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Ultrasound-assisted extraction of capsaicinoids from peppers

G.F. Barbero, A. Liazid, M. Palma *, C.G. Barroso

Departamento de Química Analítica, Facultad de Ciencias, Universidad de Cádiz, P.O. Box 40, 11510 Puerto Real, Cádiz, Spain

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Abstract

The development of a rapid, reproducible and simple method of extraction of the majority capsaicinoids (nordihydrocapsaicin, capsaicin, dihydrocapsaicin, homocapsaicin and homodihydrocapsaicin) present in hot peppers by the employment of ultrasound-assisted extraction is reported. The study has covered four possible solvents for the extraction (acetonitrile, methanol, ethanol and water), the optimum temperature for extraction (10–60 °C), the extraction time (2–25 min), the quantity of sample (0.2–2 g), and the volume of solvent (15–50 mL). Under the optimum conditions of the method developed, methanol is employed as solvent, at a temperature of 50 °C and an extraction time of 10 min. The repeatability and reproducibility of the method (R.S.D. < 3%) have been determined. The capsaicinoids extracted have been analysed by HPLC with fluorescence detection and using monolithic columns for the chromatographic separation. The method developed has been employed for the quantification of the various capsaicinoids present in different varieties of hot peppers cultivated in Spain.

Keywords: Capsaicinoids; Ultrasound-assisted extraction; Peppers

1. Introduction

Capsaicinoids are the compounds responsible for the hot, spicy flavour presented by many varieties of peppers. Among the many natural capsaicinoids found in hot chilli peppers, two compounds are predominant: capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) and dihydrocapsaicin (8-methyl-*N*-vanillylnonanamide) [1]; they represent around 90% of the total capsaicinoids present in the hot spicy varieties of peppers. In addition to these two major capsaicinoids, other minor capsaicinoids are found in hot peppers, including nordihydrocapsaicin, norcapsaicin, homocapsaicin I and II, homodihydrocapsaicin I and II, nornorcapsaicin, nornornorcapsaicin, and nonivamide, among others [2,3]. The structural characteristic of capsaicinoids that determines their spicy properties is associated with the presence of an amide bond connecting a vanillyl ring and an acyl chain [4].

Hot peppers are one of the most important species cultivated widely around the world. The properties of colour, aroma, flavour and pungency presented by these peppers account for their extensive usage. In addition to these culinary properties, capsaicinoids present many biological activities. Among these activities, capsaicinoids act as powerful antioxidants [5], present anti-mutagenic and anti-tumoral properties [6,7], function as topical analgesics against pain [8], have anti-inflammatory properties [9] and stimulate the cardiovascular and respiratory systems [10].

Many different techniques have been employed for the extraction of capsaicinoids from pepper, such as maceration [11], magnetic stirring [12], enzymatic extraction [13], ultrasound-assisted extraction [14], Soxhlet [15], extraction by supercritical fluids [16], extraction by pressurized liquids [17] and microwave-assisted extraction [18,19]. The conventional extraction methods, like Soxhlet extraction, which have been employed for decades, need long extraction times and require relatively large quantities of solvent [20]. Recent years have seen increasing demand for extraction techniques that shorten extraction times and reduce the consumption of organic solvents. Among these more efficient extraction techniques are ultrasound-assisted extraction (UAE), microwave-assisted extraction, supercritical fluid extraction and accelerated solvent extraction. The UAE technique is particularly attractive because of its simplicity and low equipment cost; it is based on the employment of the energy derived from ultrasounds (sound waves with frequencies higher than 20 kHz) to facilitate the extraction of analytes from the solid sample by the organic

* Corresponding author. Tel.: +34 956 016775; fax: +34 956 016460.
E-mail address: miguel.palma@uca.es (M. Palma).

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solvent, which is selected in function of the nature of the solutes to be extracted [21]. This technique has been employed to extract various organic compounds from different matrices, including phenolics in cosmetic creams [22], chlorinated pesticides in bird livers [23], organic acids in grapes [24], phenolic compounds from strawberries [25] or isoflavones from soybeans [26].

The enhancement of extraction efficiency of organic compounds by ultrasound is attributed to the phenomenon of cavitation produced in the solvent by the passage of an ultrasonic wave. Cavitation bubbles are produced and compressed during the application of ultrasound. The increase in the pressure and temperature caused by the compression leads to the collapse of the bubble. With the collapse of bubble, a resultant "shock wave" passes through the solvent enhancing the mixing [27].

Ultrasound also exerts a mechanical effect, allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between solid and liquid phase. This, coupled with the enhanced mass transfer and significant disruption of cells, via cavitation bubble collapse, increases the release of intracellular product into the bulk medium. The use of higher temperatures in UAE can increase the efficiency of the extraction process due to the increase in the number of cavitation bubbles formed [27–29].

Although studies have been published on the employment of UAE for the recovery of capsaicinoids from peppers [14], these not have evaluated the influence of the extraction variables nor has a systematic study for the optimisation of the method been carried out; therefore no specific protocol for the UAE of capsaicinoids in peppers has been produced. Thus, the object of the work reported here is to perform the optimisation of the various extraction parameters, particularly the appropriate solvent, temperature, extraction time, quantity of sample, etc. It is also intended to utilise the method developed to quantify the capsaicinoids present in several varieties of hot peppers cultivated in Spain.

2. Experimental

2.1. Chemical and reagents

The solvents utilised: ethanol (Panreac, Barcelona, Spain), methanol, acetonitrile and glacial acetic acid (Merck, Darmstadt, Germany), are of HPLC grade. The water was obtained by a Milli-Q water purification system, from Millipore (Bedford, MA, USA). The capsaicinoid standards: capsaicin (97%) and dihydrocapsaicin (90%), and the internal standard 2.5 dihydroxybenzaldehyde utilised were obtained from Sigma–Aldrich (Steinheim, Germany).

2.2. Plant material

The hot Cayenne pepper (*Capsicum frutescens* L.) was employed for the development of the ultrasound-assisted extraction method. They were obtained from local markets. The peppers were peeled, and the peduncle and seeds were separated. Only the pericarp and the placenta of the pepper were studied. Both the pericarp and the placenta were triturated with a conventional beater, until a homogeneous sample was obtained for the analysis. The triturated sample obtained was conserved in a freezer at −20 °C until its analysis.

2.3. Extraction procedure

The extraction of capsaicinoids originating from peppers by means of ultrasound was performed employing various different extraction conditions—solvents: methanol, ethanol, acetonitrile and water; percentage of water in methanol: 0–100%; temperature: 10–60 °C; volume of solvent: 15–50 mL; quantity of sample: 0.2–2 g; extraction time: 2–25 min. A volume of 0.5 mL of internal standard was added to the extracts obtained (1300 ppm). The extracts were filtered through a 0.45 μm nylon syringe filter (Millex-HN, Ireland) before the chromatographic analysis.

The extraction by ultrasound was performed in an ultrasonic bath of 360 W (J.P. Selecta, Barcelona, Spain) coupled to a temperature controller, which allowed the water in the bath to be renewed.

2.4. HPLC-fluorescence analysis

The HPLC-fluorescence analysis was carried out in a Dionex chromatographic system (Sunnyvale, CA, USA), consisting of an automated sample injector (ASI-100), pump (P680), thermostated column compartment (TCC-100), a photodiode array detector (PDA-100), a fluorescence detector (RF 2000), a universal chromatography interface (UCI-50) and Chromeleon 6.60 software. Capsaicinoids were separated using a Chromolith Performance PR-18e (100 mm × 4.6 mm) monolithic column (Merck).

The chromatographic separation was performed with extracts of the hot Cayenne pepper (*C. frutescens* L.). The wavelengths employed for the detection were 278 nm (excitation) and 310 nm (emission).

The method of chromatographic separation utilised a gradient of two solvents: acidified water (0.1% acetic acid, solvent A) and acidified methanol (0.1% acetic acid, solvent B), working at a flowrate of 6 mL/min. The gradient method utilised is the following: 0 min, 10% B; 2 min, 50% B; 4 min, 50% B; 4.5 min, 55% B; 5.5 min, 55% B; 6 min, 60% B; 7 min, 60% B; 9 min, 70% B; 10 min, 100% B; 15 min, 100% B. The temperature of the column was held constant at 30 °C. The chromatogram obtained by utilising this separation method is represented in Fig. 1.

2.5. Calibration

Using the method developed, calibration curves were prepared for capsaicin and dihydrocapsaicin, which are the two capsaicinoid standards commercially available. The results obtained are presented in Table 1. The limits of detection and quantification were calculated using the ALAMIN software [30].

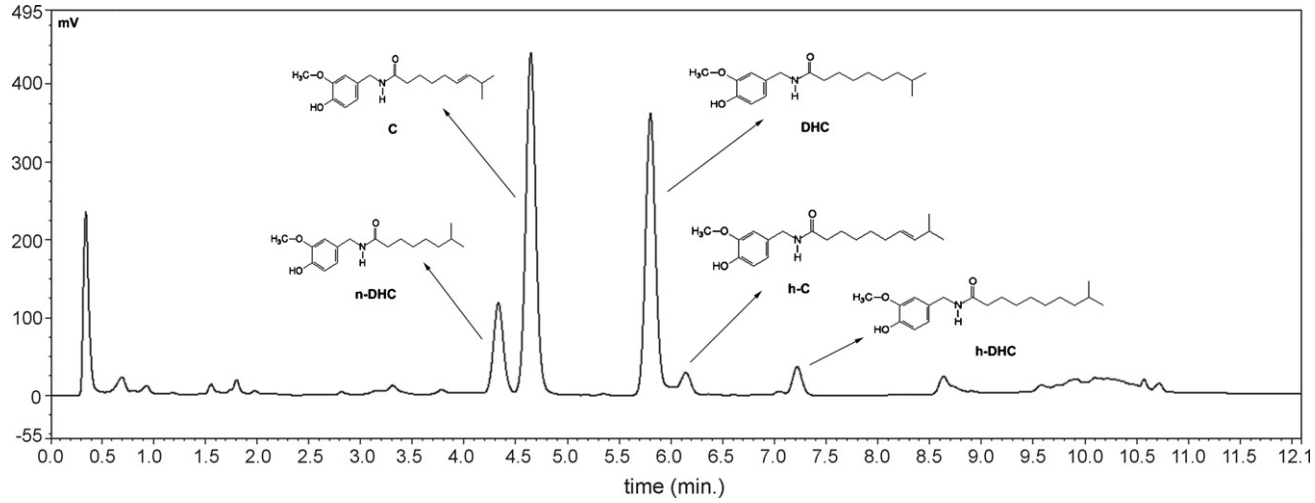

Fig. 1. Chromatogram of separation of the capsaicinoids extracted from hot Cayenne pepper. n-DHC: nordihydrocapsaicin, C: capsaicin, DHC: dihydrocapsaicin, h-C: homocapsaicin and h-DHC: homodihydrocapsaicin.

3. Results and discussion

3.1. Selection of the solvent

The selection of the most appropriate solvent for extracting the analytes of interest from the matrix of the sample is a basic step in the development of any method of extraction. First, the effectiveness of the ultrasound-assisted extraction is going to depend on the extraction solvent's capacity for absorbing and transmitting the energy of the ultrasounds. Second, the capsaicinoids should be soluble in the solvent that is employed for the extraction.

The four solvents that have been studied for extracting capsaicinoids from the matrix of the sample are methanol, ethanol, acetonitrile and water. Methanol [11,14], ethanol [13,17], and acetonitrile [12,31] are solvents that are normally employed for the extraction of capsaicinoids in various extraction techniques, such as Soxhlet extraction, maceration, and extraction by magnetic stirring. Water is not a good solvent for extracting capsaicinoids, but it has been observed that sometimes the addition of small percentages of water to the extraction solvent helps to increase the effectiveness of extraction of the analytes of interest from the sample [26].

The extractions have been performed with a quantity of triturated hot Cayenne pepper of about 1 g of the sample, in 25 mL of solvent, at a temperature of 50 °C for an extraction period of 20 min. All the assays were performed in triplicate.

Table 1
Analytical properties ($n = 3$) of the calibration curve of capsaicin and dihydrocapsaicin

	Capsaicin	DHC
Equation	$y = 112{,}901x + 187$	$y = 151{,}770x + 4589$
r^2	0.9995	0.9995
LD (mg/L)	0.008	0.011
LQ (mg/L)	0.028	0.036

Relative recoveries were calculated by calculating the relative area to the area found for each compound in the extraction showing the highest amount. The relative areas of the different capsaicinoids extracted with the four solvents studied, in the extraction conditions previously described, are represented in Fig. 2.

In the light of Fig. 2, it can be observed that both methanol and ethanol extract similar quantities of capsaicinoids; no significant differences ($p > 0.05$) are observed in the recoveries obtained with these two solvents, in the extraction conditions studied. Acetonitrile is a fairly efficacious solvent for extracting the capsaicinoids present in samples of hot peppers, but is less efficacious than ethanol and methanol, and in these extraction conditions it gives recoveries of only about 80% of those obtained with methanol and ethanol. It was decided to employ methanol as solvent for the development of the extraction method, since this solvent is more compatible with the solvents employed in the chromatographic method.

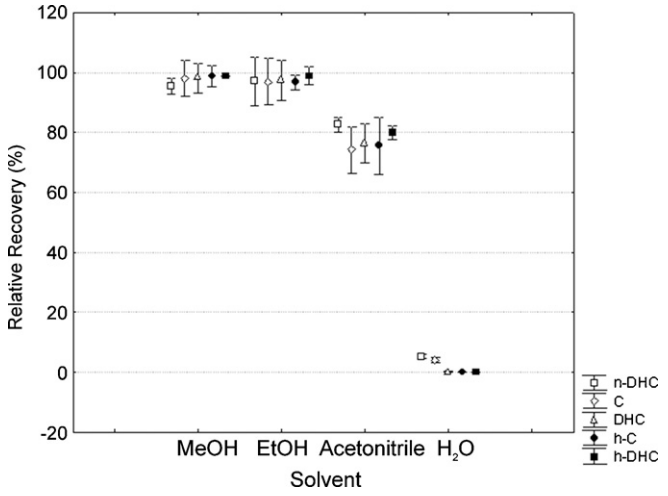

Fig. 2. Relative recoveries of capsaicinoids from hot Cayenne pepper employing different pure solvents.

Table 2
Relative recovery of capsaicinoids extracted from hot Cayenne pepper employing as solvents different mixtures of methanol and water (0, 10, 25, 50 and 100% of water)

Solvent	n-DHC	C	DHC	h-C	h-DHC
0% methanol	5.71	4.20	0.21	0	0
50% methanol	71.66	71.00	67.46	65.01	63.10
75% methanol	79.34	79.52	79.36	79.37	79.19
90% methanol	86.61	86.81	87.14	86.98	87.23
100% methanol	100	100	100	100	100

In Fig. 2 it can also be observed that water, which is a very polar solvent, has a poor capacity of extraction of capsaicinoids. This reduced effectiveness is accentuated in the case of the less polar capsaicinoids such as dihydrocapsaicin, homocapsaicin and homodihydrocapsaicin, where it extracts a lower percentage of these capsaicinoids than of the other more polar capsaicinoids like nordihydrocapsaicin and capsaicin.

On the other hand, in many instances, it has been shown that the addition of differing percentages of water to other extraction solvents improves their extractant properties. For this reason, our study has included the addition of particular percentages of water (0, 10, 25, 50 and 100%) to methanol, as the optimum solvent for extraction, to evaluate how the properties of extraction of this solvent are modified. The extraction conditions were the same as those employed for the selection of the optimum extraction solvent. Similarly, all the assays were performed in triplicate.

The relative recoveries of capsaicinoids extracted from hot Cayenne pepper employing as solvents different mixtures of methanol and water (0, 10, 25, 50 and 100% of water) are represented in Table 2.

It was found that the addition of varying quantities of water to the methanol did not produce any improvement in extracting the capsaicinoids present in the fresh samples of peppers. An addition of water, such as 10% of water in the methanol, in these extraction conditions, has the effect of reducing the recoveries obtained to around 87% of those obtained with undiluted methanol. Higher percentages of water, such as 25%, reduce even further the recoveries obtained, to around 79% of those with undiluted methanol; and the recoveries obtained continue to decline in line with increases in the percentage of water added to the methanol. Therefore, the development of the method was continued based on employing undiluted methanol as the extraction solvent.

3.2. Extraction temperature

Temperature is a fundamental parameter in extracting compounds. Generally speaking, the higher the extraction temperature, the higher the velocity and the efficacy of the extraction process. However some degradation processes can occur at high temperature, then lower recoveries can be obtained. In this study the aim was to evaluate temperatures ranging from 10 to 60 °C (10, 20, 30, 40, 50 and 60 °C). It was not proposed to perform extractions at higher temperatures because 64.7 °C is the boiling point of methanol, working at atmospheric pressure.

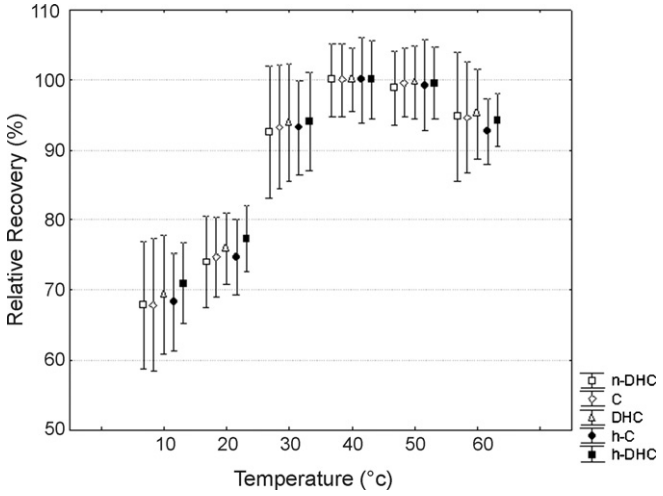

Fig. 3. Relative area of the capsaicinoids extracted at the different temperatures of the assay.

The following were the extraction conditions employed in this study—extraction solvent: methanol; extraction time: 20 min; volume of solvent: 25 mL; quantity of sample: approximately 1 g. All the assays have been carried out in triplicate.

The relative area of the capsaicinoids extracted at the different temperatures of the assay is represented in Fig. 3.

From Fig. 3 it can be observed that, in these extraction conditions, the highest recoveries are obtained at 40 and 50 °C, although the differences are not significant ($p > 0.05$) between 30 and 60 °C. At temperatures lower than 30 °C, the method is not able to extract the same quantity of capsaicinoids as are extracted at higher temperatures, probably because the extraction kinetics take place more slowly the lower the temperature.

The optimum temperature can be considered to be between 30 and 60 °C, at this range of temperatures no degradation of capsaicinoids is observed, in the extraction conditions studied. For later experiments, 50 °C was used as extraction temperature.

3.3. Extraction time

Until saturation, by increasing the extraction time, the quantity of analytes extracted is increased, although there is the risk that degradation may occur. To determine the time needed to obtain complete extractions, extractions from samples of peppers were performed for different lengths of time. Extraction times of 2, 5, 10, 15, 20 and 25 min were evaluated. The rest of the variables employed were: temperature of 50 °C, methanol as extraction solvent, 25 mL of solvent and approximately 1 g of sample. All the assays were performed in triplicate.

The results obtained are given in Fig. 4, in which the relative quantities of capsaicinoids extracted with different times of extraction (2, 5, 10, 15, 20 and 25 min), in the extraction conditions previously indicated, are represented.

It can be observed that, at extraction times longer than 5 min, there are no significant differences ($p > 0.05$) in the recoveries of the capsaicinoids, and quantitative recoveries are obtained. However, a considerable increase in the variability of the recovery is observed by employing as few as 5 and as many as 25 min;

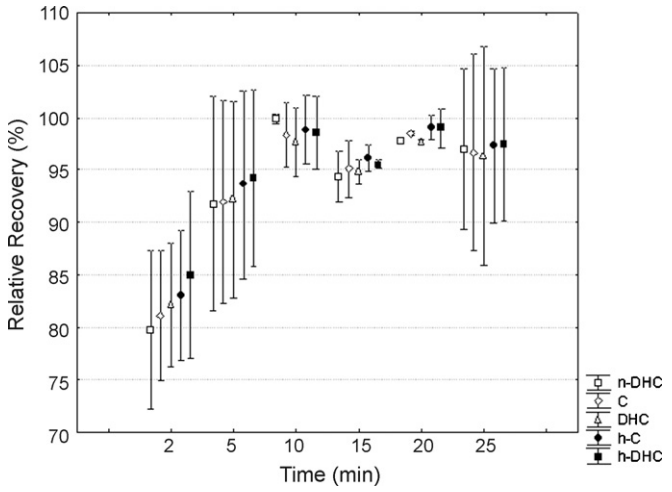

Fig. 4. Relative recoveries of capsaicinoids from hot Cayenne pepper employing different times of extraction.

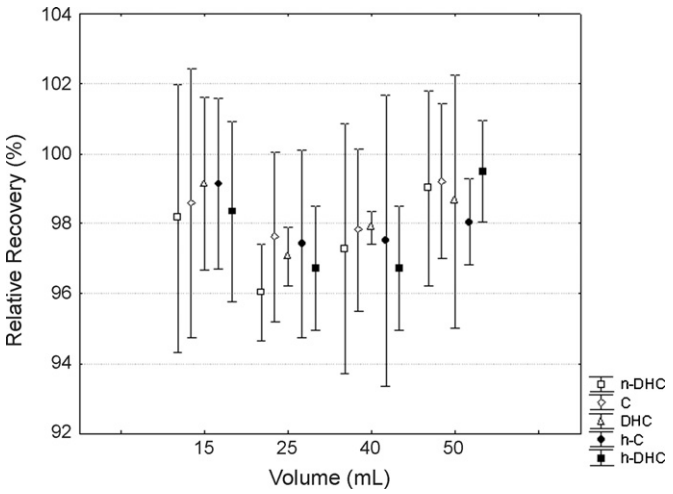

Fig. 5. Relative quantities of capsaicinoids extracted with different volumes of methanol.

therefore it is considered that an adequate time of extraction should fall between 10 and 20 min.

3.4. Volume of solvent

The mass/volume ratio of solvent is a factor that must be studied to increase the efficacy of extraction of capsaicinoids employing ultrasound-assisted extraction. For the conventional techniques of solid–liquid extraction, the tendency is to reduce the ratio of mass/volume of solvent, and in many instances this increases the extraction volume obtained. When this happens, the improvement is due to there being a greater volume of solvent to extract the same quantity of solute.

To evaluate the effect of the volume of the solvent on the extraction, a series of extractions were carried out with different volumes of solvent (15, 25, 40 and 50 mL). The rest of the extraction conditions were: temperature of 50 °C, approximately 1 g of sample, 10 min of extraction and methanol as solvent. All the assays were performed in triplicate.

The relative quantities of capsaicinoids extracted with different volumes of solvent are represented in Fig. 5.

In Fig. 5 it can be observed that there are no significant differences ($p > 0.05$) when the volume of the extraction solvent is varied. Therefore, the variable of solvent volume will not be a determining factor when extracting capsaicinoids in these conditions. It was decided to work with a volume of 25 mL since this enables compounds to be found in levels higher than the LOQ of the chromatographic method.

3.5. Quantity of sample

Once the volume of extraction solvent had been optimised, the next step was to optimise the quantity of sample, the other factor influencing the ratio of mass/volume of solvent previously mentioned. In general, by reducing the quantity of sample while holding the volume constant, the quantities of analytes extracted are increased, since the ratio of mass/volume of solvent is diminished; but the disadvantage of this practice is the decrease of the signal in the subsequent chromatographic system.

In this study sample quantities of 0.2, 0.5, 1, 1.5 and 2 g of peppers have been employed while maintaining the solvent volume constant at 25 mL of methanol. The rest of the extraction parameters utilised were: temperature of 50 °C, 25 mL of methanol as extraction solvent, and 10 min of extraction time. All the assays were performed in triplicate.

The results obtained are given in Fig. 6, in which the relative quantities of capsaicinoids extracted with different sample quantities (0.2, 0.5, 1, 1.5 and 2 g), in the extraction conditions previously indicated, are represented.

In the light of Fig. 6 it can be observed that the quantity of sample is not a relevant parameter. Thus, it was decided to employ 1 g, since this quantity of sample produces compounds found in levels higher than the LOQ of the chromatographic method.

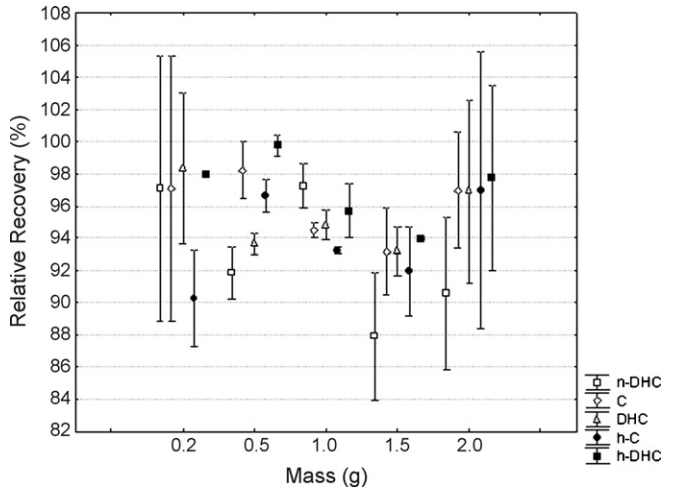

Fig. 6. Recoveries of capsaicinoids from hot Cayenne pepper employing different quantities of sample.

Table 3
Repeatability ($n = 9$) and reproducibility ($n = 18$) of the method developed for the capsaicinoids analysed

	n-DHC	C	DHC	h-C	h-DHC
R.S.D. (%) intraday	1.97	1.72	1.84	2.35	1.83
R.S.D. (%) interday	2.42	2.46	2.56	2.37	2.58

Table 4
μmol of capsaicinoid per kilogram of fresh pepper in the samples analysed

Pepper	n-DHC	C	DHC	h-C	h-DHC
Cayenne	94 ± 6	448 ± 28	265 ± 15	30 ± 1	47 ± 2
BTR	40 ± 3	370 ± 23	190 ± 11	n.d.	20 ± 1
BTL	25 ± 2	275 ± 17	122 ± 7	n.d.	14 ± 1

n.d.: not detected. BTR: Bolilla Redondo pepper and BTL: Bolilla Largo pepper.

3.6. Repeatability and reproducibility of the method

The repeatability and reproducibility of the method developed have been studied. For this a total of 21 extractions were performed, distributed as follows: 9 extractions performed on the first day of the study, and 6 more extractions on each of the two consecutive days. The resulting R.S.D.s are given in Table 3. Similar results were found for all capsaicinoids, all of them lower than 3%.

3.7. Quantification of the capsaicinoids present in different samples of peppers

The amounts of capsaicinoids (nordihydrocapsaicin, capsaicin, dihydrocapsaicin, homocapsaicin and homodihydrocapsaicin) present in three varieties of peppers have been quantified using this method. Samples of hot Cayenne pepper (*C. frutescens*), Bolilla Largo pepper (*Capsicum annuum*) and Bolilla Redondo pepper (*C. annuum*) were employed. Capsaicin and dihydrocapsaicin were quantified from the calibration curves obtained from the standard solutions. Since there are no commercial standards of nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin, these were quantified from the calibration curve of dihydrocapsaicin (for nordihydrocapsaicin and for homodihydrocapsaicin) and from the calibration curve of capsaicin (for homocapsaicin), given the structural similarities between these molecules. Table 4 gives the quantities of capsaicinoids present in the different varieties of peppers studied.

It can be observed that, of the three varieties of peppers studied, it is hot Cayenne pepper that contains the largest amount of capsaicinoids, followed by the Bolilla Redondo pepper, and lastly by the Bolilla Largo pepper. Homocapsaicin was only found in hot Cayenne pepper; neither of the other two varieties studied (Bolilla Redondo and Bolilla Largo) contained this compound.

4. Conclusions

Ultrasound-assisted extraction, by means of the method developed, allows the quantitative and reproducible (R.S.D. < 3%) extraction of the capsaicinoids present in peppers, in a short time (10 min), employing methanol as extractant solvent. Given its low instrumental requirement, its simplicity and its analytical capabilities, the method developed can be applied for the routine analysis of capsaicinoids in peppers.

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