blocking platelet aggregation and the activity of clotting factors VIII and IX (Govindarajan & Sathyanarayana, 1991). Capsaicin also inhibits oxidation of LDL (low density lipoproteins) and it reduces total serum cholesterol and lipid peroxide levels in rat models (Kempaiah, Manjunatha, & Srinivasan, 2005).

The study reported here concerns the Naga Jolokia pepper, an interspecies hybrid of *Capsicum chinense* and *Capsicum frutescens*. Naga Jolokia is mainly cultivated in Bangladesh and the Indian States of Assam, Nagaland and Manipur (Meghvansi et al., 2010). This pepper is rated at more than 1 million Scoville Heat Units (SHUs). It is mainly used as a spice in both fresh and dried form. Due to its high pungency, in India it is used as a weapon; it is incorporated in smoke bombs to keep wild elephants at distance (Moirangthem et al., 2012).

Due to the presence of capsaicinoid in many foods, forensic and pharmaceutical products, many different techniques for extraction and analysis of these compounds have been proposed. The oldest technique is the Scoville Heat Test, an organoleptic test that is used to determine the pungency of peppers (Sweat, Broatch, Borror, Hagan, & Cahill, 2016). Methods such as these have been replaced by analytical methods that include spectrophotometry (Perucka & Oleszek, 2000), capillary electrophoresis (Liu et al., 2010), gas chromatography (GC) (Cisneros-Pineda et al., 2007), supercritical fluid chromatography (SFC) (Sato et al., 1999), high-performance liquid chromatography

(HPLC) (Bae, Jayaprakasha, Jifon, & Patil, 2012; Poyrazoglu, Yemis, Kadakal, & Artik, 2005), high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) (Garcés-Claver, Arnedo-Andrés, Abadía, Gil-Ortega, & Álvarez-Fernández, 2006; Morales-Soto, Gómez-Caravaca, García-Salas, Segura-Carretero, & Fernández-Gutiérrez, 2013) and ultra high-performance liquid chromatography (UHPLC) (Coutinho, Barbero, Godoy, Palma, & Barroso, 2016; Ha et al., 2010). However, the most common technique used for the determination and quantification of capsaicinoids is reverse-phase HPLC due to its efficiency and reliability (Barbero, Liazid, Ferreiro-González, Palma, & Barroso, 2015; De Aguiar et al., 2013). HPLC of capsaicinoids is able to separate these compounds in a time between 20 and 40 minutes, or longer times to achieve optimal resolutions (Arroso, 2006; Choi et al., 2006). On using monolithic columns the analysis time for capsaicinoids can be shortened with HPLC, but this leads to an increased consumption of solvent because of the higher flows required. On using this approach separation times for major capsaicinoids of less than 8 minutes can be achieved (Barbero, Liazid, Palma, & Barroso, 2008a). The use of UHPLC can further shorten the analysis time for capsaicinoids (even less than 3 minutes), but this technique requires expensive equipment that is not available to many users (Barbero et al., 2015). Nevertheless, the HPLC methods reported in the literature have not fully exploited the recent advances in HPLC column technology such as fused core columns (Nováková & Vlčková, 2009). These columns, which were first commercially introduced in 2007, are packed with fused-core particles that are formed of a solid core surrounded by a porous silica shell (Gritti & Guiochon, 2007). In this way, the analyte does not penetrate the solid core but can only diffuse into the porous silica shell, thus leading to a shorter diffusion path while maintaining a sufficiently large overall diameter to avoid the generation of high back pressure (González-Ruiz,

Olives, & Martín, 2015). Consequently, this structure allows the mass transfer to be reduced, which increases peak efficiency, sensitivity and resolution to achieve analysis in shorter times compared to traditional HPLC particles with 3–5 µm diameters. Thus, the main advantage of this technology is the ability to perform as well as columns packed with sub-2-µm particles without the need to use higher cost ultra-high performance instrumentation and consumables (McCalley, 2010). Furthermore, this technique is particularly suited to complex food matrixes where high throughput and resolution are essential.

To the best of our knowledge a method has not been developed for the analysis of major capsaicinoids using fused core column technology. Consequently, the aim of this study was to develop and validate an HPLC method with a fused core column for the analysis of major capsaicionoids in Naga Jolokia. With this new method, both laboratories, researchers and industries will be able to separate the major capsaicinoids present in peppers and spicy samples with retention times and resolutions similar to those obtained with UHPLC. The use of HPLC has the advantage of being a cheaper technique and more available for laboratories and industries.

2. Materials and methods

2.1. Chemicals and solvents

Methanol (MeOH), acetonitrile and acetic acid (Fisher Scientific, Loughborough, UK) were HPLC grade. Ultra-pure water was obtained from a Milli-Q water purification system from Millipore (Bedford, MA, USA). The capsaicinoid standards, namely capsaicin (97%) and dihydrocapsaicin (90%), were obtained from Sigma-Aldrich (Steinheim, Germany).

2.2. Plant material

The Naga Jolokia peppers were obtained from the Agrifood and Technology Centre of Aragón (CITA-Zaragoza). The pericarp and placenta of the chillies were subsequently ground together in a conventional mill to obtain a completely homogeneous sample. Once the peppers had been milled, they were frozen at –20 °C and stored until extraction and chromatographic analysis.

2.3. Extraction procedure

The extracts were obtained using ultrasound-assisted extraction employing the method developed by Barbero et al. with modifications (Barbero, Liazid, Palma, & Barroso, 2008b). Ultrasonication was carried out using a UP200S sonifier (200 W, 24 kHz) (Hielscher Ultrasonics gmbh, Teltow, Germany), with the sample immersed in a water bath coupled to a temperature controller (Frigiterm-10, J.P. Selecta, S.A., Barcelona,

Spain). The following extraction parameters were used to extract the capsaicinoids: extraction solvent: methanol; temperature: 40 °C; transducer nominal output amplitude: 65% (200 W); duty cycle: 0.7 seconds; solvent volume: 15 mL; extraction time: 5 min; amount of sample: 1 g. The extract was then filtered through a filter paper and the volume was made up to 25 mL with methanol. The extracts were filtered through a 0.22 µm nylon syringe filter (Membrane Solutions, Dallas, USA) prior to chromatographic analysis. Recovery of the method is above 95% for capsaicinoids.

2.4. Identification of capsaicinoids by liquid chromatography coupled to mass spectrometry

The six major capsaicinoids present in Naga Jolokia peppers (nornordihydrocapsaicin (nn-DHC), nordihydrocapsaicin (n-DHC), capsaicin (C), dihydrocapsaicin (DHC), homocapsaicin (h-C) and homodihydrocapsaicin (h-DHC)) were identified by ultra-performance liquid chromatography (UHPLC) coupled to a quadrupole-time-of-flight mass spectrometer (Q-ToF-MS) (Synapt G2, Waters Corp., Milford, MA, USA). 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, 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 data processing. The protonated molecular ions $[M + H]^+$ for the capsaicinoids found showed the following *m*/*z* ratios: nornordihydrocapsaicin, 280; nordihydrocapsaicin, 294; capsaicin, 306; dihydrocapsaicin, 308; homocapsaicin, 320; and homodihydrocapsaicin, 322. In the mass spectra of these six capsaicinoids the characteristic *m*/*z* peak (137) due to the fragmentation of capsaicinoids was clearly observed.

For the identification of capsaicinoids, solvent A (water, 0.1% formic acid) and solvent B (methanol, 0.1% formic acid) were used as mobile phases at a flow rate of 0.5 mL min^{-1}. The elution gradient employed was as follows: 0 min, 0% B; 0.85 min, 55% B; 1.60 min, 55% B; 1.95 min, 60% B; 2.45 min, 63% B; 2.80 min, 70% B; 3.00 min, 70% B; 6.00 min, 100% B; 8.00 min, 100% B. The 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).

2.5. Separation and quantification of capsaicinoids by HPLC-UV-Vis

The separation and quantification of capsaicinoids were performed on an Elite HPLC LaChrom Ultra System (VWR Hitachi, Tokyo, Japan) consisting of an L-2200U Autosampler, an L2300 Column Oven, an L-2160U Pump and an L-2420U UV-Vis Detector. The column oven was adjusted to 50 ºC for the chromatographic separation. The UV-Vis Detector was set at 280 nm for the analysis. Capsaicinoinds were analyzed on a Kinetex™ C18 column (50 × 2.1 mm i.d.; 2.6 µm particle size; Phenomenex, Torrance, CA, USA). EZChrom Elite Version 3.3.2. Software was used to obtain the chromatographic parameters of the developed method.

A gradient method, using acidified water (0.1% acetic acid, solvent A) and acidified acetonitrile (0.1% acetic acid, solvent B), working at a flow rate of 0.7 mL min^{-1} was used. The gradient employed was as follows: 0 min, 0% B; 0.4 min, 55% B; 1.5 min, 55% B; 1.8 min, 60% B; 2.3 min, 65% B; 3.0 min, 65% B; 3.5 min, 100% B; 6 min,

100% B; 7.0 min, 0% B; 10.0 min, 0% B. Peaks 4 and 5 (dihydrocapsaicin and homocapsaicin) do not appear to be baseline separated (Fig. 1). Dihydrocapsaicin and homocapsaicin are capsainoids difficult to separate due to their similar polarity. The integration of capsaicinoids has been done manually. It has been carried out in the form "Valley-to-Valley". Peaks 4 and 5 have been integrated together "Valley-to-Valley" from the start of peak 4 to the end of peak 5. Finally a "split peak" in the valley formed between peak 4 (DHC) and peak 5 (h-C) has been done.

2.6. Validation procedure

A validation protocol was carried out in order to ensure appropriate detection, identification and quantification of the analytes. The evaluated parameters were: linearity, limits of detection (LODs), limits of quantification (LOQs), precision (repeatability and intermediate precision), selectivity and robustness.

A calibration curve for capsaicin ($y = 2162.73\ x + 113.28$) and dihydrocapsaicin ($y = 2323.61\ x + 73.02$), which are the commercially available standards for capsaicinoids, were constructed. The linearity of the calibration curves was evaluated by the determination of regression coefficients ($r = 0.9998$ for capsaicin and $r = 0.9999$ for dihydrocapsaicin), which were calculated using Microsoft Office Excel 2010. Since commercial standards are not available for nn-DHC, n-DHC, h-C and h-DHC, these compounds were quantified from the calibration curves of DHC (for nn-DHC, n-DHC and for h-DHC) and C (for h-C), based on the structural similarities between these molecules and taking into account their molecular weights. All analyses were carried out in duplicate. The limits of detection (LOD) and limits of quantification (LOQ) were obtained by dividing respectively 3 and 10 times the signal-to-noise ratios by the

angular coefficients of the analytical curves obtained, using Microsoft Office Excel 2010. Precision was estimated by performing repeatability and intermediate precision studies and values are expressed as the coefficient of variance (CV). Repeatability was evaluated using 12 replicates, whereas intermediate precision was studied using 30 replicates over 3 different days. The mobile phase was freshly prepared for each set of determinations. The robustness of the method was evaluated by testing a variation of ± 5–10% range of: flow rate (5%), injection volume (5%), column temperature (10%) and mobile phase composition (5%). For each parameter 6 repetitions were carried out. For the statistical analysis of the robustness, a two tailed T-test was used assuming equal variances and a level of significance of 0.05. Calculations were performed using Microsoft Office Excel 2010.

3. Results and discussion

3.1. Selection of conditions

The chromatographic method was developed using Naga Jolokia methanol extracts obtained by ultrasound-assisted extraction. Several trial-and-error experiments were conducted to optimize the method. Column efficiency was evaluated on the basis of retention time (RT), selectivity (α), symmetry factor, retention factor (k*) and resolution (Rs) of the six peaks studied: nornordihydrocapsaicin (nn-DHC), nordihydrocapsaicin (n-DHC), capsaicin (C), dihydrocapsaicin (DHC), homocapsaicin (h-C) and homodihydrocapsaicin (h-DHC).

The criterion utilized to find the best chromatographic separation was based on reaching an optimal resolution (Rs > 1.5) for n-DHC and DHC (capsaicinoids that are difficult to

separate from C and h-C, respectively) in less than 10 minutes including elution, clean-up and re-equilibration time and with a column backpressure of less than 8000 psi (55.158 MPa).

3.1.1. Mobile phase

The mobile phase selection was based on a previous series of experiments using acidified water (0.1% v/v acetic acid) as solvent A and acidified methanol or acetonitrile (0.1% v/v acetic acid) as solvent B. For the initial separation a series of runs using a linear gradient of solvent A to solvent B (0–100%) were tested by modifying the time of the linear gradient (4–8 min) and maintaining the flow rate at 0.5 mL min^{-1}. Acidified acetonitrile was chosen as solvent B due to its lower viscosity in comparison with methanol, thus leading to lower backpressure and allowing the use of higher flow rates in order to reduce the analysis time. Additionally, an overall better partial separation and peak shape was obtained with acetonitrile when compared to methanol and the separation of the peaks was also faster. In several studies acetonitrile has been used as the main solvent in the mobile phase for the separation of capsaicinoids, generally in gradient flow (Al Othman et al., 2011). Moreover, since a UV-Vis was detector used, acetonitrile is a better option because it has high sensitivity at short UV wavelengths, thus lowering the noise in the UV detection (Sganzerla et al., 2014).

3.1.2. Column temperature

The next step was to study the effect that the column temperature had on retention times and the chromatographic resolution of the peaks. Based on the principles of column temperature changes, it was gradually increased from 35 to 55 ºC in 5 ºC intervals. These changes led to a significant reduction in the retention time of the six

capsaicinoids. A clear trend was also observed on increasing the temperature of the column and this gave increased peak heights, narrower peaks, and better resolution in the separation of the six capsaicinoids present in the sample. A temperature of 55 ºC was selected since this gave the lowest retention time (RT mean for the last peak of 4.42 minutes). Indeed, on increasing the temperature the viscosity of the mobile phase decreases, thus allowing the use of higher flow rates, which further reduces the retention time. The highest flow rate that could be used to stay safely within the system pressure limit of 8000 psi (55.158 MPa) was 0.7 mL/min.

3.1.3. Flow rate

Once the optimum temperature had been selected, the reduced column back pressure allowed the flow-rate to be increased to shorten the analysis time. The flow rate was increased step-by-step from 0.5 to 0.7 mL min^{-1}. The maximum flow rate was determined by the system pressure limitation, which was set to 8000 psi (55.158 MPa). As the flow rate was increased, a proportional reduction in the gradient was applied in order to maintain the separation of the six capsaicinoids.

After establishing the best flow rate and temperature, the gradient was optimized using various trial-and-error experiments. The best separation of the six capsaicinoids was achieved in approximately 2.8 min. The best separation gradient profile was 0 min, 0% B; 0.4 min, 55% B; 1.5 min, 55% B; 1.8 min, 60% B; 2.3 min, 65% B; 3.0 min, 65% B. This method allows the separation of the six major capsaicinoids in a very short time by employing fused core columns in a conventional HPLC system. These times are very similar to – or even lower than – those obtained with ultra-high performance liquid chromatography equipment using a smaller particle size (sub 2 µm) and higher pressures (15,000 psi; 103.421 MPa) (Barbero et al., 2015; Sganzerla et al., 2014).

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309 *3.1.4. Clean-up and re-equilibration*

310 It should be noted that the chromatographic method involves a clean-up step. The
311 gradient requires 0.5 min to reach 100% of mobile phase B, 2.5 min for column clean-
312 up (3.5–6.0 min) and 1 min to return to the initial conditions (6.0–7.0 min). The
313 cleaning stage is an important aspect that is often overlooked when developing gradient
314 methods.

315 All previous sets of experiments were carried out with a time of 5 min between runs,
316 which is equivalent to approximately 42% of the total method duration (including
317 elution, clean-up and re-equilibration times) and equivalent to 12.8 volumes of the
318 column. In order to keep this equilibration time as low as possible to minimize the total
319 method duration, shorter re-equilibration times (1–4 min) were evaluated.

320 The use of 5 min to re-equilibrate the column between runs provided a mean (n = 18;
321 interday) area and retention time variability lower than 1.8% and 1.3%, respectively. A
322 reduction in the equilibration time to 4, 3, 2 and 1 min resulted in mean area variability
323 values of less than 1.9%, 2.1%, 3.2% and 3.8%, and mean retention time variability
324 values below 1.3%, 1.4%, 1.8% and 2.1%, respectively. The variability was within the
325 normal range on using of very short re-equilibration times, but a slightly higher
326 reproducibility was achieved for the analysis of the six capsaicinoids on using
327 equilibration times of less than 3 min. Therefore 3 min was considered as the most
328 appropriate re-equilibration time in order to achieve the highest possible reproducibility
329 while not having an overly long total run time. This equilibration time is equivalent to
330 7.6 times the column volume and is slightly lower than the recommended level. This
331 situation is consistent with the results of previous studies in which it was found that for

other compounds present in natural products very short equilibration times could be used with fused-core columns (González-Ruiz et al., 2015; Osorio-Tobón et al., 2016).

3.2 Characteristics and validation of the method

The method developed in this study is as follows: 0 min, 0% B; 0.4 min, 55% B; 1.5 min, 55% B; 1.8 min, 60% B; 2.3 min, 65% B; 3.0 min, 65% B; 3.5 min, 100% B; 6.0 min, 100% B; 7.0 min, 0% B; 10 min; 0% B. The column temperature was maintained at 55 ºC and the flow rate was 0.7 mL min^{-1}. These conditions provide the best balance between analysis time and separation of the six capsaicinoids (nn-DHC, n-DHC, C, DHC, h-C and h-DHC). The developed method gave retention times for nn-DHC, n-DHC, C, DHC, h-C and h-DHC of 1.80, 1.98, 2.03, 2.23, 2.27 and 2.59 min, respectively. The total analysis time (sample-to-sample) is 10.0 minutes, including the return to the initial conditions and the requilibration of the column, while the separation of the six capsaicinoids is achieved in less than 3 min. The chromatographic analytical procedure used to determine the compounds of interest was carried out according to the ICH Guideline Q2 (R1) and suggestions made in ISO 17025 (ICH, 2006 and ISO, 2005). The linearity, precision, limits of detection and quantification were evaluated along with the robustness of the method.

The retention times, width of peaks, symmetry factor, selectivity (α) and resolutions were calculated by EZChrom Elite Software. The apparent gradient retention factor at the column midpoint (k^*) was calculated according to Snyder and Dolan (Snyder & Dolan, 2006). A representative chromatogram of the methanolic extract of capsaicinoids in Naga Jolokia is presented in Fig. 1 and the chromatographic properties of the developed method are reported in Table 1. These results also indicate an excellent

chromatographic performance of the fused core column for the separation of capsaicinoids. It can be seen from the results in Table 1 that the width of the peaks, symmetry factors and selectivity have optimum values for a chromatographic method. With respect to the resolution, good resolutions can be observed despite the fact that capsaicinoids like C and h-DHC are the most difficult to separate.

3.2.1. Linearity and range

The linearity of the method was confirmed by the regression coefficients for C (r = 0.9998) and DHC (r = 0.9999) obtained from the calibration curve constructed using seven points (0.0693, 0.1386, 1.386, 6.93, 13.86, 69.3, 346.5 ppm for C; and 0.0576, 0.1152, 1.152, 5.76, 11.52, 57.6, 288.0 for DHC; in triplicate) for the two commercially available capsaicinoid standards, C and DHC. The calibration curve for h-C was calculated using the corresponding curve for C, whereas the calibration curves for nn-DHC, n-DHC and h-DHC were obtained from the corresponding one for DHC, using the molecular mass ratio for the corresponding compounds. This procedure is the usual way to quantify capsaicinoids because very few of these compounds are commercially available. Good linearity was observed in the range studied both for C and DHC (Table 2).

3.2.2. Limits of detection and quantification

The LOQ and LOD (Table 2) for capsaicin and dihydrocapsaicin were estimated as 3 and 10 times the signal-to-noise ratio, respectively. LOQ and LOD for h-C were calculated using the corresponding values for C, whilst the LOQ and LOD for nn-DHC, n-DHC and h-DHC were obtained from the corresponding one for DHC. This process

was carried out using the molecular mass ratio for the corresponding compounds as these capsaicinoids are not commercially available.

3.2.3. Repeatability and reproducibility

The repeatability and intermediate precision of the developed methods were established by considering the peak area, the chromatographic resolution and the apparent gradient retention factor for each capsaicinoid (k^*). This process involved performing a total of 26 analyses of the same sample distributed as follows: 14 analyses performed on the first day of the study and 6 more analyses on each of the next two consecutive days.

The retention time (RT) reproducibility and intermediate precision expressed as coefficient of variance (CV) were less than 2% for all of the peaks while the area reproducibility was less than 3%. Regarding the area intermediate precision, the CV was less than 2% except for nnDHC (first peak), for which the area CV was 5% – as can be seen in Table 2. It can be confirmed that in all cases the CV is below 5% for the area and RT and this shows that the method has high reproducibility and intermediate precision.

3.2.4. Robustness of the method

The robustness of the method was evaluated by testing a variation of ± 5–10% in flow rates (5%), injection volumes (5%), column temperatures (10%) and % of acetic acid in the mobile phase compositions (5%). Each parameter was tested at three different levels and for each level a total of 6 repetitions were carried out. The statistical comparison was performed using the T-test assuming equal variances. Results with a p value < 0.05 were considered to be statistically different. The effect of these variables on three parameters, i.e., retention time, chromatographic resolution of the peaks, and area of the

406 chromatographic peaks, was checked. The results for the robustness evaluation are
407 reported in Table 3.
408 As far as the column temperature is concerned, significant differences ($p > 0.05$) were
409 not found between the values of the retention time and areas for the different peaks,
410 from which it can be concluded that the retention times and areas were not influenced
411 by varying the temperature from 45 ºC to 55 ºC. With respect to the influence on the
412 peak area, the method proved to be robust ($p > 0.05$) on modifying the temperature from
413 45 ºC to 55 ºC, the injection volume (taking into account the area references for an
414 injection volume of 15 µL) and % of acetic acid in the mobile phase compositions.
415 Regarding resolution there is a statistically significant difference in this temperature
416 range. For the robustness of the flow rates all of the parameters considered
417 demonstrated that there was a statistical difference when varying the flow rate by ± 5%
418 ($p < 0.05$). The method was highly robust on changing the injection volume from 12 µL
419 to 18 µL in terms of retention time, resolution and peak area. The method was highly
420 robust when changing the percentage of acetic acid from 0% to 0.2%.
421
422 *3.3. Sample solvent*
423
424 The influence of the percentage of solvent (MeOH) present in the sample on the method
425 robustness was analyzed. The percentages of methanol were 25, 50, 75 and 100%
426 diluted with water. The extracts were analyzed using the chromatographic method
427 developed to test whether the extraction solvent affected the chromatographic separation
428 of the peaks. The studied parameters were the retention time, area CV, retention factor,
429 resolution and symmetry factor for the six chromatographic peaks of the capsaicinoids
430 in question. The results are shown in Table 4. It can be observed that there is no

statistical difference in the retention time for any percentage of methanol in the sample ($p > 0.05$). However, the best area reproducibility was obtained on using 100% to 75% of methanol while the samples with 25% methanol presented the lowest reproducibility in terms of area. In terms of chromatographic resolution the method proved to be highly robust when the percentage of methanol was varied from 75% to 25%. However, the highest extraction of capsaicinoids was achieved with 100% methanol. Since the use of 100% of methanol also produced good chromatographic resolution and symmetry of the peaks, this was selected as the best extraction solvent.

3.4. Quantification of the capsaicinoids present in different commercial products containing hot peppers

Having developed the optimum method for the analysis of capsaicinoids on employing a fused core column, a further study was carried out to quantify the major capsaicinoids present in several pepper varieties and spicy products (three paprikas, six sauces and three ketchups). The peppers were obtained from the Agrifood and Technology Centre of Aragón (CITA-Zaragoza). Commercial spicy foods were obtained from local supermarkets in Cádiz (Spain). The extracts were obtained using ultrasound-assisted extraction employing the method developed by Barbero et al. with modifications (Barbero et al., 2008b). The results are shown in Table 5.

Capsaicin and dihydrocapsaicin are the major capsaicinoids in both peppers and spicy foods. Capsaicin is generally present in greater concentrations than dihydrocapsaicin, except in the Fatalli pepper, in two sauces and a ketchup. The highest concentration of capsaicinoids was found in hot peppers, especially in the Naga Jolokia variety. Spicy paprika also has a high concentration of capsaicinoids (183.1–352.5 µg/g). A wide

range of capsaicinoid concentrations were observed in the sauces (6.4–268.1 µg/g). The spicy ketchups had a low content of capsaicinoids (1.3–3.9 µg/g). Nornordihydrocapsaicin was only found above its limit of quantification in the Naga Jolokia pepper and in the Malagueta pepper.

4. Conclusion

A rapid and reproducible method for the separation of capsaicinoids using a fused core column has been developed. A notable shortening of the analysis time (< 3 min) has been achieved for the six major capsaicinoids (nornordihydrocapsaicin, nordihydrocapsaicin, capsaicin, dihydrocapsaicin, homocapsaicin and homodihydrocapsaicin) present in hot peppers when compared with the existing methods described in the bibliography that utilize a conventional C-18 column in reversed-phase HPLC. The method developed here has a working temperature of 50 ºC in the column and a flow rate of 0.7 mL min^{-1}. These conditions provide a rapid method for the separation and analysis of these capsaicinoids, with high repeatability and intermediate precision (CV < 4%) for peak areas, retention time and resolution. It has been demonstrated that the method is robust with respect to the area of the peaks and to their capacity factor by modifying the sample injection volumes and temperature; however, the method was not robust for changes in the flow rate if the resolutions are considered. The method was also found to be robust with respect to the capacity factor and resolution of the chromatographic peaks on modifying the percentage of methanol in the extracts from 75% to 25%. The combination of state-of-the art column technology and optimized conditions significantly increased sample throughput in standard chromatographic systems when compared to conventional methods. This study demonstrates that fused-core column technology has great potential to deliver faster and more sensitive methods for the analysis of capsaicinoids and other natural products.

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Figure caption

Fig. 1. Chromatogram of the capsaicinoids obtained using a fused-core column. 1- nornordihydrocapsaicin; 2- nordihydrocapsaicin; 3- capsaicin; 4- dihydrocapsaicin; 5- homocapsaicin; 6- homodihydrocapsaicin. $\lambda = 280$ nm.

Table 1
Chromatographic characteristics of the developed method.

	RT (min)	Width	Symmetry Factor	Retention factor (*k**)	Selectivity (α)	Resolution
nn-DHC	1.80	0.04	1.11	8.64	-	-
n-DHC	1.98	0.04	1.21	9.61	1.11	7.36
C	2.03	0.08	1.09	9.86	1.03	1.86
DHC	2.23	0.06	1.11	10.92	1.11	7.44
h-C	2.27	0.06	1.12	11.15	1.02	1.51
h-DHC	2.59	0.07	1.09	14.25	1.28	18.17

Table 2
Validation parameters for the developed method.

	Calibration curve	r	LOD (ppm)	LOQ (ppm)	Retention time		Peak area		Peak resolution	
					Intraday*	Interday**	Intraday*	Interday**	Intraday*	Interday**
nn-DHC	y = 2563.41 x + 73.02	0.9999	0.0998	0.3327	1.79	1.93	2.72	3.94	-	-
n-DHC	y = 2440.92 x + 73.02	0.9999	0.1048	0.3493	1.69	1.82	2.92	1.70	2.61	3.55
C	y = 2162.73 x + 113.28	0.9998	0.1242	0.4140	1.68	1.80	0.85	1.24	2.25	2.87
DHC	y = 2323.61 x + 73.02	0.9999	0.1098	0.3660	1.47	1.60	0.89	1.11	2.03	3.42
h-C	y = 2067.81 x + 113.28	0.9998	0.1299	0.4330	1.43	1.55	2.26	1.60	1.68	3.60
h-DHC	y = 2228.01 x + 73.02	0.9999	0.1148	0.3827	0.91	1.45	1.71	2.08	1.48	3.52

* Intraday coefficient of variance (%) (n = 12).
* Interday coefficient of variance (%) (n = 30).

Table 3

Method robustness. Same letter in the same column mean there are not significant differences by T test ($p < 0.05$).

		Retention time						Area						Resolution					
		nn-DHC	n-DHC	C	DHC	h-C	h-DHC	nn-DHC	n-DHC	C	DHC	h-C	h-DHC	nn-DHC	n-DHC	C	DHC	h-C	h-DHC
Column temperature (ºC)	45	1.837^{a}	2.023^{a}	2.071^{a}	2.271^{a}	2.316^{a}	2.648^{a}	166210^{a}	669038^{a}	8095964^{a}	4454683^{a}	647693^{a}	702312^{a}	-	8.05^{a}	1.97^{a}	7.66^{a}	1.53^{a}	18.54^{a}
	50	1.809^{a}	1.987^{a}	2.033^{a}	2.225^{a}	2.268^{a}	2.561^{b}	169659^{a}	708639^{a}	8031821^{a}	4351528^{a}	645099^{a}	699901^{a}	-	7.26^{b}	1.84^{b}	7.39^{b}	1.49^{b}	18.11^{b}
	55	1.771^{a}	1.949^{a}	1.996^{a}	2.193^{a}	2.237^{a}	2.557^{b}	163722^{a}	704235^{a}	7997022^{a}	4408881^{a}	640343^{a}	698978^{a}	-	7.66^{c}	1.91^{c}	7.53^{c}	1.51^{a}	18.33^{b}
Flow rate (mL/min)	0.665	1.885^{a}	2.075^{a}	2.123^{a}	2.325^{a}	2.370^{a}	2.692^{a}	174383^{a}	749376^{a}	8467770^{a}	4577903^{a}	677241^{a}	742349^{a}	-	8.13^{a}	1.94^{a}	7.75^{a}	1.54^{a}	18.52^{a}
	0.700	1.803^{b}	1.984^{b}	2.031^{b}	2.229^{b}	2.273^{b}	2.592^{b}	163801^{b}	712693^{b}	8064116^{b}	4364944^{b}	645440^{b}	705927^{b}	-	7.36^{b}	1.86^{b}	7.44^{b}	1.49^{b}	18.17^{b}
	0.735	1.710^{c}	1.882^{c}	1.927^{c}	2.119^{c}	2.162^{c}	2.476^{c}	154058^{c}	681184^{c}	7660534^{c}	4135756^{c}	612567^{c}	675563^{c}	-	8.11^{a}	1.92^{a}	7.66^{c}	1.53^{a}	18.59^{a}
Injection volume (µL)	12	1.802^{a}	1.983^{a}	2.030^{a}	2.227^{a}	2.271^{a}	2.588^{a}	162449^{a*}	712519^{a*}	8037489^{a*}	4363380^{a*}	646007^{a*}	699834^{a*}	-	7.86^{a}	1.89^{a}	7.58^{a}	1.53^{a}	18.37^{a}
	15	1.808^{a}	1.990^{a}	2.037^{a}	2.234^{a}	2.279^{a}	2.597^{a}	165765^{a}	713762^{a}	8073747^{a}	4418526^{a}	650210^{a}	704816^{a}	-	7.68^{a}	1.88^{a}	7.53^{a}	1.51^{a}	18.32^{a}
	18	1.813^{a}	1.995^{a}	2.042^{a}	2.240^{a}	2.284^{a}	2.601^{a}	167938^{a*}	715652^{a*}	8036829^{a*}	4384546^{a*}	647220^{a*}	705554^{a*}	-	7.55^{b}	1.86^{a}	7.54^{a}	1.52^{a}	18.39^{a}
Mobile phase composition (%)	0	1.807^{a,b}	1.990^{a,b}	2.037$^{a.b}$	2.236^{a}	2.280^{a}	2.609^{a}	164253^{a}	716443^{a}	8064308^{a}	4363743^{a}	665327^{a}	713115^{a}	-	7.81^{a}	1.93^{a}	7.65^{a}	1.52^{a}	18.07^{a}
	0.1	1.782^{a}	1.961^{a}	2.007^{a}	2.204^{b}	2.248^{b}	2.566^{b}	165919^{a}	719494^{a}	8082794^{a}	4347424^{a}	660684^{a}	710625^{a}	-	8.40^{b}	1.94^{a}	7.74^{a}	1.53^{a}	18.59^{b}
	0.2	1.814^{b}	1.996^{b}	2.043^{b}	2.242^{c}	2.286^{c}	2.600^{c}	162729^{a}	720323^{a}	8103907^{a}	4388959^{a}	657184^{a}	714356^{a}	-	7.69^{a}	1.94^{a}	7.74^{a}	1.53^{a}	18.46^{c}

* Areas are referenced to an injection volume of 15 µL.

Table 4

Effect of the sample solvent on the chromatographic performance of the developed method. Same letter in the same column mean there are not significant differences by T test ($p < 0.05$).

	MeOH/H_2O (%)	nn-DHC	n-DHC	C	DHC	h-C	h-DHC
RT (min)	100	1.806^a	1.987^a	2.033^a	2.230^a	2.275^a	2.592^a
	75	1.794^a	1.974^a	2.021^a	2.219^a	2.264^a	2.583^a
	50	1.802^a	1.982^a	2.028^a	2.224^a	2.269^a	2.585^a
	25	1.804^a	1.984^a	2.030^a	2.227^a	2.272^a	2.589^a
Area (CV)	100	1.02^a	1.18^a	1.21^a	1.11^a	1.34^a	1.32^a
	75	2.06^b	0.97^b	0.51^b	0.55^b	2.24^b	0.52^b
	50	2.26^b	2.74^b	2.11^b	1.78^b	1.84^b	1.78^c
	25	3.30^c	2.07^c	1.26^c	1.30^c	4.87^c	4.09^d
Retention factor (k*)	100	8.26^a	9.19^a	9.43a	10.44^a	10.66^a	13.62^a
	75	8.01^b	8.92^b	9.16^b	10.15^b	10.38^b	13.29^b
	50	7.90^c	8.79^c	9.01^c	9.98c	10.20^c	13.05^c
	25	8.16^d	9.07^d	9.30^d	10.30^d	10.53^d	13.46^d
Symmetry factor	100	1.20^a	1.20^a	1.08^a	1.00^a	1.12^a	1.07^a
	75	1.04^a	1.11^b	1.11^b	1.13^b	1.08^a	1.09^a
	50	1.04^a	1.12^b	1.10^b	1.13^b	1.10^a	1.08^a
	25	1.07^a	1.28^c	1.11^b	1.14^b	1.10^a	1.09^a
Resolution	100	-	7.36^a	1.86^a	7.44^a	1.50^a	18.17^a
	75	-	7.76^b	1.88^b	7.67^b	1.56^b	18.52^b
	50	-	8.01^c	1.91^b	7.71^b	1.57^b	18.53^b
	25	-	8.20^d	1.89^b	7.69^b	1.57^b	18.62^b

Table 5
Individual capsaicinoids content (μg/g ± sd)[a] of different spicy samples.

Sample	nn-DHC (μg/g)	n-DHC (μg/g)	C (μg/g)	DHC (μg/g)	h-C (μg/g)	h-DHC (μg/g)	Total (μg/g)
Habanero pepper	< LOQ	11.4 ± 0.4	1128.3 ± 38.5	321.6 ± 12.2	24.9 ± 1.0	8.6 ± 0.3	1494.8 ± 52.5
Cumari pepper	< LOQ	8.7 ± 0.4	1302.7 ± 46.0	196.2 ± 7.0	12.3 ± 0.5	7.1 ± 0.3	1527.0 ± 54.3
Fatalli pepper	< LOQ	72.1 ± 2.8	312.0 ± 11.2	512.1 ± 19.4	29.1 ± 1.2	< LOQ	925.3 ± 34.6
Naga Jolokia pepper	91.3 ± 3.9	416.3 ± 16.6	4469.4 ± 156.0	2319.9 ± 87.2	342.4 ± 14.4	363.2 ± 14.9	8002.5 ± 293.1
Baiana pepper	< LOQ	7.2 ± 0.3	832.9 ± 29.9	192.7 ± 6.8	6.5 ± 0.3	11.3 ± 0.5	1050.6 ± 37.8
Murupi pepper	< LOQ	10.8 ± 0.4	1776.7 ± 61.4	226.4 ± 8.8	7.7 ± 0.3	14.3 ± 0.6	2035.9 ± 71.5
Dedo-de-Moça pepper	< LOQ	17.1 ± 0.7	274.5 ± 9.8	120.2 ± 4.7	< LOQ	< LOQ	411.8 ± 15.3
Malagueta pepper	11.2 ± 0.3	111.6 ± 4.2	969.1 ± 34.4	490.6 ± 18.3	30.6 ± 1.3	21.9 ± 0.9	1625.0 ± 59.4
Spicy paprika 1	< LOQ	9.4 ± 0.4	200.9 ± 5.6	112.3 ± 3.5	20.7 ± 0.8	8.7 ± 0.4	352.5 ± 10.8
Spicy paprika 2	< LOQ	8.7 ± 0.3	103.9 ± 3.0	59.4 ± 1.9	4.3 ± 0.2	1.5 ± 0.1	183.1 ± 5.4
Spicy paprika 3	< LOQ	6.0 ± 0.2	104.5 ± 2.9	53.2 ± 1.7	12.5 ± 0.5	11.4 ± 0.5	193.1 ± 5.8
Spicy sauce 1	< LOQ	1.4 ± 0.1	26.1 ± 0.7	14.1 ± 0.4	2.5 ± 0.1	0.5 ± 0.0	45.9 ± 1.3
Spicy sauce 2	< LOQ	0.6 ± 0.0	40.5 ± 1.1	11.5 ± 0.4	0.9 ± 0.0	0.3 ± 0.0	55.2 ± 1.6
Spicy sauce 3	< LOQ	0.3 ± 0.0	2.2 ± 0.1	3.6 ± 0.1	< LOQ	0.1 ± 0.0	6.4 ± 0.2
Spicy sauce 4	< LOQ	0.4 ± 0.0	8.2 ± 0.2	2.6 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	12.3 ± 0.4
Spicy sauce 5	< LOQ	5.7 ± 0.2	57.3 ± 1.6	158.3 ± 5.0	35.6 ± 1.4	2.8 ± 0.1	268.1 ± 8.4
Spicy sauce 6	< LOQ	1.8 ± 0.1	7.0 ± 0.2	6.8 ± 0.2	0.3 ± 0.0	0.4 ± 0.0	16.9 ± 0.5
Spicy ketchup 1	< LOQ	< LOQ	0.8 ± 0.0	0.5 ± 0.0	< LOQ	< LOQ	1.3 ± 0.1
Spicy ketchup 2	< LOQ	< LOQ	1.2 ± 0.0	1.0 ± 0.0	< LOQ	< LOQ	2.2 ± 0.1
Spicy ketchup 3	< LOQ	0.1 ± 0.0	0.6 ± 0.0	2.3 ± 0.1	0.2 ± 0.0	0.6 ± 0.0	3.9 ± 0.7

[a]Mean composition of three replicates ± sd (standard deviation). < LOQ = Below the limit of quantification.

Figure 1

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