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Pressurized Liquid Extraction of Capsaicinoids from Peppers

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A method has been developed for the extraction of capsaicinoids from peppers by pressurized liquid extraction (PLE); these compounds are determined by reverse phase high-performance liquid chromatography (HPLC), with detection by fluorescence spectrophotometry and mass spectrometry (MS). The stability of capsaicin and dihydrocapsaicin has been studied at different temperatures (50−200 °C), and several extraction variables have been assayed: solvent (methanol, ethanol, and water), different percentages of water in the methanol (0−20%) and in the ethanol (0−20%), and the number of extraction cycles. The study has evaluated the repeatability (RSD < 7%) and the reproducibility (RSD < 7%) of the method. Finally, the PLE method developed has been applied to quantify the capsaicinoids present in three varieties of hot peppers cultivated in Spain, quantifying five capsaicinoids: nordihydrocapsaicin, capsaicin, dihydrocapsaicin, an isomer of dihydrocapsaicin, and homodihydrocapsaicin.

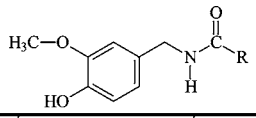
KEYWORDS: Capsaicinoids; pungent compounds; pressurized liquid extraction; peppers

INTRODUCTION

Hot or spicy peppers are savory food additives that are widely used in many parts of the world and highly valued for their attributes of color, pungency, and aroma. The capsaicinoids are the compounds responsible for the hot, spicy flavor imparted by many peppers. The two major capsaicinoids present in most varieties of hot peppers are capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) and dihydrocapsaicin (8-methyl-*N*-vanillylnonanamide) (*1*). In addition to these two major capsaicinoids, other minor capsaicinoids are found in hot peppers, such as nordihydrocapsaicin, norcapsaicin, homocapsaicin, homodihydrocapsaicin, nornorcapsaicin, nornornorcapsaicin, and nonivamide, among others (*2*, *3*) (**Figure 1**).

To date, capsaicinoids have been described in only the fruits of plants of the *Capsicum* genus. The concentrations of capsaicinoids in the hot spicy pepper varieties vary significantly one from another. The less spicy varieties of peppers have capsaicinoid concentrations that range from 0.003 to 0.01% in dry weight of the pepper. The concentrations of capsaicinoids in the mildly hot spicy varieties range from 0.01 to 0.3%, and the very hot spicy varieties are characterized by a content in capsaicinoids in excess of 0.3% of the total weight, and this can reach 1% (*4*).

Many techniques for the extraction of capsaicinoids from peppers have been studied, including maceration (*5*), magnetic stirring (*6*), Soxhlet (*7*−*9*), ultrasonic assisted extraction (*10*), extraction by supercritical fluids (*11*, *12*) extraction by microwaves (*13*), and enzymatic extraction (*14*).



Compounds	Symbol	R
Capsaicin	C	
Dihydrocapsaicin	DHC	
Norcapsaicin	n-C	
Nordihydrocapsaicin	n-DHC	
Homocapsaicin	h-C	
Homodihydrocapsaicin	h-DHC	
Nornordihydrocapsaicin	nn-DHC	
Nornornordihydrocapsaicin	nnn-DHC	
Nonivamide	Non	

Figure 1. Major capsaicinoids present in hot peppers.

Extraction by means of pressurized liquids (PLE) is a relatively novel extraction technique in which temperature and pressure are utilized to accelerate the extraction of compounds originating from solid or semisolid samples. High temperature and pressure can modify considerably the physical properties

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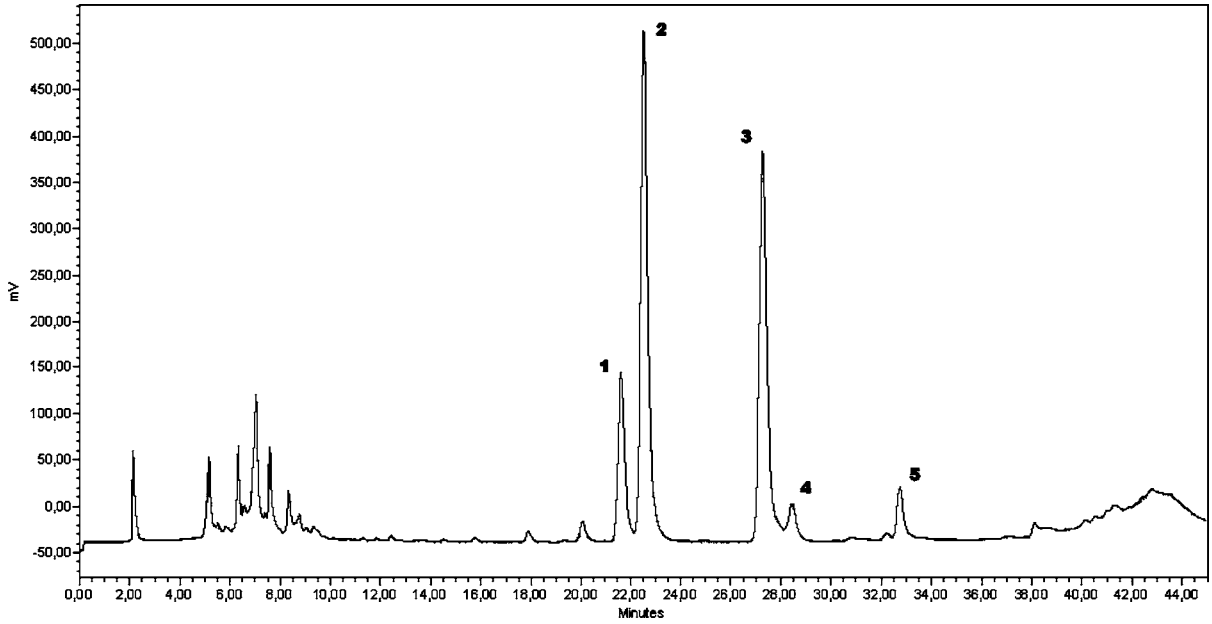

Figure 2. Chromatogram of a cayenne pepper extract (fluorescence signal, emission 310 nm). Peaks: 1, nordihydrocapsaicin; 2, capsaicin; 3, dihydrocapsaicin; 4, i-dihydrocapsaicin; 5, homodihydrocapsaicin.

of the extraction solvents, with the effect of increasing selectivity in the extraction (*15*).

This technique is attractive for many reasons. The technique offers a substantial reduction of the quantity of organic solvent needed in the extraction. The time of analysis of extraction is also considerably reduced in comparison with traditional extraction techniques. In addition, extraction by PLE utilizes highly automated equipment, which translates into much more precise results. In addition, with PLE, extractions can be performed under an inert atmosphere and in the absence of light, which represents a great advantage in the extraction of compounds that may be sensitive to oxidative degradation through the action of the air or the light (*16*).

Several studies have successfully used the technique of PLE for the extraction of different natural products from vegetable matrices, such as polyphenolic compounds from grape seeds (*17*) and apples (*18*), proanthocyanidins from malt (*19*), and isoflavones from soybeans (*20*). Other bioactive natural compounds have been extracted successfully by means of PLE (*21*, *22*).

Prior to this paper, the use of PLE to extract capsaicinoids from pungent hot peppers has not been reported. Moreover, no stability studies have been performed with this technique. Therefore, in this study we evaluate the feasibility of PLE for isolating capsaicinoids present in hot peppers and provide a reliable analytical extraction method.

EXPERIMENTAL PROCEDURES

Samples. This PLE method has been developed with spicy cayenne pepper. Once the method had been developed, three varieties of peppers from the varieties cultivated in Spain (cayenne pepper, long marble pepper, and round marble pepper) were analyzed. Commercially available fresh peppers were used. The peppers were peeled fresh, and the seeds and peduncle were removed. Once the pepper was separated from the seeds and peduncle, it was triturated using an electric mixer to obtain a homogeneous paste. Subsequently, the sample was stored at −20 °C until its analysis.

Chemical and Solvents. Ethanol (Panreac, Barcelona, Spain) and methanol (Merck, Darmstadt, Germany) used were of HPLC grade. Water was supplied by a Milli-Q water purifier system from Millipore (Bedford, MA). The standards of capsaicinoids, capsaicin (99%) and dihydrocapsaicin (90%), were obtained from Sigma Chemical Co.

Extraction of Hot Pepper. Extractions were performed on a Dionex ASE 200 extractor (Dionex Corp., Sunnyvale, CA). The sample was mixed with sea sand (Panreac) and placed in an 11-mL stainless steel extraction cell. A cellulose filter (Dionex Corp.) was placed at the bottom of the extraction cell.

The extraction chamber was filled with the extraction solvent [water, ethanol, or methanol (0−20% in water)], pressurized to 100 atm of pressure, and heated to temperatures ranging from 50 to 200 °C. The extractions were done with pepper sample quantities of ∼0.7 g. The extracts of peppers (volumes of <50 mL) were topped up to 50 mL, and these were the samples that were analyzed by HPLC with fluorescence detection and mass spectrometry.

Analysis of the Capsaicinoids by HPLC with Fluorescence and Mass Spectrometry Detection. The extracts obtained from all of the extractions performed were analyzed by HPLC in a Waters system, comprising an autoinjector, a pump controller, a pump, and a fluorescence detector using a C-18 column (Luna 5 μm, 150 × 3 mm, Phenomenex). A gradient method, using acidified water (0.1% acetic acid, solvent A) and acidified methanol (0.1% acetic acid, solvent B), working at a flow rate of 0.4 mL min^{-1}, was utilized for the chromatographic separation. The gradient employed was the following: 0 min, 0% B; 1 min, 0% B; 5 min, 30% B; 8 min, 50% B; 16 min, 70% B; 20 min, 70% B; 28 min, 90% B; 30 min, 90% B; 32 min, 100% B; 42 min, 100% B.

The volume of sample injected was 20 μL. Detection by excitation/emission in fluorescence was employed for the quantification; specifically an excitation wavelength of 278 nm was utilized, and the emission was recorded at a wavelength of 310 nm. **Figure 2** shows a typical chromatogram.

Capsaicin and dihydrocapsaicin were quantified by means of calibration curves obtained from commercial standards of these compounds. The nordihydrocapsaicin, the isomer of dihydrocapsaicin, and the homodihydrocapsaicin were quantified by comparison with the calibration curve of dihydrocapsaicin. **Table 1** presents the properties of the calibration curves of capsaicin and dihydrocapsaicin. The limits of detection and quantification have been calculated using ALAMIN software (*23*).

The capsaicinoids were identified in the samples of peppers by HPLC-MS. The analyses of the extracts were performed in a Finnigan LCQTM coupled LC-MS system, of Finnigan MAT (Thermo Electron Co., San Jose, CA). This equipment is fitted with a Spectra System 2000 model gradient pump (Thermo Separation Products, Fremont, CA)

Table 1. Parameters of the Calibration Curves of Capsaicin and Dihydrocapsaicin

	capsaicin	dihydrocapsaicin
calibration range (mmol L^{-1})	3.25–0.08	3.25–0.08
regression equation	$y = 38072 + 1725945.64x$	$y = 50349 + 2374245.41x$
regression coefficient	0.9998	0.99993
detection limit (mmol L^{-1})	0.112	0.036
quantification limit (mmol L^{-1})	0.375	0.121

and a mass detector (model LCQ) that consists of an electrospray interface and an ion trap mass analyzer. The software for the control of the equipment and for the acquisition and treatment of data is Xcalibur, version 1.2. The same gradient as in LC-UV was applied. The sample injection volume was 25 μL. The interface conditions were as follows: positive ionization mode; temperature of the capillary, 220 °C; spray voltage, 20 kV; capillary voltage, −5 V; focus gas flow, 80 (arbitrary units); and auxiliary gas flow, 10 (arbitrary units). API-MS spectra were acquired in the *m*/*z* range of 50−400.

RESULTS AND DISCUSSION

Analysis of Capsaicinoids by HPLC-MS. The extracts of peppers obtained by PLE were analyzed by HPLC-MS. By means of this technique five capsaicinoids have been determined in the extracts of cayenne pepper. Capsaicin and dihydrocapsaicin have been identified by both retention data and fragmentation modes of these compounds in comparison with those of standards using HPLC-MS. The other three capsaicinoids found (nordihydrocapsaicin, an isomer of dihydrocapsaicin, and homodihydrocapsaicin) have been identified using their molecular ion and their fragmentation model, similar to that of capsaicin and dihydrocapsaicin.

The molecular ions for the capsaicinoids found presented the following *m*/*z* ratios: nordihydrocapsaicin, 294; capsaicin, 306; dihydrocapsaicin, 308; isomer of dihydrocapsaicin, 308; and homodihydrocapsaicin, 322. In the mass spectra of these five capsaicinoids, the *m*/*z* peak (137) characteristic of the fragmentations of capsaicinoids appears clearly.

The isomer of dihydrocapsaicin, which appears at a retention time different from that of dihydrocapsaicin, has been determined through its possessing the same *m*/*z* ratio as dihydrocapsaicin, possessing a pattern of fragmentation similar to that of dihydrocapsaicin, and the identification of this compound in the literature (*24*).

In **Figure 3** the mass spectra of the capsaicinoids found in the peppers studied are presented, together with the structure of the molecules and their fragmentation.

Stability of Capsaicin and Dihydrocapsaicin with Temperature. The stability of standards of capsaicin and dihydrocapsaicin with temperature has been studied. For this standard, solutions of capsaicin (22.1 mg L^{-1}) and dihydrocapsaicin (27.72 mg L^{-1}) in methanol as solvent were prepared. The experiments by pressurized liquids were performed at a pressure of 100 atm and with an extraction time of 30 min. The solvent employed for the extraction was methanol. The working temperatures studied were 50, 100, 150, 175, and 200 °C.

To carry out the study, 0.5 mL of the prepared standard solution of capsaicin and of dihydrocapsaicin was introduced into the extraction chamber and mixed with the solid phase of the chamber filling (sea sand), and the experiment was then performed under the conditions previously stated, for 30 min. Volumes of solvent of <25 mL were obtained, and this volume was later topped up to 25 mL for the subsequent analysis by HPLC coupled to a fluorescence spectrophotometer.

To ensure that the standards (capsaicin and dihydrocapsaicin) introduced into the extraction chamber were not washed out by the automatic flushing of the equipment that takes place when an overpressure is reached inside the chamber, the shut-off valves of the system were controlled manually, thus avoiding the automatic flushing. All of these experiments were performed in duplicate.

Figure 4 shows the amount of capsaicin and dihydrocapsaicin for each temperature studied with respect to the reference sample, that is, 0.5 mL of the prepared standard solution of capsaicin and of dihydrocapsaicin diluted to 25 mL. It can be observed that there is no apparent degradation of the capsaicin or of the dihydrocapsaicin as increasing temperatures were assayed, from 50 to 200 °C; most likely this is due to the absence of air during the process, avoiding oxidation reactions, which are accelerated at high temperatures. The stability of capsaicins during PLE conditions makes it feasible, therefore, for extractions of the real samples of peppers to be performed at any temperature between 50 and 200 °C for at least 30 min.

Selection of Extraction Temperature. After the stability of commercially available capsaicinoids with temperature had been checked, the extraction of nordihydrocapsaicin, capsaicin, dihydrocapsaicin, an isomer of dihydrocapsaicin, and homodihydrocapsaicin from real pepper samples at different temperatures from 50 to 200 °C was studied. Approximately 0.7 g of cayenne pepper was extracted using the same PLE method (1 cycle, 5 min, 100 atm). The resulting relative amount found (**Figure 5**) showed that extractions run at 200 °C produced the highest recoveries for the five studied capsaicinoids. Higher temperatures are not allowed by the instrument used in this study. Therefore, 200 °C was selected as extraction temperature.

Selection of Extraction Solvent. At first, three extraction solvents were assayed: methanol, ethanol, and water, all in their pure state. The extraction conditions in this assay were the following: extraction pressure, 100 atm; extraction temperature, 200 °C; sample quantity, ∼0.7 g of cayenne pepper; three extraction cycles of 5 min each, which gives an overall extraction time, including the preheating, of ∼30 min. Results obtained are shown in **Figure 6**.

From **Figure 6**, which shows the quantity of capsaicinoids (micromoles per kilogram) extracted with each extraction solvent assayed (ethanol, methanol, and water), it can be seen that the largest quantities of capsaicinoids were extracted with methanol. With ethanol it is possible to extract quantities of capsaicinoids similar in some cases to those using methanol, although generally somewhat lower. Of the three solvents studied, water was the one that produced the lowest extraction values of capsaicinoids from fresh samples of peppers using the technique of PLE.

After the assays with the pure solvent, it was decided to evaluate the capacity of ethanol/water and methanol/water mixtures, because in the literature there are descriptions of PLE methods that produce better results with solvents in which small quantities of water are incorporated (*20*). For this, extractions of samples of peppers have been performed utilizing as extraction solvents diluted ethanol (0−20% H_2O) and methanol (0−20% H_2O). The extraction conditions in this assay were the same as those described previously in this same part of the paper. The results are shown in **Figures 7** and **8**.

It can be observed that the highest recovery is obtained using ethanol (**Figure 7**) with a water content of 10%. However, the

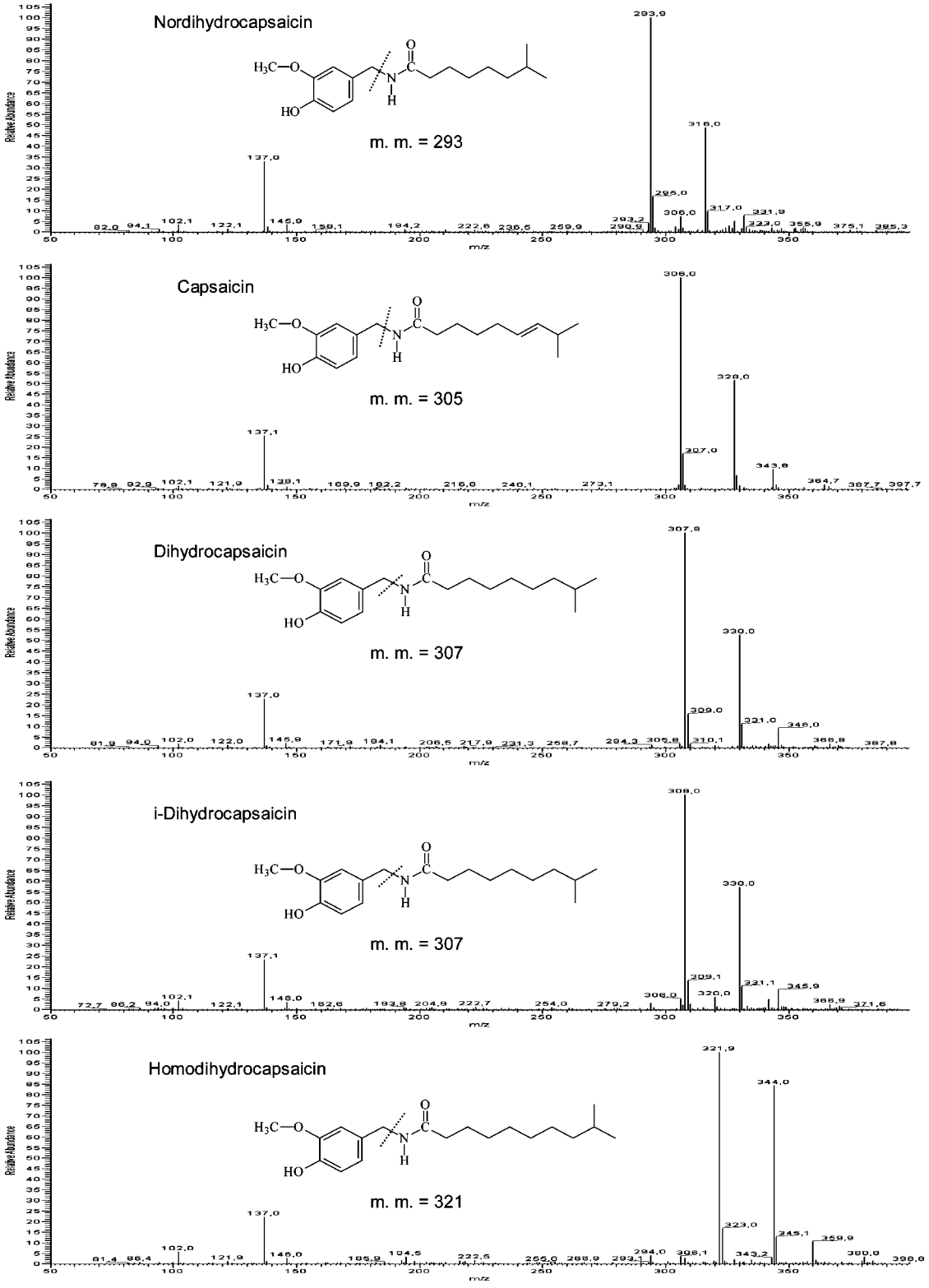

Figure 3. Mass spectrum, molecular structure, and fragmentation of (**a**) nordihydrocapsaicin, (**b**) capsaicin, (**c**) dihydrocapsaicin, (**d**) isomer of dihydrocapsaicin, and (**e**) homodihydrocapsaicin found in the peppers studied.

highest recovery with methanol (**Figure 8**) is obtained using the solvent in its pure form, without the addition of water.

In light of all the results, it can be confirmed that the greatest extraction yields are obtained with 100% methanol, and for this reason this solvent was chosen for performing the extraction method.

Effect of the Number of Extraction Cycles on the Recovery of Capsaicinoids. A study was conducted to observe the quantity of capsaicinoids extracted with one, two, and three extraction cycles of 5 min each. The extraction conditions in this assay were the following: extraction pressure, 100 atm; extraction temperature, 200 °C; quantity of sample for extraction, ~0.7 g.

From **Figure 9** it can be seen that there are no significant differences in the quantity of capsaicinoids extracted with one, two, or three cycles of extraction of 5 min of duration; therefore,

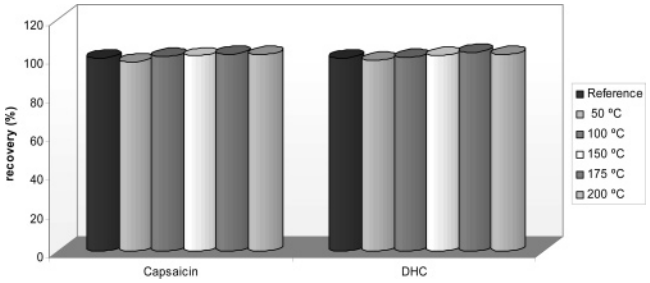

Figure 4. Percentage of capsaicin and dihydrocapsaicin recovered from the standard solution by PLE at different temperatures.

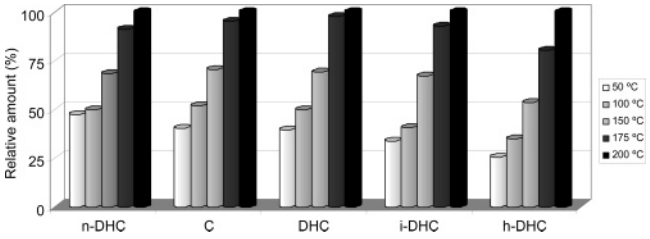

Figure 5. Relative average ($n = 3$) amount of capsaicinoids extracted using different extraction temperatures.

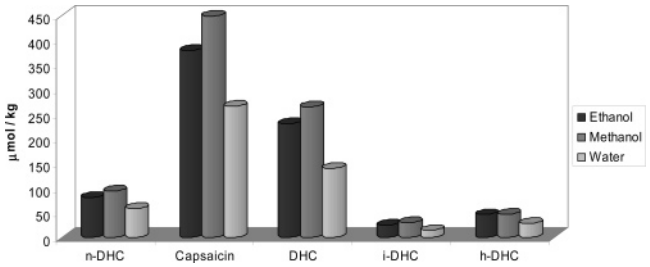

Figure 6. Quantity of capsaicinoids (micromoles per kilogram) extracted with ethanol, methanol, and water.

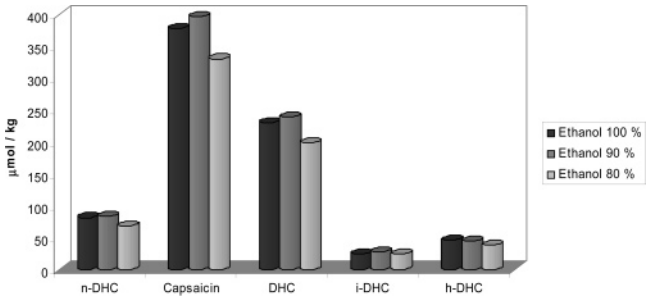

Figure 7. Quantity of capsaicinoids (micromoles per kilogram) extracted with ethanol/water mixtures.

a single extraction cycle was considered to be sufficient. This means that the full extraction method is going to take only 12 min, including the preheating step.

Re-extractions. To check that the extraction method developed using PLE produces quantitative extractions, a sample re-extraction study was conducted. For this, extractions were made from a series of samples by the method of PLE extraction developed. Once these samples had first been extracted by PLE, they were then extracted a second time using this same method. The recoveries of capsaicinoids observed in the re-extractions are presented in **Table 2**.

From **Table 2** it can be deduced that the re-extractions of the samples produce a recovery of capsaicinoids ranging from 2.4% for nordihydrocapsicin to 3.2% for homodihydrocapsaicin.

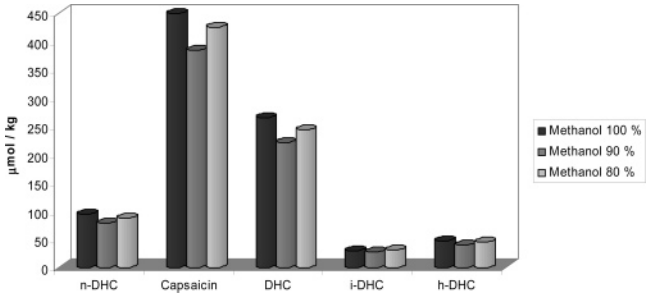

Figure 8. Quantity of capsaicinoids (micromoles per kilogram) extracted with methanol/water mixtures.

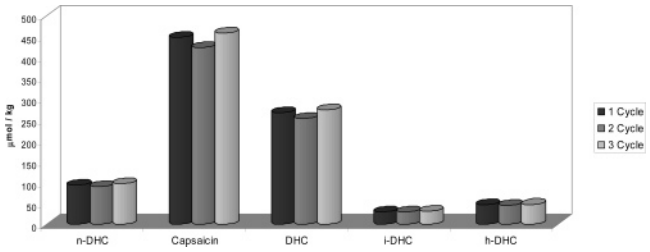

Figure 9. Quantity of capsaicinoids (micromoles per kilogram) extracted with one, two, and three cycles of extraction of 5 min each.

Table 2. Results of Extraction and Re-extraction Yields of Capsaicinoids with the PLE Method Developed

	yield (μmol kg^{-1})				
capsaicinoid	n-DHC	C	DHC	i-DHC	H-DHC
first extraction	91.2	432.9	262.0	29.3	46.8
second extraction	2.2	10.0	6.2	nd[a]	1.5

[a] Not detected.

Table 3. Average and Relative Standard Deviation (RSD) for the Study of Intraday and Interday Results for a Total of 12 Extractions

	av (μmol kg-1)				
	n-DHC	C	DHC	i-DHC	H-DHC
intraday RSD (%)	6.73	6.30	5.73	4.50	4.73
interday RSD (%)	6.18	5.92	5.67	6.08	4.51

Therefore, quantitative recoveries can be obtained for all the capsaicinoides assayed in the first extraction using the developed method.

Repeatability and Reproducibility of the Method. The repeatability and reproducibility of the method have been studied. For this, six extractions were made on the same day, and nine extractions were made on three different days. The results obtained are presented in **Table 3**.

From **Table** 3 it can be checked that the values of RSD for repeatability and reproducibility are below 7% for the five capsaicinoids analyzed; therefore, the method developed can be considered to offer high repeatability and reproducibility.

Quantification of the Capsaicinoids Present in Different Samples of Peppers. Quantification has been performed to determine the five capsaicinoids (nordihydrocapsaicin, capsaicin, dihydrocapsaicin, the isomer of dihydrocapsaicin, and homodihydrocapsaicin) present in three varieties of peppers (cayenne pepper, long marble pepper, and round marble pepper). Capsaicin and dihydrocapsaicin were quantified from the calibration curves obtained from the standard solutions prepared from the standards of these compounds.

Table 4. Concentration of Capsaicinoids in the Samples Analyzed

	concn (μmol kg^{-1} of fresh pepper)				
pepper	n-DHC	C	DHC	i-DHC	h-DHC
cayenne	93.8 ± 6.3	448.4 ± 28.3	265.1 ± 15.2	29.7 ± 1.3	46.8 ± 2.2
long marble	40.3 ± 2.7	369.8 ± 23.3	190.1 ± 10.9	nd[a]	19.7 ± 0.9
round marble	25.3 ± 1.7	275.2 ± 17.3	122.5 ± 7.0	nd	14.5 ± 0.7

[a] Not detected.

Because there are no commercial standards available for nordihydrocapsaicin, the isomer of dihydrocapsaicin, and homodihydrocapsaicin, quantification was performed from the calibration curve of dihydrocapsaicin.

The capsaicinoids present in the samples of peppers were quantified using HPLC coupled to fluorescence spectrophotometry.

From **Table 4**, it can be seen that cayenne pepper is the variety that possesses the largest quantity of capsaicinoids of the three studied, followed by round marble pepper and finally long marble pepper. Neither of the latter types of pepper was found to contain any of the isomer of dihydrocapsaicin, which appears in cayenne pepper; therefore, the presence or absence of this compound could be of help in taxonomic studies.

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