ABSTRACT

Capsaicinoids are organic compounds present in several foods like chili peppers. This group of molecules are responsible of fruit pungency, as well as, of several healthy properties. The present work reports an optimized and validated liquid chromatography method coupled to fluorescence and mass spectrometry detectors for a selective determination of capsaicin in Chili peppers (*Capsicum annuum* and *C. pendulum*). To the best our knowledge this is the first report about capsaicin determination in chili peppers commercialized in Chile. Chromatographic conditions were optimized giving the following optimal conditions: 76% organic phase (MeOH: ACN: acetic acid (50:50:0.1 v/v/v)) at 9 min of gradient program and column temperature of 35°C. With these conditions capsaicin, dihydrocapsaicin and nordihydrocapsaicin were separated in 15 min. Data calibration curve (0.01-2.00 mg L$^{-1}$) fitted a linear regression model with a determination coefficient ($R^2$) of 0.9986. Repeatability (relative standard deviation, RSD) and intermediate precision (RSD) showed values of 1.51% ($n=6$) and 1.04% ($n=3$), respectively. Recovery ($n=3$) at three levels ranged from 94.80 to 109.40%, (RDS <2.39%). The method was applied to analyze 10 chili peppers varieties commercialized in Chile. A broad range of capsaicin and dihydrocapsaicin contents were observed, finding values from 0.1 up to 127.3 μg g$^{-1}$.

Keywords: Capsaicinoids, capsaicin, fluorescence, mass spectrometry, chromatography

1. INTRODUCTION

Worldwide overweight and obesity have become a major health threat. In 2016 more than 1.9 billion adults (≥18 years old) presented overweight, from which 650 million were obese 1. Both conditions are one of the major risk factors for several diseases like diabetes type 2 and cardiovascular diseases. These diseases are classified as chronic non-communicable diseases (CNCD), which are pathologies characterized by showing long duration and slow progression. The main CNCD risk factors are tobacco use, physical inactivity, harmful use of alcohol and unhealthy diets. Regarding the latter, it has been clearly established the relation between health and diet. Since CNCD incidence has continuously increased, a new kind of food has been developed/evaluated called functional foods. A simple definition describes the functional foods as processed or unprocessed foods that contain biologically active components that exert healthy effects beyond of intrinsic nutritional effect, that may reduce the risk of suffer from CNCD 2. In this regard, chili peppers could be classified as a functional food considering the healthy effects ascribed to its consumption. Chili peppers are fruits from the genus *Capsicum* and belong to the family Solanaceae. The genus *Capsicum* L. comprises five main species: *C. annuum*, *C. frutescens*, *C. chinense*, *C. baccatum* and *C. pubescens*. These peppers varieties are worldwide used and valued for their sensory properties, i.e. color, pungency and aroma. Pungency, a commercially important attribute of peppers, is produced by particular group of molecules denominated capsaicinoids 3. Capsaicin and dihydorcapsaicin are the two most abundant capsaicinoids in chili peppers, constituting about 90% 4-6. Other minor ones are nordihydrocapsaicin, norcapsaicin, homocapsaicin, nornorcapsaicin, nornorcapsaicin and noniavamide 5-8. Capsaicinoids content depend on the genotype and maturation stage 10. Capsaicin and other capsaicinoids produce a number of physiological and pharmacological effects on the cardiovascular system 11 and gastrointestinal tract 12. Capsaicin has been used in neurological research to stimulate sensory nerves and also to treat bladder inflammation. In topical ointments it has been used for arthritis and neuralgia treatment 15. Regarding its consumption, in Latin America, Mexico is the country with highest intake of *Capsicum* spices corresponding to 20 g per day 14 (equivalent to one chili pepper) 15. In USA and Europe the maximum daily intake of capsaicin from mild chilies and paprika is about 1.5 mg per day 16. According to a recent estimation, the mean and maximum intakes of capsaicin from industrially prepared food products that contains the recommended general limit of 5 μg g$^{-1}$ is 0.77 and 2.64 mg per day, respectively 14,17. The pungency level has been evaluated using the Scoville Organoleptic Test 18, which express the pungency level in a scale called the Scoville Heat Unit (SHU) 19. Today this test has since been replaced by instrumental methods that measure the capsaicinoids (capsaicin) content in chili
peppers, e.g. gas chromatography (GC) 18, liquid chromatography (HPLC) 20, and thin layer chromatography (TLC) 21, among others. By far the most common technique used for the determination and quantification of this type of compound is reversed phase HPLC due to its reliability. Several extraction methods has been described for capsaicinoids extraction from chili peppers, i.e. solid-liquid extraction 22, ultrasound-assisted extraction 23, microwave 24 and pressured liquid extraction 25. Due to its functional properties, it is relevant to determine the capsaicin and dihydrocapsaicin content in chili peppers commonly consumed by the population. For this reason, the objective of this work was to optimize and validate a liquid chromatographic method with fluorescence and mass spectrometry detection for capsaicin and dihydrocapsaicin determination in chili peppers. To the best our knowledge this is the first report about capsaicin determination in chili peppers commercialized in Chile.

2. MATERIAL AND METHODS

2.1 Reagents, chemicals and samples

Capsaicin [8-metil-N-vanillyl-trans-6-nonenamide, MW 305.41 g mol⁻¹ ≥95%] was purchased from Sigma (St. Louis, MO, USA). LC-grade methanol (MeOH) and acetoniitile (ACN) and acetic acid (99.8 %) were obtained from Merck (Darmstadt, Germany). Ultrapure water (18.2 MΩ cm) was produced by means of Simplicity system from Millipore (Bedford, MA, USA). Filter paper Nº4 was obtained from Whatman (NJ, USA) and 13 mm PVDF syringe filters (0.45 μm pore size) were purchased from Millipore. Capsaicin stock solution was prepared in mobile phase (MeOH: ACN : acetic acid; 50:50:0.1 v/v/v) for a given concentration of 10 mg L⁻¹. Working solutions were prepared by aliquot from stock solutions. All the solutions kept refrigerated at 4°C were stable for at least seven days. Twelve different chili peppers samples from three Capsicum species were purchased in local market and supermarket. Cultivated varieties (cv.) “square green pepper”, “Camuyo”, “Sweet banana” and green and mature “Cacho de cebra” from C. annum and “green chili”, “Anahiem”, “Hungarian”, “Cristal”, “Escabeche” and “Cayena” from C. baccatum var Pendulum and “Putamadre” from C. chacoense, were analyzed.

2.2 Sample preparation

First peduncle and seeds were separated, then, the rest of the chili pepper was homogenized using mortar and pestle. Capsaicinoids were extracted from homogenized samples applying the method described by Collins et al. 25 with slight modifications. Briefly, 120 mL of methanol were added to 12 g of sample, the extraction was carried out by shaking in a KS 125 basic shaker from IKA (Staufen, Germany) at 150 rpm for 4 hours at 40°C. The extract was filtered through filter paper Nº4 and 10 mL of filtrated evaporated to dryness with a slight nitrogen stream at 60°C. The residue was dissolved with 4 mL of organic solvent at 9 min of gradient phase (X1), expressed as the percentage of organic solvent at 9 min of gradient program and column temperature (X 2). According to preliminary chromatographic assays, a range was established for organic mobile phase percentage at 9 min (70–100 % v/v) and column temperature (35–55°C), which resulted in an experimental plan with 10 runs (Table 1). All experiments were randomly conducted in triplicate (n=3) in order to minimize the effects of uncontrolled factors. Experimental data from peak height response and resolution fitted a second-degree model with a cubic experimental domain. An analysis of variance (ANOVA) with a significance level of 0.05 was carried out to determine which experimental factors significantly affect the chromatographic performance regarding peak height and resolution.

| Table 1. Experimental runs for a central composite design with the corresponding responses (means) for resolution and peak height. |
|---|---|---|---|---|
| Experiments (Runs) | Temperature (°C) | MoOH:ACN/acetic acid (50:50:0.1 v/v/v) | Resolution | Peak Height (EU)* |
| 1 | 45 | 85 | 0.80 ± 0.01 | 766283±3.53 |
| 2 | 45 | 100 | 0.74 ± 0.02 | 534627±5.65 |
| 3 | 55 | 70 | 0.50 ± 0.04 | 489252±4.24 |
| 4 | 35 | 85 | 0.47±0.03 | 929672±4.94 |
| 5 | 45 | 70 | 0.80±0.07 | 538878±2.12 |
| 6 | 45 | 85 | 0.65±0.04 | 885623±7.77 |
| 7 | 35 | 100 | 0.72±0.07 | 673380±12.02 |
| 8 | 55 | 85 | 0.74±0.03 | 568568±7.07 |
| 9 | 35 | 70 | 1.10±0.23 | 549022±8.28 |
| 10 | 55 | 100 | 0.77±0.02 | 539048±4.24 |

*aEmission units
*mean= standard deviation (n=3).
According to the observed results, none of the factors significantly (P>0.05) influenced the variables response. The quadratic coefficient of percentage of MeOH : ACN to 9 min gradient mobile phase affected significantly the peak height (P<0.05). This result has not implications from the standpoint of the analytical method. Using the individual optimum, a multiple response optimization was done in order to determine the optimal conditions for all responses, giving desirability conditions. Thus, the optimal conditions calculated were: 76% organic mobile phase at 9 min of gradient program and column temperature of 35°C, with these chromatographic conditions clear and well-resolved chromatograms were obtained (Fig 1).

### 3.2 Validation

Validation of method was carried out according to ICH recommendations 28. Calibrations with and without matrix were compared in order to evaluate a possible matrix effect. No statistical difference was observed among slopes (F= 0.008, P=0.92); hence, calibration was established with pure standard in the range of 0.01 to 2.00 mg L⁻¹ with six levels in triplicate. Calibration data fitted a linear regression model with a determination coefficient (R²) of 0.998 (Table 2). Method precision was evaluated through repeatability and intermediate precision. Repeatability was studied injecting in sextuplicate (n=6) a 0.5 mg L⁻¹ Capsaicin standard showing a RSD of 1.51%. Intermediate precision was established measuring in triplicate (n=3) a 0.5 mg L⁻¹ capsaicin standard during three days (n=3), showing a RSD of 1.04%. Method accuracy was determined through recovery evaluation. Chili pepper samples were spiked with three capsaicin concentration levels, i.e. 0.01, 0.50 and 2.00 mg L⁻¹, defined according to the calibration range and the reported values. Each level was prepared daily and measured in duplicate during three days. Recovery in all matrices was adequate with values ranging from 94.80 to 109.40 % with RSD lower than 2.39 %. Detection and quantification limits were calculated using signal-to-noise ratios (S/N) of 3 and 10, respectively. Considering an injection volume of 20 µL, the detection and quantification limits in chili peppers were 0.003 and 0.010 mg L⁻¹, respectively. Robustness was evaluated simultaneously with optimization using a response surface design. According to ANOVA results the proposed chromatographic method is robust for the percentage of organic solvent at 9 min of gradient program and column temperature (P>0.05). ICH describes the term specificity, but due to the general agreement and the IUPAC recommendation 29, the preferred and promoted term is selectivity.

Capsaicin identification was established by retention time 30 comparison with pure standard and using the standard addition method. Selectivity was studied by mass spectrometry. Each chromatographic peak showed only one protonated molecule corresponding to each capsaicinoids evaluated finding m/z 294 for nordihydrocapsaicin, m/z 306 for capsaicin and m/z 308 for dihydrocapsaicin (Fig 2), which are in agree with the values already described in literature 31. Comparatively, this optimized method showed similar validation results than others methods for capsaicinoides determination in peppers using liquid chromatography. Detection and quantification limits were much lower than those reported by Stipcovich et al 32 using UHPLC/MS method (LOD 0.1 mg L⁻¹; LOQ 0.3 mg L⁻¹). Linear range (0.01 to 2.00 mg L⁻¹) is lower than reported by Sganzerla et al 5 (0.0055–66 mg L⁻¹) and Cisneros-Pineda 30 (0.25-2.50 mg mL⁻¹). Recovery results (>90%) are similar to those reported by Sganzerla et al 5 (88 to 112%), and Sweat et al 33 (101 to 115%). In terms of precision showing RSD value the 1.04% lower than reported by Sganzerla et al 5 (6.11%) and Sweat et al 33 (6.9%).

### Table 2. Summary of validation results of analytical method applied to evaluated capsaicin in chile peppers

<table>
<thead>
<tr>
<th>Range (mg L⁻¹)</th>
<th>Regression equation y = ax±SD + bx±SD</th>
<th>R²</th>
<th>Confidence interval a ±b±SD</th>
<th>Rp (%)</th>
<th>IP (%)</th>
<th>Recovery (%)</th>
<th>LOD (mg L⁻¹)</th>
<th>LOQ (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01-2.00</td>
<td>y=4987.70x±46.86 +102.81 ± 52.43</td>
<td>0.998</td>
<td>4888 ± 5087</td>
<td>-8.34 ± 214.0</td>
<td>1.51</td>
<td>1.04</td>
<td>94.85 ± 2.39-108.48 ± 1.04</td>
<td>0.003</td>
</tr>
</tbody>
</table>

a range: 0.01 to 2 mg L⁻¹.  
b n = 3 three injections for each level  
c Rp: repeatability, n = 6  
d IP: intermediate precision, n = 3  
e mean ± SD, n = 3  
S/N = 3  
S/N = 10

### Fig 1. Chromatogram capsaicin standard of 1 mg L⁻¹ and extract of green pepper spiked with 1 mg L⁻¹ of capsaicin standard in optimal conditions.

### Fig 2. Mass spectrum of nordihydrocapsaicin (A), capsaicin (B) and dihydrocapsaicin (C).
3.3 Samples analysis

Twelve samples were analyzed using the optimized and validated method. The analytical method developed permitted the separation of 3 capsaicinoids in 15 min run. Since no commercially available dihydrocapsaicin standard was found, both, capsaicin and dihydrocapsaicin contents were quantified using capsaicin calibration. For the case of nordihydrocapsaicin it was not possible to quantified using capsaicin calibration because the levels found were below calibration range. Samples showed a capsaicin content from 0.10 to 127.30 mg kg⁻¹ and dihydrocapsaicin from 1.46 to 100.20 mg kg⁻¹ (Table 3). The corresponding capsaicin contents were converted to Scoville heat units in order to classify them according to their pungency levels (Table 3). The use of the SHU parameter is the recommended method for pepper evaluation as it provides a better indicator of the pungency level, but is considered less precise 5. Capsicum cv. Pimiento cuadrado verde, Sweet banana, Anaheim, Hungaro, Cristal and Escabeche presented the lowest capsaicinoids content and, therefore, the lowest pungency. Instead, cv. Cacho de cabra, showed the highest capsaicin content and, therefore, the highest pungent level. Since this is the first report about capsaicin content in chili peppers commercialized in Chile it was not possible to compare or discuss the values found. However, these results are in agree with those reported by Othman et al 13 for the same capscisic species. Regarding the maturity effect over capsaicinoids content, Capsicum cv. Cacho de cabra showed the typical behavior observed during ripening 33. Capsaicin and dihydrocapsaicin content decreases according to the ripeness of the fruit, increasing the content of other capsaicinoids. In the same way some harvest period also influences capsaicin concentration 33.

The contents of capsaicin and dihydrocapsaicin found in the present work (2 to 127 µg g⁻¹ and 1.5 to 100 µg g⁻¹ respectively) for the different pepper varieties are in good agreement with those found by other authors who reported that a variation in capsaicin concentration is observed in the different peppers 33. Sganzerla 3 found that levels for capsaicin to 156-1442 µg g⁻¹ and for dihydrocapsaicin 26-478 µg g⁻¹. Dueldum et al 36 found ranges to 0.69-131 jumole g⁻¹ for capsaicin and 0.66 to 28.35 jumole g⁻¹ for dihydrocapsaicin. Stipovich et al 12 in 7 samples of peppers found concentrations for capsaicin 274-4469 µg g⁻¹ and for dihydrocapsaicin 120-2319 µg g⁻¹.

Table 3. Capsaicinoids content (µg g⁻¹) in Chilean peppers.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chili</th>
<th>Capsaicin content (µg g⁻¹ ± SD)</th>
<th>Dihydrocapsaicin content (µg g⁻¹±SD)</th>
<th>SHU</th>
<th>Pungency level</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Square green pepper (Capsicum annum)</td>
<td>2.00±0.41</td>
<td>2.80±0.27</td>
<td>320.00±47.37</td>
<td>No pungent</td>
</tr>
<tr>
<td>2</td>
<td>Camuyo (Capsicum annum)</td>
<td>6.83±2.49</td>
<td>6.30±0.31</td>
<td>1092.80±71.15</td>
<td>Midly pungent</td>
</tr>
<tr>
<td>3</td>
<td>Sweet banana (Capsicum annum)</td>
<td>12.20±3.18</td>
<td>9.38±0.18</td>
<td>195.20±31.96</td>
<td>No pungent</td>
</tr>
<tr>
<td>4</td>
<td>Puta madre (Capsicum chacoense)</td>
<td>43.26±14.30</td>
<td>24.12±0.33</td>
<td>692.16±69.67</td>
<td>Midly pungent</td>
</tr>
<tr>
<td>5</td>
<td>Green chili (Capsicum pendulum)</td>
<td>14.31±0.00</td>
<td>7.07±3.05</td>
<td>2289.60±63.82</td>
<td>Midly pungent</td>
</tr>
<tr>
<td>6</td>
<td>Anaheim (Capsicum pendulum)</td>
<td>0.30±1.85</td>
<td>3.77±3.31</td>
<td>48.00±21.21</td>
<td>No pungent</td>
</tr>
<tr>
<td>7</td>
<td>Hungarian (Capsicum pendulum)</td>
<td>0.10±0.21</td>
<td>1.46±0.30</td>
<td>16.00±55.15</td>
<td>No pungent</td>
</tr>
<tr>
<td>8</td>
<td>Green Cacho de cabra (Capsicum annum)</td>
<td>127.30±1.20</td>
<td>100.24±9.04</td>
<td>20368.00±19.09</td>
<td>Highly pungent</td>
</tr>
<tr>
<td>9</td>
<td>Mature Cacho cabra (Capsicum annum)</td>
<td>15.65±2.03</td>
<td>7.47±2.05</td>
<td>2504.00±77.78</td>
<td>Midly pungent</td>
</tr>
<tr>
<td>10</td>
<td>Cristal (Capsicum pendulum)</td>
<td>1.10±0.31</td>
<td>1.81±0.48</td>
<td>160.00±21.92</td>
<td>No pungent</td>
</tr>
<tr>
<td>11</td>
<td>Cayena (Capsicum pendulum)</td>
<td>12.07±6.48</td>
<td>20.13±4.51</td>
<td>1931.20±17.53</td>
<td>Midly pungent</td>
</tr>
<tr>
<td>12</td>
<td>Escabeche (Capsicum pendulum)</td>
<td>33.51±62.50</td>
<td>30.47±60.70</td>
<td>536.16±36.65</td>
<td>No pungent</td>
</tr>
</tbody>
</table>

CONCLUSIONS

This work report an optimized and validated method for a reliable quantification of capsaicinoids in peppers. The optimization via central composite design of the chromatographic conditions allowed a well-resolved separation of three capsaicinoids in 25 min without matrix interferences. Considering validation results the method proved to be reliable, accurate and precise. To the best of our knowledge, this study shows for the first time the evaluation of capsaicinoids in peppers commercialized in Chilean market. All 12 chili pepper samples analyzed showed the presence of nordihydrocapsaicin, capsaicin, and dihydrocapsaicin, which were the main capsaicinoids in the different chili and pepper samples. The variety “Cacho de cabra” green was the one with the major pungency. Variations in capsaicinoids quantity could be attributed to genetic factors to each cultivar or alternatively to the environmental conditions where they were cultivated.

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