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# Determination of nordihydrocapsaicin, capsaicin, dihydrocapsaicin, and pungency levels in pepper sauces by RP-HPLC: Capsaicinoid levels and pungency classification of commercial pepper sauces

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**Abstract**

Capsaicinoids are a class of compounds that confer various pungency levels to peppers, and have a range of applications as dietary supplements, medications, pain relievers, and sprays for repelling animals and personal attackers. Although analyses and classifications of peppers have been extensively reported in the literature, data describing and classifying the pungency of Brazilian pepper sauces are scarce. The objective of the present work was therefore to measure the levels of nordihydrocapsaicin, capsaicin, and dihydrocapsaicin in commercial pepper sauce samples, classify their pungency, and compare them with the recommended daily intake limits. Solvent extraction was performed using ethanol, and capsaicinoids were identified and quantified using high-performance liquid chromatography (HPLC). Most of the samples had mild to moderate pungency expressed in Scoville Heat Units (SHU). There were no significant differences between sauces with and without milk on the pungency of green or red pepper sauces. Capsaicin levels were below the recommended daily intake limits. The capsaicin levels found in all but two of the pepper sauces were below the recommended limits for capsaicin daily intake in industrial foods samples. According to the United States Department of Agriculture (USDA) specifications, the classification of pungency is not a valid criterion for classifying pepper sauces; therefore, a new classification was proposed.



## Introduction

Capsaicinoids are derived from members of the Solanaceae family. They belong to a class of compounds that confer various pungency levels to peppers (*Capsicum* spp.), thus giving them economic value (Bernal *et al.*, 1993). *Capsicum* spp. are found in tropical and humid areas of Central and South America. The three most widely consumed hot pepper varieties are *C. annuum*, *C. frutescens*, and *C. chinense*. Mature pepper fruits are traditionally used as colorants and natural food additives. The importance of peppers is that the consumption continues to grow because of their high nutritional value; they are rich sources of vitamins C and E, provitamin A, and carotenoids, compounds with well-known antioxidant properties.

Peppers contain two major compounds that confer pungency; capsaicin (N-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methyl-6-nonenamide) and dihydrocapsaicin (N-[(4-hydroxy-3-methoxy-phenyl)methyl]-8-methylnonanamide), which confer approximately 90% of the pungency in peppers. The difference between these compounds involves the saturation of acyl groups (Reyes-Escogido *et al.*, 2011). Other compounds are also present, although at low amounts, including nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin (Bernal *et al.*, 1993). About 20 known capsaicinoids are amides produced by condensation of vanillylamine with fatty acids of varying chain lengths, and with various degrees of unsaturation (Reyes-Escogido *et al.*, 2011).

These products have several applications including dietary supplements, medications, pain relievers, sprays for repelling animals, and personal defense (Rouhi, 1996). Their biological and physiological activities confer antioxidant (Materska and Perucka, 2005), anticancer (Macho *et al.*, 2003), energy synthetic, fat mobilisation (Ohnuki *et al.*,

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2001; Zheng *et al*., 2017), and anti-inflammatory activities (Sancho *et al*., 2002). They also improve metabolic syndrome (Panchal *et al.*, 2018), and possess antimicrobial properties mediated by microbiota alteration (Rosca *et al*., 2020). Studies have also shown that capsaicin and other capsaicinoids act as agonists for molecules that inhibit neurogenic inflammation, *i.e.*, the mode of inflammation mediated by the efferent (motor) functions of sensory neurons (Chiu *et al*., 2012). Inhibitors of this inflammation rely on capsaicin to activate transient receptor potential V1, which is found in neuronal membranes, preventing entry into the cell (Fattori *et al*., 2016).

High levels of capsaicin intake can also exert adverse health effects. One study revealed that individuals who consumed peppers had a 5.4-fold higher risk of developing gastric cancer than those who did not, while individuals eating large amounts of peppers had a 17-fold greater risk of developing this disease (Othman *et al*., 2011). Therefore, it is recommended that the mean and maximum capsaicin intake from processed foods be limited to 0.77 - 2.64 mg/day (EC, 2002). Capsaicin and other members of the capsaicinoid group promote many physiological and pharmacological effects in the gastrointestinal, cardiovascular, respiratory, and sensory thermoregulation systems. Capsaicinoids are also potent irritants; they cause burns and pain in the skin and mucous membranes in low concentrations. When administered orally, capsaicinoids increase salivation and gastric secretion, a rapid and variable burning sensation ranging from a hot sensation to intolerable burning, in addition to promoting gastrointestinal disorders, depending on dose (EC, 2002). Despite several possible applications of capsaicinoids, they have not been widely used because of the untoward effects of these molecules and the irritation they cause as a function of pungency. This situation has prompted searches for analogous molecules devoid of undesirable characteristics (Reyes-Escogido *et al*., 2011).

The need for further research on capsaicinoids has prompted studies aimed at improving pepper farming (Sung *et al*., 2005), developing novel techniques of cultivating plant tissues and cells (Ochoa-Alejo and Ramirez-Malagón, 2001), and enhancing technologies for chemical and enzymatic syntheses (Castillo *et al*., 2007). Capsaicin and capsaicinoid extraction have been improved and diversified recently, thus generating growing interest. The most widely used extraction methods are liquid-liquid extraction (Tapia *et al*., 1993; Kaale *et al*., 2002), enzymatic extraction (Santamaría *et al*., 2000), supercritical fluid extraction (SFE) (Sato *et al*., 1999; Gnayfeed *et al*., 2001; Kaale *et al*., 2002; Duarte *et al*., 2004; Perva-Uzunalić *et al*., 2004), pressurised liquid extraction (Barbero *et al*., 2006a), magnetic stirring extraction (Kaale *et al*., 2002), solid phase microextraction (Tapia *et al*., 1993), reflux (Peusch *et al*., 1997), microwave assisted extraction (Barbero *et al*., 2006b), maceration (Kirschbaum-Titze *et al*., 2002), ultrasound-assisted extraction (Kaale *et al*., 2002), and supercritical fluid extraction coupled to ultrasound (SFE-US), which consists of a coupled technique which is environmentally friendly with reduced energy and produces extracts with high purity; the application of ultrasonic waves in SFE processes increases the extraction yield and rate (Santos *et al*., 2015; Dias *et al*., 2016).

Following capsaicinoid extraction, qualitative and quantitative analyses can be carried out using gas chromatography (Thomas *et al*., 1998; Spicer and Almirall, 2005) or high-performance liquid chromatography (HPLC) (Peusch *et al*., 1997; Higashiguchi *et al*., 2006; Schweiggert-Weisz *et al*., 2006; Chanthai *et al*., 2012). Although the analysis and classification of peppers have been extensively reported in the literature (Othman *et al*., 2011), few reports have classified the pungency of Brazilian pepper sauces. Pungency influences the acceptance of capsaicin-containing products, thus limiting the clinical application of this promising molecule that has attracted interest from the scientific community (Reyes-Escogido *et al*., 2011). Therefore, the objective of the present work was to measure levels of capsaicin, dihydrocapsaicin, and nordihydrocapsaicin in pepper sauces sold in Brazilian supermarkets, and to classify their pungency.

## Materials and methods

### *Sampling*

Pepper sauces from several national and international manufacturers (22 samples) were purchased from local supermarkets in 2015. The samples were weighed (12.5 g), transferred into flat-bottomed flasks, and subjected to extraction using 100 mL of ethanol (Sigma-Aldrich, Saint Louis, USA) under reflux for 1.5 h. Then, the samples were removed from reflux and cooled. Aliquots of 4 mL were withdrawn and filtered through 0.45-µm membranes.

*HPLC*

Capsaicinoids were quantified using HPLC as per the method described by Association of Official Analytical Chemists (AOAC, 1995). This method uses an external standard and response factor for capsaicinoid quantification. The extracts were injected in triplicate into a liquid chromatograph (Shimadzu) equipped with a UV-VIS detector (SPD-10 AV). A Hypersil Gold $C_{18}$ analytic column (Thermo Scientific, 150 × 4.6 mm) packed with 5-µm particles was used. The mobile phase was a solution of 1% acetic acid (Dinâmica, Indaiatuba, Brazil) in water and acetonitrile (J. T. Baker, City of Mexico, Mexico) at a ratio of 35:65 isocratically. The flow rate was 1.5 mL/min, and the wavelength was 280 nm. The injection loop was 20 µL with a UV detector at 280 nm, and the temperature was 40°C. The chromatograph was calibrated using a solution of capsaicin standard (Sigma-Aldrich, Saint Louis, USA) in ethanol at 1.5 mg/mL.

The pungency levels of the capsaicinoids in Scoville Heat Units (SHU) was determined using HPLC, a method that provides the required precision and accuracy. The method described by the AOAC (1995) for the determination of capsaicinoid levels in SHU and HPLC was employed to determine capsaicin, dihydrocapsaicin, and nordihydrocapsaicin levels in the sauces. The quantification of capsaicinoids in sauce samples was performed in triplicate as prescribed by AOAC (1995) using external standards and response factors for each capsaicinoid. After obtaining chromatograms and identifying the peaks corresponding to nordihydrocapsaicin (N), capsaicin (C), and dihydrocapsaicin (D) (Figure 1A), their mean areas were used to calculate the pungency in SHU for each of the capsaicinoids and total capsaicinoid levels (N + C + D) for each sauce.

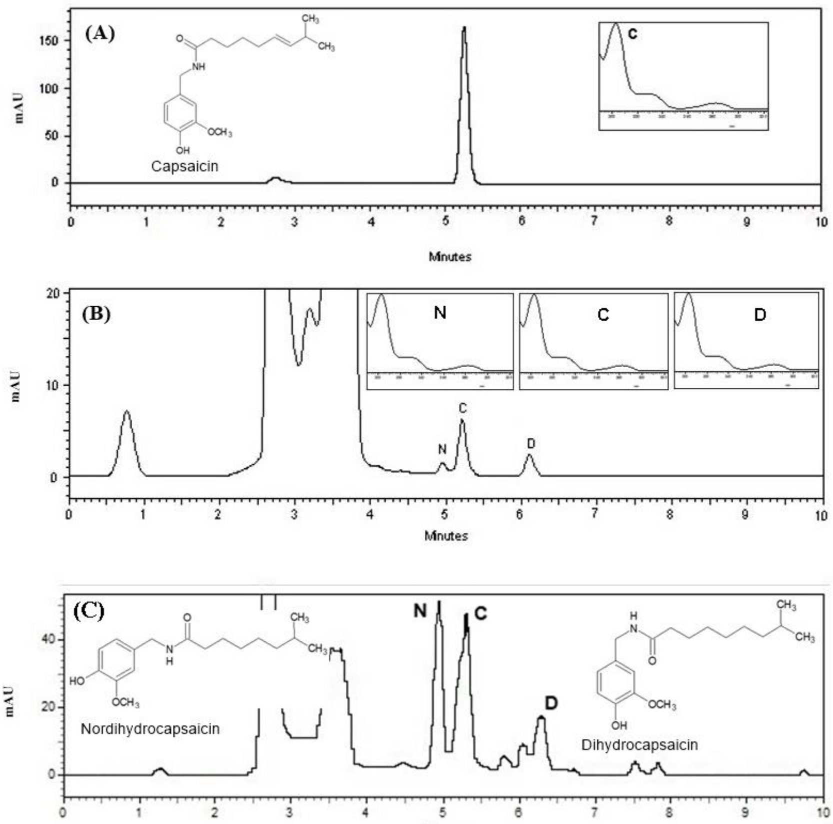


**Figure 1. (A)** HPLC chromatogram obtained by injection of capsaicin (C) standard at 280 nm showing a peak with a retention time of 5.2 min, absorption spectrum in UV region for peak, and the chemical structure of capsaicin. **(B)** Chromatogram obtained by injection of sauce 10 showing peaks for nordihydrocapsaicin (N), capsaicin (C), and dihydrocapsaicin (D) at 280 nm, and absorption spectrum in UV region of nordihydrocapsaicin (N), capsaicin (C), and dihydrocapsaicin (D). **(C)** Chromatogram obtained by injection of sauce 15 showing peaks for nordihydrocapsaicin (N), capsaicin (C), and dihydrocapsaicin (D) at 280 nm, and chemical structure of nordihydrocapsaicin and dihydrocapsaicin.

The following equations were used to calculate nordihydrocapsaicin (N), capsaicin (C), and dihydrocapsaicin (D) capsaicinoids:

$$N = (Pn/Ps).(Cs/wt).(100/0.95).9300 \quad \text{(Eq. 1)}$$

$$C = (Pc/Ps).(Cs/wt).(100/1.00).16100 \quad \text{(Eq. 2)}$$

$$D = (Pd/Ps).(Cs/wt).(100/0.99).16100 \quad \text{(Eq. 3)}$$

where, Pn, Pc, and Pd = mean areas of peaks for nordihydrocapsaicin, capsaicin, and dihydrocapsaicin, respectively; Ps = mean area of the standard solution, Cs = concentration of the standard solution (mg/mL), and Wt = mass of the sample in grams (g). The total capsaicinoid level was the sum of N + C + D, expressed in SHU and µg/g (AOAC, 1995).

Sauces were classified by pungency in SHU based on the following criteria: 0 - 700 SHU, no pungency; 700 - 3000 SHU, mild pungency; 3000 - 25000 SHU, moderate pungency; 25000 - 70000 SHU, high pungency; and > 80000, very high pungency (Baser, 2004).

In addition to the capsaicinoid level, the sauces were also analysed and classified following the methods and conditions described by the United States Department of Agriculture (USDA, 2010) for non-volatile solids, percentage salt, total titratable acidity, and pH.

*Statistical analysis*

The Kolmogorov-Smirnov test was performed to confirm the data's normality of distribution. Subsequently, a descriptive analysis was performed on the values for nordihydrocapsaicin, capsaicin, and dihydrocapsaicin. Values obtained by injection in triplicate for each sample and total capsaicinoids for each sauce type were expressed as mean and standard deviations. The Student's *t*-test was applied to compare the means of red and green pepper sauce groups and compare the means of groups with and without milk. The statistical program GraphPad Prism® 5.0 (San Diego, CA, USA) was used to apply the tests and presentation of the graphs.

## Results

After analysing 22 sauces for non-volatile residues, salt, titratable acidity, and pH, an attempt was made to classify the sauces according to USDA specifications (USDA, 2010). Although some results allowed classification under the sauce types outlined in Table 1, most occurred outside these specifications. Another classification based on pungency in SHU (Table 2) was compared with the USDA pungency specifications (Table 1). We observed that the second method more appropriately classified the pepper sauces.

**Table 1.** Classification and specifications for pepper sauces according to Commercial Item Description (CID) (USDA, 2010).

| Type | Description | pH | Total titratable acidity (%) | Salt (%) | Non-volatile solids | Pungency (SHU) |
|---|---|---|---|---|---|---|
| I- Hot | Each type of hot sauce shall be a red to reddish-brown coloured liquid, and possess a pungent odour and a good flavour that is well balanced and peppery. | ≤ 3.40 | 2.5 - 5.0 | 4.9 - 12.0 | 7.5 - 18.0 | ≥ 650 |
| II- Extra hot | Each type of hot sauce shall be a red to reddish-brown coloured liquid, and possess a pungent odour and a good flavour that is well balanced and peppery. | 2.7 - 3.4 | 3.6 - 9.5 | 1.2 - 12.0 | 3.6 - 17.0 | ≥ 2500 |
| III- Green | The green hot sauce shall be a pale green coloured liquid, and possess a mild jalapeño flavour and odour with vinegar background notes. | 2.7 - 3.0 | 4.5 - 5.0 | 7.0 - 8.0 | 8.5 - 14.0 | 600 - 1200 |

**Table 2.** Capsaicinoid levels and classification of pepper sauce samples according to pungency in Scoville Heat Units (SHU).

| Sauce | N ± SD* (SHU) | C ± SD* (SHU) | D ± SD* (SHU) | N + C + D* (SHU) | Pungency classification** |
|---|---|---|---|---|---|
| 1[b] | 4.3 ± 0.13 | 884.0 ± 32.06 | 342.6 ± 12.43 | 1231.0 | Mild |
| 2[a] | 16.0 ± 1.80 | 589.4 ± 66.65 | 199.8 ± 28.75 | 805.1 | Mild |
| 3[b] | 33.5 ± 3.85 | 723.4 ± 53.85 | 396.5 ± 29.52 | 1153.4 | Mild |
| 4[b] | 16.4 ± 2.03 | 2827.2 ± 291.54 | 1637.1 ± 53.23 | 4480.7 | Moderate |
| 5[b] | 4.57 ± 0.17 | 766.9 ± 23.20 | 326.3 ± 6.26 | 1097.8 | Mild |
| 6[b] | 41.1 ± 1.64 | 1465.4 ± 177.94 | 679.97 ± 50.61 | 2186.5 | Mild |
| 7[b] | 6.8 ± 1.02 | 973.5 ± 54.17 | 813.3 ± 93.26 | 1793.6 | Mild |
| 8[b] | 17.3 ± 1.62 | 2596.9 ± 193.29 | 1793.4 ± 133.49 | 4407.6 | Moderate |
| 9[b] | 2.9 ± 0.34 | 748.5 ± 21.54 | 436.1 ± 50.01 | 1187.5 | Mild |
| 10[b] | 85.6 ± 0.85 | 716.2 ± 22.14 | 303.4 ± 22.82 | 1105.2 | Mild |
| 11[a] | 277.7 ± 4.57 | 1576.1 ± 21.81 | 680.2 ± 16.35 | 2534.1 | Mild |
| 12[b] | 216.3 ± 16.1 | 480.0 ± 55.05 | 233.9 ± 21.12 | 930.23 | Mild |
| 13[b] | 4525.2 ± 185.40 | 7257.9 ± 419.24 | 2604.5 ± 8.95 | 14387.6 | Moderate |
| 14[b] | 20.3 ± 0.31 | 864.7 ± 6.37 | 350.2 ± 15.15 | 1235.2 | Mild |
| 15[b] | 3243.9 ± 292.7 | 8343.4 ± 621.02 | 2514.1 ± 287.83 | 14101.4 | Moderate |
| 16[b] | 428.1 ± 11.44 | 1512.8 ± 29.28 | 712.9 ± 3.27 | 2.653.8 | Mild |
| 17[b] | 87.6 ± 7.99 | 3381.5 ± 312.18 | 1825.5 ± 146.53 | 5294.7 | Moderate |
| 18[a] | 62.1 ± 2.26 | 1681.7 ± 39.24 | 1221.9 ± 55.14 | 2965.8 | Mild |
| 19[b] | 95.7 ± 3.47 | 3.470.3 ± 3.00 | 1624.0 ± 115.21 | 5190.0 | Moderate |
| 20[c] | 0.3 ± 0.02 | 257.5 ± 7.81 | 187.9 ± 6.81 | 445.7 | None |
| 21[b] | 1396.3 ± 173.78 | 2128.1 ± 276.75 | 727.5 ± 61.54 | 4252.0 | Moderate |
| 22[c] | 46.2 ± 6.29 | 1663.5 ± 235.98 | 799.4 ± 58.55 | 2509.1 | Mild |

(*) Nordihydrocapsaicin (N), Capsaicin (C), and Dihydrocapsaicin (D) in Scoville Heat Units. (**) Classification of pungency (Baser, 2004). ([a]) red pepper sauce with milk, ([b]) red pepper sauce, and ([c]) green pepper sauce.

Among the samples, 90.9% were red pepper, and 9.1% were green pepper; 13.6% contained milk. There was no statistically significant difference in capsaicinoid levels between red and green pepper sauces. Similarly, there were no significant differences between the group of sauces with and without milk (Table 3).

Based on values displayed in Table 2 and the method of Othman *et al*. (2011), SHU values of sauces classified as having moderate pungency (highest pungency level detected) were converted into µg/g to quantify capsaicin, and converted into mg/day according to average Brazilian intake of pepper (Table 4).

**Table 3.** Comparison of capsaicinoid level in pepper sauce groups.

| Sauce type | Mean capsaicinoid level (SHU) | *p** |
|---|---|---|
| Sauce without milk | 3665.4 (4039.7) | 0.097 |
| Sauce with milk | 2101.7 (1143.4) | |
| Red pepper sauce | 3649.7 (3920.2) | 0.103 |
| Green pepper sauce | 1477.4 (11459.1) | |

***Capsaicin levels are expressed as mean and standard deviation. Student's *t*-test was used to compare means between groups, adopting $p < 0.05$ as level of significance.

**Table 4.** Capsaicin (C) levels in Scoville Heat Units (SHU) and μg/g, also capsaicin availability in pepper sauces with moderate pungency according to average Brazilian intake.

| Sauce | C (SHU) | C (μg/g) | C average intake (mg/day) |
|---|---|---|---|
| 4 | 2827.2 ± 291.54 | 176.7 | 0.35 |
| 8 | 2596.9 ± 193.29 | 162.3 | 0.32 |
| 13 | 7257.9 ± 419.24 | 453.6 | 0.91 |
| 15 | 8343.4 ± 621.02 | 521.5 | 1.04 |
| 17 | 3381.5 ± 312.18 | 211.3 | 0.42 |
| 19 | 3470.3 ± 3.00 | 216.9 | 0.43 |
| 21 | 2128.1 ± 276.75 | 133.0 | 0.27 |

Capsaicin in μg/g = capsaicin in SHU/16 (Kaale *et al*., 2002). Average intake (mg/day) = capsaicin in (μg/g / $10^3$) × 0.5.

A chromatogram was obtained by injecting solutions of the sample from sauce number 10, showing that nordihydrocapsaicin (N), capsaicin (C), and dihydrocapsaicin (D) were eluted at 4.8, 5.2, and 6.1 min, respectively (Figure 1B). Samples 1 - 12, 14, 16 - 20, and 22 exhibited similar chromatographic profile, while samples 13, 15, and 21 exhibited different chromatographic profile. The chromatograms obtained for all other sauces revealed that the compounds conferring pungency—capsaicin and dihydrocapsaicin—predominated, and were responsible for 90% of the pungency. The chromatographic profile for the three sauces differed from the other sauces (Figure 1C), containing nordihydrocapsaicin levels ranging from 17.5 to 32.8% for total capsaicinoids, thus suggesting that these sauces were prepared using a different variety of pepper or a mixture of different peppers.

## Discussion

The diverse compositions, manufacturing processes, and varieties of peppers precluded our method as a standard for classifying pepper sauces. Todd *et al*. (1977) reported the contribution of capsaicinoids with pungency determined using the Scoville method. Through these organoleptic experiments, the contribution of each capsaicinoid to the oral sensation produced by peppers or their products was determined. Capsaicin, dihydrocapsaicin, and nordihydrocapsaicin produce a short-lived sensation of pungency near the back of the palate and throat. In contrast, homocapsaicin and homodihydrocapsaicin tend to produce a long-lasting sensation of low-intensity pungency in the middle region of the mouth and palate.

Studies concerning the content of capsaicinoids in pepper sauces are scarce in the literature. However, a recent study sought to integrate some studies to build a database of capsaicinoid content in foods commonly consumed in Korea. The capsaicinoid content in the spicy-sweet pepper sauce was estimated to be 1.6 mg/100 g; as a reference, tabasco sauce contains 20 mg/100 g (Cho and Kwon, 2020).

The minimum level of capsaicinoids obtained in the samples assessed in the present work was 445.7 SHU, while the maximum was 14387.6 SHU. Thus, most of the sauces had mild pungency (63.6%), according to the classification by Baser (2004). Only one sauce, a green pepper type, exhibited no pungency (Table 2). This method was superior to the USDA classification for the classification of pepper sauces (Table 1) because most fell outside the specifications of the latter. The final amount of capsaicinoids in pepper depends on a complex balance between factors that stimulate synthesis and degradation. Capsaicinoid levels are therefore highly plastic, and most likely determined by a complex set of ecological and physiological trade-offs with solid input from the environment (Naves *et al*., 2019).

Regarding the effect of milk on the bioavailability of capsaicinoids, there was no difference between sauces with and without milk (Table 3). This can be explained by the fact that casein blunts the burning sensation caused by pepper only in the mouth as casein breaks the link between capsaicin and taste receptors, thus reducing the spicy sensation associated with pepper (Corrêa *et al*., 2016).

Regarding pepper consumption, the recommendations in the United States and Europe on maximum capsaicin intake derived from industrially prepared food products is 0.77 mg/day (average daily limit) (EC, 2002). Per capita direct or indirect consumption of pepper in Brazil is approximately 2 g/day, equivalent to approximately 6 mg of capsaicinoids, since 1 g of dry red pepper contains 3 mg of capsaicinoids (Rodrigues *et al*., 2015). We found that the level of capsaicin in 2 g of the sauces was below the recommended intake limit of 0.77 mg/day, except for samples 13 and 15 (Table 4).

Capsaicinoids have been extensively studied for their hypocholesterolaemic (Zhang *et al*., 2013), anti-inflammatory and antioxidant properties (Liu and Nair, 2010), and their weight management activities (Whiting *et al*., 2014). Nevertheless, high intake levels of these substances can have deleterious effects on health, including an increased risk of developing gastric cancer. A recent meta-analysis evaluated the association between chili consumption and risk for gastric cancer with a sample size of over 7800 from 13 case-control studies published between 1985 and 2018. The results suggested significant associations between chili consumption and gastric cancer, with a 1.96-fold increased risk with moderate-high (> 90 mg/day) chili consumption (Du *et al*., 2020).

Therefore, it is essential to establish the safe levels of capsaicin in foods because this information helps consumers to establish balanced consumption to prevent excessive pepper intake while obtaining the benefits of their biological activities.

## Conclusion

Most samples had mild to moderate pungency in terms of SHU. No significant differences were detected in the pungency of green or red pepper sauces, and between the group of sauces with and without milk. The capsaicin levels found in all but two pepper sauces were below the recommended limits for capsaicin daily intake in industrial food samples. These findings suggested that the classification of pungency according to USDA specifications does not help classify pepper sauces; therefore, a new classification based on the Baser method was proposed. Further studies are needed to establish criteria for assessing and validating this parameter, and establish a safe consumption limit for consumers.

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