LUND UNIVERSITY

Master thesis

Natural Extraction of Pungency

Beatriz Sánchez Santana

May 2017

MSc Food Technology and Nutrition

Lund University

Supervisors: Björn Bergenståhl and Thomas Adner

Examiner: Lars Nilsson (Faculty of Engineering, Lund University)

Abstract

Chilli extract is used in the food industry for multiple applications, such as a flavour for hot sauces or spicy chocolates. The demand for organic products has grown in the recent years, while changes in demography make it necessary to obtain and aroma that can fulfil the market needs. Hence, the purpose of this project was to develop an extraction method to obtain an organic and halal chilli extract and a quantification method to further establish the spiciness of the aroma.

Dried and cut chilli fruits were introduced with different ethanol/water solvent mixtures at 65°C with magnetic stirring for one hour. In order to quantify the capsaicinoids, HPLC was the method used. The mobile phase used was a gradient of water/methanol and ethyl paraben was employed as internal standard. The results showed that capsaicinoids were unevenly distributed in the different parts of the chilli peppers, where the amount found was 37 times higher in placenta than in pericarp. Solvent mixtures >20% ethanol increased the extraction yield compared to just water, but both can be used to obtain a halal extract. Nonetheless, if ethanol was used it was evaporated and this process did not affect the capsaicinoid content.

Around 90% of the total capsaicinoids extracted in one hour were obtained in the first 20 minutes, but the same fruit was not depleted after two extraction cycles. Hence, these results could be used as a basis to further investigate a two-step counter-current extraction to use the least amount of solvent possible and reduce the extraction time.

Popular Abstract

Are you a spice lover?

If that is the case, you should continue reading because you are on fire! Not so long ago, the only thing that you could buy was Tabasco and hot sauces to experience that amazing heat, but nowadays you have the opportunity to taste sweet products with chilli flavour, such as chocolate or ice cream. And you may ask, how is that possible if there are no chilli chunks inside? Well, here is the secret: chilli extracts are added to food products to give that spicy sensation.

More and more people are concerned about what they eat and how it has been produced. To obtain this natural elixir made for Gods, we have used organic chillies blended with hot water and low ethanol content. As a result, we obtained a natural and organic aroma appealing for everyone, yes! For everyone, with independence of age or religion, since the alcohol is removed to have an aroma with <0.05% while preserving all the spiciness of the flavour. The hotness of the extract has been checked by developing a reliable method, which will allow a correct aroma dosage in food products to have the final desired heat.

Welcome to the latest trend in chilli pepper extracts. Welcome to a world full of organic spicy food opportunities.

Preface

The present project was done at Lund University in the department of Food Technology as a master thesis. The project was conducted from November 2016 until May 2017.

I would like to express my gratitude to all the people that have contributed to this project in one way or another. Thanks to:

My supervisor, Dr Björn Bergenståhl for his guidance and valuable knowledge provided not only for this project but also from a personal perspective. Thank you so much.

Federico Gómez, coordinator of the Master in Food Technology and Nutrition for giving me the opportunity to study this master and for being always willing to help. Many thanks.

Daniel Osorio and Yi Lu, for their skilful assistance with HPLC. I am very grateful to you.

To all the personnel in the Department of Food Technology, Engineering and Nutrition from LTH, especially Åsa Håkansson, Yvonne Granfeldt, Hans Bolinsson and Ali Marefati.

To all my colleagues for so many great moments, laughs and amazing experiences together during these two years of master. I will miss you.

Table of contents

Abstract 2

Popular Abstract 3

Preface 4

1. Introduction 7

2. Objectives 7

2.1 General objective 7

2.2 Specific objectives 7

3. Background 8

3.1 Genus Capsicum 8

3.2 Anatomical structure of chilli peppers 8

3.3 Capsaicinoids 9

3.3.1 Synthesis 9

3.3.2 Degradation 10

3.4 Pungency 10

4. Regulations behind natural, organic and halal products 11

4.1 Natural requirements 11

4.2 Organic certification 11

4.3 Halal certification 11

5. Extraction parameters 11

5.1 Preparation of the sample 11

5.1.1 Drying 11

5.1.2 Freezing 12

5.1.3 Blanching 12

5.2 Solvents and their properties 12

5.2.1 Molecular forces 12

5.2.2 Effect of temperature 12

5.2.3 Effect of pressure 12

5.3 Crystals solubility 12

6. Quantification of capsaicinoids 13

6.1 HPLC principle and components 13

6.1.1 Column 13

6.1.2 Mobile phase 14

6.1.3 Temperature 14

6.1.4 Pressure and flow rate 14

6.2 Calibration method 14

7. Ethanol evaporation 14

8. Literature review of capsaicinoids extraction 15

9. Methods 20

9.1 Materials 20

9.2 Determination of dry matter 20

9.3 Extraction method **20**
9.4 Sample preparation **20**
9.4.1 Dried, fresh and blanched chillies 20
9.4.2 Processing of dried samples 20
9.4.3 Capsaicinoids content in different parts of chilli peppers 20
9.5 Optimization of extraction time **21**
9.6 Multiple step extraction **21**
9.7 Optimization of extraction solvent **21**
9.8 Capsaicinoids quantification **21**
9.8.1 Sample preparation 21
9.8.2 HPLC analysis 21
9.8.3 Capsaicinoids determination 21
9.9 Ethanol evaporation **22**
9.10 Statistical analysis **22**
10. Results and Discussion **22**
10.1 Methodology **22**
10.1.1 Extraction conditions 22
10.1.2 HPLC mobile phase composition and method 22
10.1.3 Selection of the Internal Standard (IS) 23
10.2 Water content in different anatomical parts of chilli fruits **24**
10.3 Optimization of sample preparation **24**
10.3.1 Dried versus fresh 24
10.3.2 Blanching effect 25
10.3.3 Processing of dried samples 25
10.4 Optimization of the extraction time **26**
10.5 Extraction cycles **26**
10.5.1 Fruit 27
10.5.2 Solvent 27
10.6 Distribution of capsaicinoids in different parts of chilli peppers and classification of the raw material **27**
10.7 Optimization of extraction solvent **28**
10.7.1 Variation of ethanol composition 28
10.7.2 Water extraction at different temperatures 28
10.8 Consequences of ethanol evaporation **29**
11. Conclusions **30**
12. Further perspectives **31**
13. References **32**

1. Introduction

Pungent extracts from the genus *Capsicum*, which include chilli peppers, are widely used in the pharmaceutical and food industry. Capsaicinoids are the molecules responsible for the "hotness" of these fruits and they are present in crystal form inside plant cell vacuoles. Their extraction implies the presence of lipid components that affect their crystallization, reason why the crude extract is usually called "oleoresin" (Suzuki et al, 1984). The extraction will be determined by the physical state of the fruit and the characteristics of the method employed, such as temperature and solvent selection.

Nowadays, the interest in organic products is growing and there is a need to search for extracts that fulfil the organic legislation, where the selection of the solvent is a key factor. However, not only this consumer profile is growing. Islamic adepts are expected to increase in the next 30 years, at around 30% per decade just in Sweden (Pew Research Center, 2015). Hence, obtaining a "halal" certification could allow including them as possible consumers of products made with *Capsicum* extract.

Ethanol is a widely used solvent for plant extracts and it complies with the organic legislation. However, if used it must be evaporated after the extraction to reduce it at least to 0.05% to have a "halal" extract. This process could lead to some inconveniences, such as the compromise of capsaicinoids stability due to high temperatures and ethanol related fire hazard. The latter could represent a safety risk while increasing costs in large-scale production.

The aim of this project is to extract capsaicinoids with a solvent and a method that can give the best extraction yield to have an extract that is organic and halal certified.

2. Objectives

2.1 General objective

The main objective was to have in depth knowledge of the capsaicinoids and how these could be extracted to have an organic oleoresin that could be used in food applications.

2.2 Specific objectives

-To identify capsaicinoids that can be found in chilli peppers, which belong to the genus *Capsicum*.

-To develop a method for capsaicinoids extraction that fits with the organic regulation regarding organic products and, if possible, to have an extract that can be certified as halal.

-To optimize the extraction regarding preparation of the sample, solvent composition, extraction time, extraction temperature and extraction method.

-To select the ideal extraction process, where if ethanol is needed, use the least amount possible with the highest extraction yield. Minimizing the amount of ethanol needed could favour its removal by evaporation.

-To use the optimal procedure in terms of accuracy and reliability for capsaicinoids detection and quantification.

3. Background

3.1 Genus Capsicum

The genus *Capsicum* (Family *Solanaceae)* includes both green and red chillies (Coultate, 2009b). These fruits are rich in capsaicinoids, which are alkaloids responsible for the chilli's spiciness. There are several species that can be found in this genus *Capsicum* and are described in Table 1.

Table 1: Species of the genus Capsicum and varieties. (USDA, n.d.)

Species	Variety	Synonyms	Common name
Capsicum annuum L.	*Capsicum annuum L. var. annuum*	-*Capsicum annuum* L. var. *frutescens* (L.) Kuntze -*Capsicum frutescens* L.	-Cayenne pepper -Tabasco (Zachariah et al., 2008).
	Capsicum annuum L. var. glabriusculum (Dunal) Heiser & Pickersgill	-*Capsicum annuum* L. var. *aviculare* (Dierbach) D'Arcy & Eshbaugh -*Capsicum annuum* L. var. minimum (Mill.) Heiser -*Capsicum annuum* L. var. *minus* (Fingerh.) Shinners -*Capsicum baccatum auct*. non L. -*Capsicum frutescens* sensu Standl., non L.	Cayenne pepper
Capsicum baccatum L.	*Capsicum baccatum L. var. baccatum*	-	Locoto
	Capsicum baccatum L. var. pendulum (Willd.) Eshb.	*Capsicum pendulum* Willd.	Aji
Capsicum chinense Jacq.	-	-	-Aji -Bhut Jolokia (Amruthraj,2014c) -Habanero (Pruthi, 2003) -Scotch Bonnet (Pruthi, 2003)
Capsicum pubescens Ruiz & Pav.	-	-	Rocoto

3.2 Anatomical structure of chilli peppers

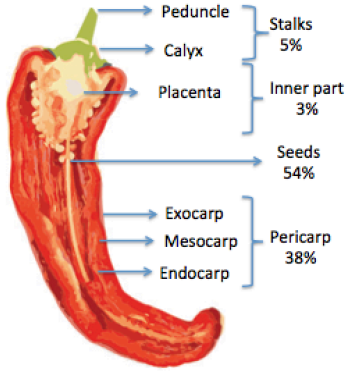


Figure 1: General structure of *Capsicum* fruit and percentages of each part in dry weight. (Vineyard, 2012)

The general structure of chilli fruit can be seen in Figure 1. Seeds make a big contribution in dry weight (DW) of the chilli pepper compared to other parts. The percentage can vary from 20 to 50%, where the smaller the chilli pepper the higher is the seed contribution to DW. This is because seeds have high lipid content compared to other parts, which is around 12-25% (Grubben et al., 2004).

The white-yellowish tissue that forms the inner part is called placenta, where capsaicinoids are synthetized. These will be further translocated to the rest of the fruit.

3.3 Capsaicinoids

Capsaicinoids are formed by vanyllamides of branched fatty acids, which can have 9 to 11 carbons (Angioi et al, 2004). The general chemical structure of capsaicinoids can be seen in Figure 2.

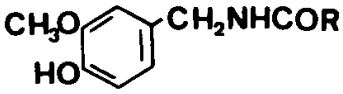


Figure 2: General structure of capsaicinoids (Suzuki et al., 1984)

There are 5 main capsaicinoids, named capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin (Suzuki et al, 1984). The commercial "Capsaicin" is normally a mixture of the first three capsaicinoids mentioned (Suzuki et al, 1984) but capsaicin is the main one in terms of abundance (70%), followed by dihydrocapsaicin (Coultate, 2009b). Some of their main characteristics can be seen in Table 2.

Table 2: Main characteristics of capsaicin, dihydrocapsaicin and nordihydrocapsaicin (Suzuki et al., 1984).

Capsaicinoid	Side chain (R)	Molecular weight	Physical form	Colour	Melting point (°C)	Absorbance (nm)	Amount in Oleoresin (%) (Barceloux, 2008)
Capsaicin	$(CH_3)_2CHCH=CH(CH_2)_4-$	305,12	Crystal	White	64,5	281	70
Dihydrocapsaicin	$(CH_3)_2CH(CH_2)_6-$	307,22	Crystal	White opaque	65,6-65,8	281	20
Nordihydrocapsaicin	$(CH_3)_2CH(CH_2)_5-$	293,12	Crystal	White	65,6	280,5	7

Capsaicinoids extraction implies the presence of impurities like lipids, fats, pigments and sterols, which affect capsaicinoids crystallization, reason why the crude extract is usually called "oleoresin" (Suzuki et al, 1984). The average melting point of capsaicinoids is around 65°C and extraction at higher temperatures can produce a vapour that could cause irritation of eyes and respiratory tract (Angioi et al, 2004).

3.3.1 Synthesis

Capsaicinoids are secondary metabolites produced in the epidermal cells of the placenta (Suzuki et al, 1984; Fattorusso et al., 2008), where 90 % of the total capsaicin content of the fruit is found (Pruthi, 2003). The enzyme responsible for their production is called capsaicinoid synthetase, located in the membrane that surrounds the vacuole or tonoplast (Bernal et al., 1993) (See Figure 3). Then, capsaicinoids are stored inside the vacuole (Bernal et al., 1993; Wink, 1993) but they can translocate to other parts of the fruit, such as seeds (Zachariah et al, 2008; Mokhtar et al, 2016), pericarp, stem and leaves (Appendino, 2008).

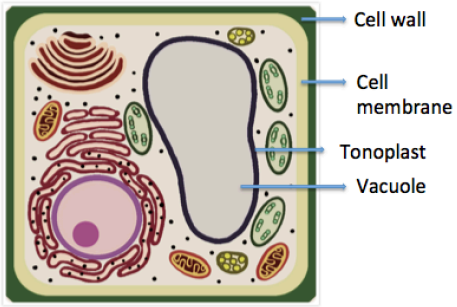


Figure 3: Schematic representation of the general structure of plant cells (Kemsley, 2008)

The amount of total capsaicinoids can change depending on the cultivar and maturation of the chilli. It is higher 2-4 weeks after flowering, decreasing due to oxidation after 50 days (Appendino, 2008). Their accumulation in the plant is affected by fertilization, light exposure and temperature,

increasing during the night at temperatures above 20°C (Suzuki et al, 1984; Appendino, 2008). Higher temperatures and other stress situations such as lack of water will further contribute to raise the production of these molecules in the fruit (Zachariah et al, 2008; Appendino, 2008). This could be explained because capsaicinoids are allelochemicals, i.e. substances produced to defend the plant against herbivores, microorganisms or competing plants. Therefore, these molecules play an important role in the survival of the plant and it could be one reason why they are present in the seeds, as a protection for the plant reproduction (Wink, 1993).

3.3.2 Degradation

The amount of capsaicin in chilli peppers is usually <1% on a dry basis (Barceloux, 2008). Nonetheless, it can be reduced due to the presence of two enzymes in the cell plant, e.g. polyphenol oxidase (PPO) and a peroxidase (POD). PPO is responsible for the enzymatic browning in the fruit and it will indirectly contribute to capsaicinoids degradation since it generates H_2O_2 as a product, which is a substrate for POD (Arnnok et al., 2010). The latter can be mainly found in the placenta (Schweiggert et al., 2006) and it is directly responsible for capsaicinoids degradation due to oxidation of the vanillyl fraction (Bernal et al., 1993). POD has optimal activity around 40°C, decreasing at temperatures above 60°C, probably due to the enzyme denaturation (Arnnok et al., 2010).

The stability of capsaicinoids will be further determined by the storage conditions. In particular, they are more stable to high temperatures and storage time in oleoresin than in dry products. In dried fruit the loss is of 1-2% /month when they are preserved in the freezer (-16°C) and it increases under ambient conditions. In ground powder the loss is of 5% /month and here also will depend on the storage temperature and grind finesses (Berke et al, 2012). These facts could be explained because the optimal temperature for PPO and POD activity is between 30-40°C (Arnnok et al., 2010) and PPO is activated in the presence of oxygen (Coultate, 2009a).

3.4 Pungency

Pungency or "hotness" is the sensation experienced in the oral cavity and other tissues due to compounds like capsaicinoids. They irritate pain receptor cells producing a heat sensation (Grubben et al., 2004) and releasing substance P, which is a neurotransmitter responsible for pain sensation (Zachariah et al., 2008). Different capsaicinoids would give different effects than can be seen in Table 3.

Table 3: Level of pungency, effects and their location in the oral cavity of different capsaicinoids

Capsaicinoids	Pungency SHU (Bobba et al., 2015)	Effect and Location (Zachariah et al, 2008)
Capsaicin	$16x10^6$	-Heat sensation -Back of the tongue, mid-mouth, mid-palate and throat
Dihydrocapsaicin	$16x10^6$	
Nordihydrocapsaicin	$9,1x10^6$	-Less heat than capsaicin and dihydrocapsaicin -Palate and front of the mouth
Homodihydrocapsaicin	$8,6x10^6$	-Sharp and lasting irritating effect -Throat, palate and back of the tongue

The amount of capsaicinoids or pungency can be expressed as Scoville Heat Units (SHU), where pure capsaicin has the highest value (See Table 3). The equivalence between the amount of capsaicinoids and SHU is 1µg/ml= 1ppm= 16SHU= 0,001mg/g= 0,0001% (Berke et al, 2012). The classification of chilli peppers depends on the SHU and can be seen in Table 4.

Table 4: Classification of chillies per Scoville Heat Units (Weiss, 2002) and approximate amount in ppm and mg/g DW.

Classification	SHU	ppm	mg/g DW
Non-pungent	0-700	0-45	0-0.045
Mildly pungent	700-3 000	45-190	0.045-0.19
Moderately pungent	3 000-25 000	190-1500	0.19-1.5
Highly pungent	25 000- 70 000	1500-4400	1.5-4.4
Very highly pungent	>80 000	>5000	>5

4. Regulations behind natural, organic and halal products

4.1 Natural requirements

European Regulation (EC) No 1334/2008 establishes the requirements for products considered natural, which relate to their origin and also the preparation method. For a flavour to be called natural, it should come from a natural source. If this source is listed, no less than 95% of the flavour should be obtained from the chilli, in this case. The flavour should be prepared by traditional methods such as: drying, freezing, cutting, filtration, maceration and distillation, among others. In addition, the extraction temperature should not exceed 180°C for 15min, increasing double the time for every decrease in 10°C. However, this regulation also suggests that capsaicin is a component that shall not be added to food. (EC, 2008b)

4.2 Organic certification

According to the Commission Regulation (EC) No 889/2008 of 5 September 2008 about Organic Production, Labelling and Control, water, ethanol, carbon dioxide and vegetable oils are the processing aids allowed for organic plant origin foods (EC, 2008a). This is also applicable to the production of organic flavours (IFOAM, 2012).

4.3 Halal certification

Halal means permitted in Arabic language. The amount of ethanol that is considered allowed in the final product is limited to 0.05% in Sweden. However, this value can vary within countries (Islamguidens Halalcertifiering AB, 2013).

5. Extraction parameters

The extraction yield can be affected by the characteristics of the raw material (dried or fresh), solvent, temperature, extraction time and pressure.

5.1 Preparation of the sample

Fruit pre-treatment is crucial to extract capsaicinoids. As mentioned before, these alkaloids are located in the vacuole of the cell plant. For this reason, it would be advisable to break down the cell wall of the plant cell and the vacuole, to favour the access of the solvent to the solute. This can be done by physical disintegration, drying, freezing or exposing the fruit to high temperatures.

5.1.1 Drying

Chilli fruit can be dried by different methods like sun drying, freeze-drying and oven drying. The temperature of desiccation can affect the capsaicinoid content. According to the AOAC protocol for fruit and vegetables analysis, the temperature should be kept under 70°C during

oven drying with convection, to minimize the risk of capsaicinoids volatilization (Chinn et al, 2011). If the fruit is dried at 60°C to a final moisture content of 8%, capsaicinoids content can decrease in approximately 10%, reaching 50% of loss if the temperature is maintained for more time afterwards (Berke et al, 2012).

5.1.2 Freezing

Freezing the chilli will lead to the formation of ice crystals that would break down the vacuole and release its content into the cytoplasm. Hence, capsaicinods would be available for the solvent but also for enzymes that will degrade them.

5.1.3 Blanching

Fruit is exposed to high temperatures for short time to inactivate catalytic enzymes. Moreover, at around 50°C and 80°C cellulases (Serva Electrophoresis, n.d.) and pectin methyl estherase respectively have their optimal temperature. These enzymes degrade the cell wall, thus depending on the blanching temperature they could increase their activity until approximately 85°C, when they are denaturated (Serva Electrophoresis, n.d.).

5.2 Solvents and their properties

Solvents are substances that have the ability to form a solution or dissolve a solute, without changing it from a chemical point of view. Solvents are affected by their physical interactions, molecular weight, temperature and pressure applied during extraction.

5.2.1 Molecular forces

There are different intermolecular forces present in solvents. According to their strength in a decreasing order are hydrogen bonding, polar interactions and dispersion forces. The interactions between molecules are broken when they make the transition to the vapour phase. Thus, the boiling point of the solvent is an indicative of the strength of the molecular forces, where the higher boiling point, the stronger they are (Aten, 1996).

5.2.2 Effect of temperature

The increase in temperature favours the extraction process because it decreases the molecular interactions by decreasing the heat of vaporisation, which is the energy needed to separate molecules and take them to vapour phase. (Aten, 1996)

5.2.3 Effect of pressure

High pressure during the extraction allows increasing the temperature above the boiling point of the solvent, which is the principle of Accelerated Solvent Extraction (ASE). An increase in temperature will make the extraction faster because it increases the diffusion rates while less solvent would be needed.

5.3 Crystals solubility

The ideal solubility of capsaicin crystals could be described by Van't Hoff equation that relates solubility to temperature (Standard, 2015).

$$\ln(X) = \frac{-\Delta H^{\circ}}{R}\left(\frac{1}{T} - \frac{1}{Tm}\right) \qquad (1)$$

Where:
X=Solubility
ΔH°= Enthalphy of fusion of capsaicinoid
R=Universal Gas Cosntant = 8,31J/mol K
Tm=Melting Point of capsaicinoid

Given that ΔH°= 160,7 J/g for capsaicin (Jincheng et al., 2010), the theoretical influence of different temperatures was calculated (See Figure 4).

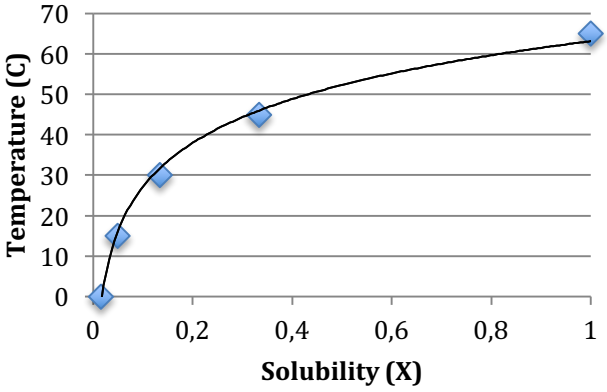


Figure 4: Influence of the increase in temperature in the solubility of capsaicin according to Van´t Hoff equation

Solubility increases slightly from 0 to 40°C. and then it will be more remarkable with small changes in temperature until it reaches 65°C, which is the melting point of capsaicin (Suzuki et al, 1984). At this point, the molecular forces would be disrupted, increasing the interaction with the solvent and hence the solubility (Aten, 1996). Nevertheless, this is a theoretical approach, since Van't Hoff equation refers to the solubility in an ideal solution and it is not known which solvent or mixture is ideal for capsaicin extraction.

6. Quantification of capsaicinoids

Capsaicinoids were traditionally quantified by organoleptic tests. These were based on the detection limit of the pungent effect of a diluted sample with capsaicin (Bergenståhl, 2015). However, this method has a low reliability, being replaced nowadays by instrumental methods, such as High-Performance Liquid Chromatography (HPLC), among others (Zachariah et al, 2008; Amruthraj et al, 2014b; Davis et al., 2007).

6.1 HPLC principle and components

HPLC is a separation technique for non-volatile compounds like capsaicinoids, where a sample is injected into a column (stationary phase) and a liquid will pass through it (mobile phase) forced by a pump. Molecules in the sample will travel in a determined time depending on the interactions with the stationary and mobile phases. When they reach the end of the column, a UV Spectrophotometer will detect them, being able to further quantify the molecules (Agilent Technologies, n.d.) because the absorbance is directly proportional to the concentration of analyte (Taylor, 2015). Each analyte will give different UV spectra, which relates their absorbance at different wavelengths, in the case of capsaicinoids from 200 to 300nm.

Main factors in HPLC are the selection of the column and the mobile phase composition. Nonetheless, there are other parameters that must be considered such as temperature of the column, pressure and flow rate.

6.1.1 Column

Capsaicinoids have been separated using reversed-phase chromatography (See Table 5), where the column is hydrophobic due to the attachment of hydrocarbons chains to the silica. As a result, non-polar substances present in the sample will have more interaction with the column, increasing the retention or elution time, which is the time since the sample is injected until it passes through the column (Clark, 2007).

6.1.2 Mobile phase

The mobile phase can have the same composition over time (isocratic) or can change (gradient) and they are usually useful for simple or complex samples respectively. Capsicum oleoresin is a complex substance due to its composition. Hence, it is advisable to use a gradient for the separation of the components. Under gradient conditions, one solvent is increased gradually, and it is used to develop the appropriate method for the sample. The peaks will be sharper and they will separate better, making it easier to identify the different components in less time than with isocratic conditions. This is due to the gradual elution of molecules that are strongly bound to the column, which will be released when solvent strength increases, i.e. rising organic solvent such as methanol in reversed-phase HPLC. (Agilent Technologies, n.d.)

6.1.3 Temperature

Temperature can be adjusted inside the column and it is important to fix it to have reproducibility in the method. This is because temperature will affect molecule retention, since if the molecules in the sample are not soluble in the mobile phase there could be some problems like precipitation at low temperatures (Agilent Technologies, n.d.). In addition, pressure inside the column can be decreased by applying temperatures between 40-60°C because it will decrease the viscosity of the mobile phase.

6.1.4 Pressure and flow rate

The pressure inside the column can vary between 30-200bar and it is affected by different factors such as temperature, flow rate, column particle size and column dimension. Generally, large particle size, short and wide columns will drop the pressure inside the system, allowing higher flow rates. Flow rate is the amount of mobile phase that will pass through the column per unit time and it is usually expressed as ml/min. The standard flow rate for a column of 4,6x250mm is 1ml/min (Guzzeta, 2016) but it can vary between 0,5 to 2 ml/min (Crawford Scientific, n.d.)

6.2 Calibration method

Oleoresin goes through different steps that could lead to volumetric problems due to processes like pipetting, evaporation or filtration, which altogether could decrease the capsaicinoids fraction of the original extract when it is injected in the HPLC. The addition of a known concentration of a substance to the oleoresin from the beginning of the sample preparation will make the loss of both proportional, since ratios between concentration and areas are constant. The substance to be added is called Internal Standard and it should have certain characteristics. It should be stable, pure and it should have similar structure to capsaicinoids. The latter is because it should be compatible with the wavelength used for the UV detector. The resolution should be >1.5 in order to have a good separation and identification of the peaks (Dolan, 2012).

7. Ethanol evaporation

Ethanol is a classified as class 2 or highly flammable liquid and vapour, since its flash point is below 23°C and the boiling point is above 35°C (EC, 2008c). Flash point is the lowest temperature at which the vapour above the liquid can be ignited when exposed to a flame or hot surface, being 13°C for ethanol (NIOSH, 2014). Thus, ethanol evaporation involves a fire hazard that should be considered during the aroma production. Similarly, the total evaporation of an extract with pure ethanol could imply precipitation of capsaicinoids and possibly carry their loss due to oxidation or high temperatures. Therefore, solvent mixtures of ethanol and water could have some advantages. Flash point of ethanol will increase when mixing it with

water, further decreasing the fire hazard. For instance, a mixture of 50:50 %(v/v) ethanol:water will have a flash point of 24°C (NIOSH, 2014).

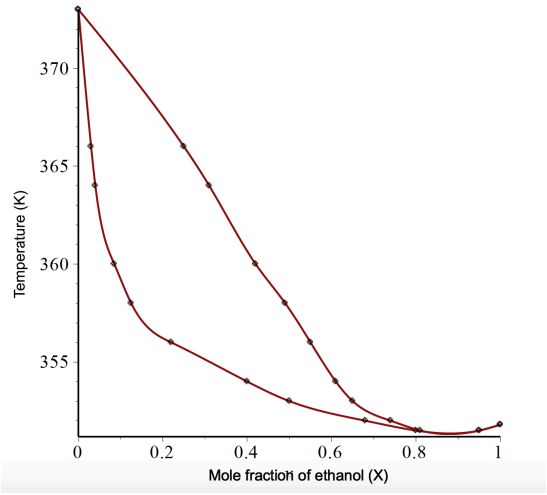


Figure 5: Variation of the boiling point and vapour composition with the mole fraction of ethanol in the liquid. (Bergenståhl, 2017)

The composition of the liquid will not only influence the flash point but it will also define the time of evaporation needed to achieve the target of 0,03% w/v (See Figure 5).

At higher ethanol concentrations, this is more difficult to eliminate. When the concentration is below 45%, there is more ethanol in the vapour and less water at the boiling point of the mixture.

To evaporate the ethanol of a solution of 40% ethanol (v/v) (X=0.17) down to 0,03% w/v (X=1.2exp-4), that mixture should be reduced 70% in volume (Bergenståhl, 2017).

8. Literature review of capsaicinoids extraction

The amount of capsaicinoids to quantify will depend on the raw material, the extraction conditions and the method selected to quantify them. Numerous studies have been done in extraction of capsaicinoids and a summary of some of them is provided in Table 5.

Most of the researchers applied temperatures over 50°C, agitation techniques and organic solvents during the extraction. Nevertheless, Amruthraj et al. (2014a) extracted capsaicinoids with vegetable oils. The outcome was very optimistic but still the extraction with organic solvents such as acetonitrile with the same cultivar gave 20 times more capsaicinoids than using vegetable oils, when quantifying them by UV-VIS Specthrophotometry. Consequently, even if vegetable oils could be used to obtain an organic certified extract, these solvents would not be as efficient as ethanol.

The combination of alcohols and water seems to have a beneficial effect for the extraction. Mokhtar et al., (2016) showed that solvent a mixture with methanol increase the yield compared to just the alcohol by itself. Chantai et al. (2012) obtained also good results with 80% ethanol, hence it could be interesting to try different ethanol mixtures to see how they could differ between them or if they have a similar pattern.

Referring to capsaicinoids distribution, Mokhtar et al. (2016) analysed different parts of the chilli peppers and found that the placenta had the highest amount, followed by the seeds and the pericarp. What is more, the relative amount of dihydrocapsaicin was higher in the placenta than in the rest of the fruit.

In terms of quantification, HPLC was the method used and in some papers this was preceded by purification of the extract with Solid Phase Extraction (SPE) or Thin Layer Chromatography (TLC). However, it is expected that by applying the appropriate gradient, using standards and

checking the spectra and the retention times, capsaicinoids will be easily identified and quantified by HPLC. Accordingly, purification will not be considered during this study.

Many studies have been carried out comparing fresh and dried samples for capsaicinoid extraction. Dried samples have given a higher extraction yield of capsaicin than fresh samples (Mokhtar et al, 2016) when using organic solvents such as methanol or ethanol. This could be because the presence of water in the fresh fruit could affect interaction with capsaicinoids and the hydrophilic properties of the solvent (Amruthraj et al, 2014a). In addition, there could be a decrease in the solvent diffusion through the fresh tissue, probably due to the waxy protective coating of the fruit (Mokhtar et al, 2016). However, these outcomes could be not only related to the solvent properties but also to its access to the solute due to the loss of the cellular structure that implies the drying process.

The comparison of the different results becomes difficult since there are multiple variables that can influence them, such as solvent composition, quantification method and sample pre-treatment. In any case, the cultivar seems to play and important role in the amount of capsaicinoids extracted.

Table 5: Concentration of Capsaicin (*) and Dihydrocapsaicin (**) obtained with different samples, extraction methods and quantification methods (The amount of capsaicinoids are approximated).

Sample characteristics		C* (ppm)	DC** (ppm)	Extraction method	Process of extraction	Processing after extraction	Quantification Method	Ref
-10 different chilli peppers -Dried -Ground		1600	800	2.5 ml/g Methanol/60°C/2h	Magnetic stirring extraction	Purification in C18 cartridge SPE	RP-HPLC - Methanol:Water (50-70% v/v) - 0.9 mL/min - Photodiode Array Detector (PDA) at 280 nm	Juangsamoot, 2012
		1200	700	2.5 ml/g 80%ethanol+Water/60°C/3h				
		1100	600	2.5 ml/g 80%ethanol+Water/90°C/1h				
-Different chilli peppers -Dried	Hot chilli	4200	4500	1 ml/g Ethanol/80°C/4h	Swirled manually every hour	Filtered through 0.45 µm filter paper	RP-HPLC - Mobile phase: Acetonitrile:Water (50:50) v/v - 1,5ml/min -UV detection at 222nm	Al Othman, 2011
	Red chilli	300	200					
Capsicum chinense (Habanero) -Freeze dried -0.5g	Seeds	-	4200	15% w/v (solvents used where Ethanol or Acetone or Acetonitrile)	Shaking water bath (50 °C). Samples taken every 20 min for 1 h.	Vacuum filtration	RP-HPLC - Acetonitrile:Water (40:60) adjusted to pH 3 with acetic acid -Column T=30 °C. - 1 ml/min -UV/vis detector at 280 nm	Chinn et al., 2011
	Shells	-	1900					
	Whole peppers	-	2400					
-Capsicum annuum L. var. Yatsubusa -Fresh/ freeze dried/ sun dried		Total capsaicinoids 3100		1 ml/g (Fresh sample) or 10ml/g (dried sample) Acetonitrile	Blended with the solvent for 2 min	Purification with SPE C18 cartridge with acetonitrile/water	HPLC	Attuquayefio et al., 1987

Continuation Table 5

Sample characteristics		C* (ppm)	DC** (ppm)	Extraction method	Process of extraction	Processing after extraction	Quantification Method	Ref
Capsicum chinense (Habanero) -Fresh product -0.5g	Seeds	-	9200	15% w/v (solvents used were Ethanol or Acetone or Acetonitrile)	Shaking water bath (50 °C)	Vacuum filtration	RP-HPLC - Acetonitrile:Water (40:60) -T=30 °C. - 1 ml/min -UV/vis detector at 280 nm	Chinn et al., 2011
	Shells	-	600					
	Whole peppers	-	3000					
Capsicum chinense (Habanero) -Oven dried 65C/24h -0.5g	Seeds	-	5200					
	Shells	-	1300					
	Whole peppers	-	2000					
Capsicum chinense (Bhut Jolokia) -Sun dried for a day -Ground		72 000	-	10 ml/g olive oil	65°C/1h	-	Phosphomolybdic acid reduction	Amruthraj et al., 2014a
		86 000	-	10 ml/g ginger oil				
		15 000	-	10 ml/g olive oil			UV-VIS Specthrophotometry	
		11 000	-	10 ml/g ginger oil				
Capsicum chinense (Bhut Jolokia) -Sun dried for a day -Ground sieved through 20-30 mesh		293 000	-	1:10 (w/v) acetonitrile	65°C/1h	Purification with TLC silica gel. Mobile phase.Petroleum ether: Chloroform: Acetonitrile (40: 45: 15)	UV-VIS Specthrophotometry	Amruthraj et al., 2014b
		-	-	1:10 (w/v) acetone				
		697 000	$300x10^3$	1:10 (w/v) acetonitrile			HPLC - Acetonitrile:Water 50:50 - 1 ml/min -UV-Vis detector at 280nm	
		370 000	$180x10^3$	1:10 (w/v) acetone				

Continuation table 5

Sample characteristics		C* (ppm)	DC** (ppm)	Extraction method	Process of extraction	Processing after extraction	Quantification Method	Ref
10 different chilli peppers -Dried -Ground		1300-6700	800-2400	2.5 ml/g Methanol/200°C	ASE 5min static time with 3 cycles	Purification with SPE C18 cartridge	RP-HPLC -Binary solvent mixtures (66-44% v/v) of methanol and water - Fluorescence detector (excitation wavelength at 278 nm and emission at 310 nm)	Chantai et al., 2012
-Capsicum annuum L. -Fresh product -10g	Pericarp	C+DC= 700		Acetone/petroleum ether 1:1	-	Purification in TLC plate developed in petroleum ether:acetate:methanol (75:20:5)	HPLC	Perucka et al.,2000
		C+DC= 700					UV-VIS Specthrophotometry	
-C. annuum L. green bell pepper var. Biskra -Fresh	Pericarp	52	13	25 ml/g Methanol 75% in water/100°C/20min	-	Filtered through Whatman paper and evaporated at 45°C	LC-MS-PDA - Water/Acetic Acid (0.075 %)(solvent A) and Acetonitrile/Acetic acid (0.075 %) (solvent B), under isocratic conditions (60 % B). -1 mL/min, -MS detection PDA at 280 nm	Mokhtar et al., 2016
	Placenta	500	220					
	Seeds	200	80					
-C. annuum L. green bell pepper var. Biskra -Oven dried 65C	Pericarp	50	15					
	Placenta	1500	800					
	Seeds	220	100					

9. Methods

9.1 Materials

Absolute Ethanol and HPLC grade Methanol used for the experiments were purchased from VWR. Capsaicin, dihydrocapsaicin and ethyl paraben were obtained from Sigma Aldrich. Different batches of Habanero chilli peppers (*Capsicum chinense Jacq.*) from Egypt were purchased from the local ICA supermarket.

9.2 Determination of dry matter

Three whole chilli peppers without stalks were introduced in an oven at 103°C for 16 hours. The difference in weight was measured to calculate the water content. In addition, the same method was applied to 3 samples of placenta, pericarp and seeds to determine the water content of the different parts separately.

9.3 Extraction method

Samples were introduced in a 25ml flask with solvent at a ratio of approximately 1:20 w/v (g:ml) (DW) (0,46g DW in 10ml solvent) with a magnetic stirrer of 1cm. These flasks were capped and placed on IKA C-MAG HS 7 (IKA-Werke GmbH & Co. KG, Germany), at a stirring rate of 3500rpm. The temperature was set at 65°C and it was further increased to 80°C only for the extraction with water. An autoclave was used for the extraction with water at 120°C, increasing the pressure to 5 bar.
All the extractions were done in 1 hour and samples were introduced when the temperature reached the desired value, which was checked with a thermocouple. Unless otherwise stated, the solvent used was 100% ethanol.

9.4 Sample preparation

Habanero peppers were cut into squares of approximately 1,5cm, removing seeds and stalks. Then the cut fruit was mixed manually to have pieces from different chillies in all the experiments conducted. Unless otherwise stated, these were oven-dried at 103°C for 16h.

9.4.1 Dried, fresh and blanched chillies

Five different batches were prepared before the extraction as follows. One was used as fresh. Another batch was frozen for 1 day. A third batch was blanched for 2 minutes in boiling water and then cooled down in ice water for the same time (Burtness, 2015) before freezing it for one day. Both frozen batches were thawed at room temperature for 1,5h before analysis. The fourth and fifth batches were oven dried at 103°C for 16 hours and at 65°C for 24 hours (Chinn et al.,2011) respectively. Between 2 to 4 replicates of each experiment were done.

9.4.2 Processing of dried samples

3 different experiments were run with 2 to 4 replicates each. A first batch was dried and then pulverized with a mortar. A second batch was dried and shredded with a grater while the third one was first grated and then dried.

9.4.3 Capsaicinoids content in different parts of chilli peppers

Three different chilli pepper fruits were separated in placenta, seeds and pericarp. Pericarp was cut into squares and placenta was dried as a whole. All the different parts were oven-dried for 16h at 103°C.

9.5 Optimization of extraction time

During the extraction, aliquots of 1 ml of the oleoresin (n=2) were taken at time 20, 40 and 60 minutes.

9.6 Multiple step extraction

Two different experiments were done. First, the same fruit was used twice with fresh ethanol in each extraction. Second, the same ethanol was employed twice using a new dried fruit sample.

9.7 Optimization of extraction solvent

Different ethanol percentages from 100 to 0% in increments of 10% were employed. From 4 to 6 replicates were done for each solvent composition. Additionally, deionized water was used for the extraction at 80°C and at 120°C.

9.8 Capsaicinoids quantification

9.8.1 Sample preparation

An aliquot of 1 ml of the different extracts obtained was mixed with 1 ml of a 100ppm solution of ethyl paraben. This mixture was passed through a 0,45µm filter and stored at 4°C until analysis in HPLC.

9.8.2 HPLC analysis

Analysis were performed by reversed phase HPLC using an Agilent Technologies 1260 Infinity. The column used was Zorbax Eclipse XDB-C18 150 x 4,6mm i.d., 5 µm particle size and detection wavelength was fixed at 280nm. The method was based on the one proposed by Waite et al. (2008) as follows. The injection volume was 25µl and the mobile phase had a flow rate of 1,5mL/min. This consisted of a gradient of methanol: water from 40:60% v/v to 85:15 % v/v (8 min) and 85:15 % v/v to 99:1% v/v (2 min), remaining isocratic for 15 min and then returning to the initial composition of 40:60% (5 min). The latter remained isocratic for 5 min, making a total run time of 35min. In addition, UV spectra were recorded to identify both capsaicinoids and the internal standard.

9.8.3 Capsaicinoids determination

Capsaicinoids were quantified using ethyl paraben as internal standard. The ratio that resulted from capsaicin and dihydrocapsaicin standards concentration and area compared to the one obtained from the internal standard gave a constant *k*.

$$k \cdot \frac{Area\ Standard}{Area\ Internal\ Standard} = \frac{Standard\ Concentration}{Internal\ Standard\ Concentration} \qquad (2)$$

This was then used for the determination of the concentration of unknown samples:

$$\frac{Area\ capsaicinoid \cdot\ Internal\ Standard\ Concentration}{Area\ Internal\ Standard} \cdot\ k =\ Capsaicinoid\ Concentration \qquad (3)$$

The amount of capsaicin and dihydrocapsaicin were expressed in mg/g (DW) (See Equation 4) of chilli fruit and total amount of capsaicinoids was obtained by the sum of both components.

$$\frac{mg}{g} DW = \frac{C \cdot V}{Grams\ DW \cdot 1000} \qquad (4)$$

Where:
C=Concentration in µg/ml
V=Volume that remains after the extraction
Grams DW= Grams of sample in dry weight used for the extraction

9.9 Ethanol evaporation

Oleoresin samples (n=3) extracted with 40% ethanol were introduced in a water bath at 95°C. The difference in weight was measured after 30, 60 and 90 minutes and aliquots of 1 ml were taken every time.

9.10 Statistical analysis

The data was analysed in *R* software with ANOVA (P<0,05). In the case of significant differences, Tukey Test (P<0,05) was performed to see where was the difference between treatments.

10. Results and Discussion

10.1 Methodology

10.1.1 Extraction conditions

Magnetic Stirring Extraction was the method selected because it was expected that during agitation the entire fruit sample would be in touch with the solvent, favouring the solute-solvent interaction.

The selection of the temperature was done with regards to some facts concerning both solute and solvent in terms of solubility, stability and safety. Increasing the temperature could not always have a positive effect. On one hand, the vapour originated could be irritant for eyes and respiratory tract (Tucker, 2001) and capsaicinoids could be degraded at high temperatures, around 125 °C (Mokhtar et al., 2016). On the other hand, the boiling point of the solvent should be considered to avoid its evaporation, being 78,3°C for ethanol (Aten, 1996). Consequently, 65 °C was selected as the initial extraction temperature. This was further increased only during the extraction with 100% water, since capsaicinoids are soluble in this solvent only at high temperatures (Suzuki et al, 1984).

10.1.2 HPLC mobile phase composition and method

The selection of the mobile phase solvents depended on their availability in the laboratory and it was based on the method proposed by Waite et al. (2008) with slight modifications. A washing cycle of 15 minutes was added to the method and the temperature was increased to 40°C to decrease the pressure from 300 to 200bar inside the column while increasing the solubility of the capsaicinoids. (Agilent Technologies, n.d.).

The method gave a good resolution, since the different molecules were separated in order to be easily identified (See Figure 6).

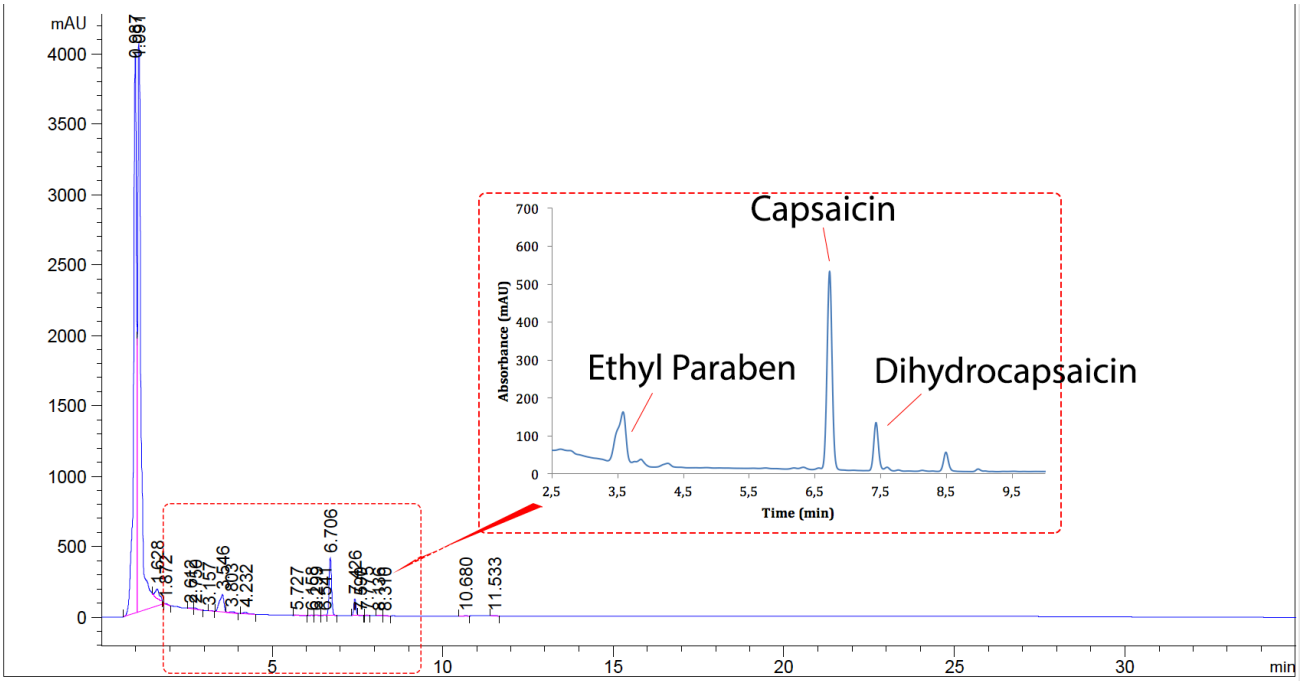


Figure 6: Chromatogram with peaks corresponding to ethyl paraben, capsaicin and dihydrocapsaicin.

Capsaicin and dihydrocapsaicin were eluted after 6.7 and 7.4 minutes respectively. The spectra of both capsainoids was checked in all the chromatographs that were obtained (See Figure 7).

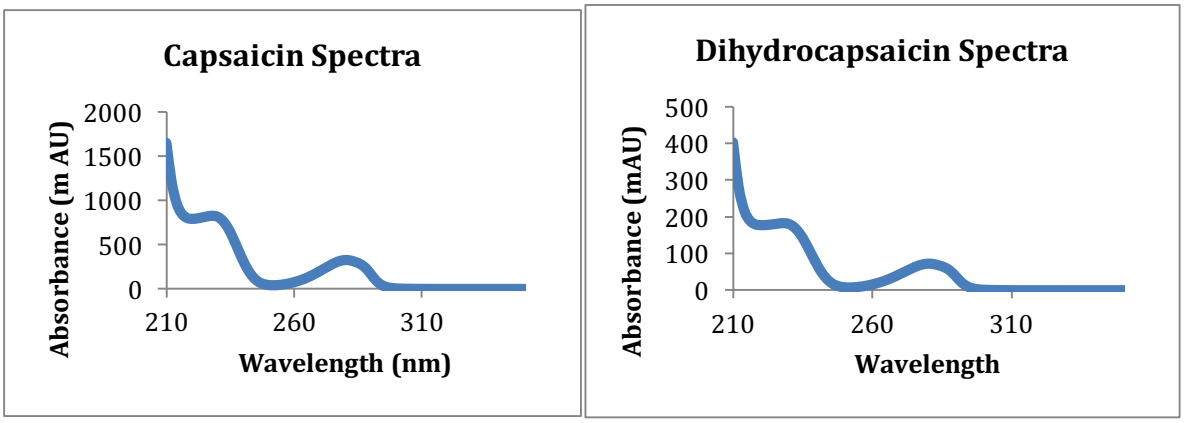


Figure 7: Capsaicin and dihydrocapsaicin spectra

Both capsaicinoids had a similar pattern since the only difference between them is a double bond in the side chain.

10.1.3 Selection of the Internal Standard (IS)

Vanillin and ethyl paraben (EP) were selected as possible IS, since they met with the criteria exposed in Section 6.2. The elution time was 1.7 minutes and 3.5 minutes respectively, which made vanillin to be rejected since it eluted too close to the void peak. This is the initial peak that comes in the chromatogram due to the solvent used for the sample and very polar substabces that are not retained in the column. Therefore, vanillin would make the measurement not trustworthy. EP eluted before the capsaicinoids but there were least 3 minutes between them. The ratio between capsaicin and dihydrocapsaicin with EP (equation 2) gave 2 constants with a value 4,25 and 3,52 respectively.

10.2 Water content in different anatomical parts of chilli fruits

Water content was different within the chilli pepper (See Table 6).

Table 6: Water content in different anatomical parts of the chilli pepper

Sample	Water content (%)	WW (%)	DW (%)
Whole chilli	89 ± 0,42	100	100
Placenta	88 ± 0,59	6 ± 0,61	6 ± 1,02
Pericarp	92 ± 0,86	90 ± 1,64	76 ± 6,07
Seeds	54 ± 3,03	4 ± 2,24	18 ± 7,08

Chilli fruits had water content of around 90%, where seeds showed the lowest value, since these have higher oil content compared to the other parts.

In addition, there was a higher variability of the data in seeds expressed by the standard deviation. This could be because some placenta could have been stuck in them, leading to a more remarkable change in weight after drying. Depending on the size of the chilli, seeds will make a smaller or greater contribution to the total DW (Grubben et al., 2004), which could make the samples that will be further analysed more uneven. Hence, seeds were removed and only pericarp and placenta were considered during this project. Both altogether had water content of 91% ± 0,15.

10.3 Optimization of sample preparation

10.3.1 Dried versus fresh

The main objective of this experiment was to check if the application of different treatments to break down the plant cells could influence the extraction compared to fresh fruit. In addition, the effect of blanching was tested as a procedure to inactivate catalytic enzymes that can affect capsaicinoids. The results are displayed in Table 7.

Table 7: Amount of capsaicinoids in mg/g DW chilli pepper obtained by different sample preparation methods.

Sample	Capsaicin	Dihydrocapsaicin	Total capsaicinoids
Fresh	4 ± 0.42	0.6 ± 0.01	4.55 ± 0.43
Frozen	4 ± 0.27	0.8 ± 0.06	4.96 ± 0.33
Dried at 103°C	5 ± 0.19	0.8 ± 0.05	5.3 ± 1.65
Dried at 65°C	7 ± 2.20	0.9 ± 0.25	7.45 ± 2.44
Blanched and Frozen	2.04 ± 0.27	0.34 ± 0.06	2.39 ± 0.31

Freezing did not make any difference compared to fresh samples ($p=0.99$). This could have been because water content could affect favourably the solubility properties of ethanol, with independence of breaking down the plant cells.

Oven dried samples gave the highest values of total capsaicinoids, finding no significant difference between both temperatures ($P=0.25$). Probably this method could be the best option to break down the cell wall of plant cells, since the loss of water will damage their structure, but these do not seem to be statistically different to fresh samples ($p>0.05$). The data reported here for dried samples had a higher variation than in the rest of the results and this could be the main reason for the statistical outcome. This could have occurred due to capsaicinoids loss that could reach 50% when the water content is below 8% (Berke et al, 2012).

Fresh fruit is susceptible to spoilage and could also lead to capsaicinoid loss over time due to the enzymatic activity. Drying the fruits could also lead to loss due to the high temperatures. Nonetheless, drying could act as a preservation method until extraction together with the reduction of microbial load and decrease in enzyme activity linked to the heat treatment (Schweiggert et al, 2005). As a consequence, drying is the selected pre-treatment process to conduct the following experiments.

10.3.2 Blanching effect

Blanching gave the lowest results compared to all the methods and it was only significantly different from drying methods, both at 65°C (p=4e-3) and at 103°C (p=0,04). This result agrees with the one obtained by Schweiggert et al, (2005), who suggested that blanching had more direct influence in the decrease of the amount of total capsaicinoids, rather than drying or mincing the fruit. Blanching could have positive effect before freezing when storing samples over time, since it leads to the denaturation of catalytic enzymes (Orak et al., 2005). But POD is a heat resistant enzyme, which is inactivated after 6min at 100°C (Ismail et al., 2006), and it can be further regenerated at pH 5-9, range in which fresh and dried pulverized chilli peppers lie (pH 5-6,5) (Schweiggert et al, 2005). Even though Orak et al. (2005) suggested that the increase in POD during storage time was related to decreasing capsaicin content, Schweiggert et al. (2005) suggested that a high POD activity is not always related to capsaicinoid loss. Other facts could be taking part, such as non-enzymatic degradation, with independence of exposure to light.

Capsaicinoids loss is more remarkable when increasing the blanching time up to 10 min and at a temperature over 80°C (Orak et al., 2005; Schweiggert et al., 2005). Thus, blanching at 100°C could have been an extremely high temperature even if the time of exposure was 2 minutes. The loss could have been caused by the heating process (Schweiggert et al. 2005), still another assumption could be that they were solubilized in blanching water. For this reason, water was further analysed (n=2) and the results can be seen in Table 8.

Table 8: Capsaicinoids (mg/g DW) quantified in water used for blanching.

Sample	Capsaicin	Dihydrocapsaicin	Total capsaicinoids
Blanching water	0.77 ± 0.5	0.21 ± 0.1	0.98 ± 0.6

If 0.98 is added to the amount obtained of "Blanched and frozen" it will give a total amount of capsaicinoids of 3.37 mg/g DW.

Thus, without accounting the possible loss due to high temperature, 29% of capsaicinoids will be lost due to their solubility in boiling water. This could be high considering that during the blanching process the time of exposure of the solute to the solvent was two minutes, whilst one standard extraction was done in one hour. In this respect, this result will be further analysed with the outcome of the extractions with 100% water.

10.3.3 Processing of dried samples

To avoid changes in the solvent's polarity and to favour the access of the solvent to the plant material the fruit is usually dried and ground (Juangsamoot, 2012; Amruthraj, 2014a, 2014b).
Several experiments were conducted to see the effect of decreasing the sample particle size in the extraction. The results are shown in Table 9.

Table 9: Effect of different procedures applied to dried samples in the amount of capsaicinoids extracted.

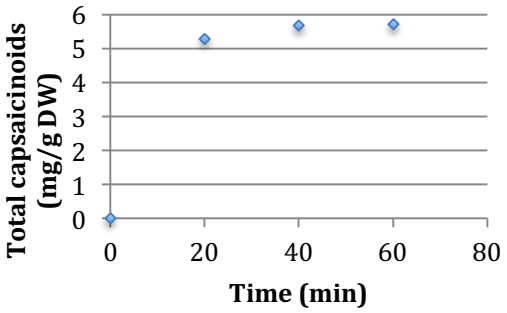
Sample	Capsaicin	Dihydrocapsaicin	Total capsaicinoids
Powder	3.37 ± 1.24	0.58 ± 0.34	3.96 ± 1.58
Dried and Grated	2.33 ± 0.01	0.55 ± 0.08	2.88 ± 0.08
Grated and Dried	1.92 ± 0.40	0.44 ± 0.07	2.36 ± 0.47
Dried at 103°C	5 ± 0.19	0.8 ± 0.05	5.3 ± 1.65

The highest yield corresponded to the samples that were just cut into squares and dried but no statistically significant difference was found between the different procedures. This could have been due to large errors shown in the standard deviation. If a less demanding evaluation was used, such as lowering the confidence interval from 95% to 90%, a significant difference could be seen between dried at 103°C and powder ($p=0.013$). It cannot be excluded that grinding increases the fruit surface in touch with O_2, thus increasing the activity of PPO and hence POD, having a negative effect in capsaicinoids content. In this sense, Schweiggert et al. (2005) found that this POD activity is higher in dried powder compared to fresh fruit, decreasing considerably when blanching before drying.

Likewise, it cannot be said that in the dried fruit cut into squares there is no degradation, since just by mincing fresh fruit capsaicinoids content could decrease 6% (Schweiggert et al, 2005). Additionally, samples were totally dried, not taking into consideration the recommended 8% of moisture that should be left in the dried fruit to avoid losses (Berke et al., 2012).

10.4 Optimization of the extraction time

Mokhtar et al. (2016) suggested that 20 minutes was the ideal extraction time for capsaicinoids, since these could be further degraded for longer time at 100°C. However, the temperature during this study was fixed at 65°C and this could be less damaging for the solutes. Hence, trials were conducted and the results can be seen in Figure 8.



92% of the total capsaicinoids present in the oleoresin after 1 hour of extraction were obtained in the first 20 minutes. This result agreed in part with Mokhtar et al. (2016), but in this case, more capsaicinoids were obtained after 20 minutes, suggesting that there was no degradation.

Figure 8: Total amount of capsaicinoids extracted in ethanol after 20,40 and 60 minutes.

10.5 Extraction cycles

The outcome of 10.4 may suggest that more capsaicinoids could be extracted after one hour, so that the fruit could be still useful for extraction. What is more, if the solvent could be used for more than one extraction without being saturated, less solvent will be needed, which may increase the efficiency in large-scale production of the aroma. To check these assumptions, 2 different sets of experiments with consecutive extractions were conducted checking both fruit and solvent.

10.5.1 Fruit

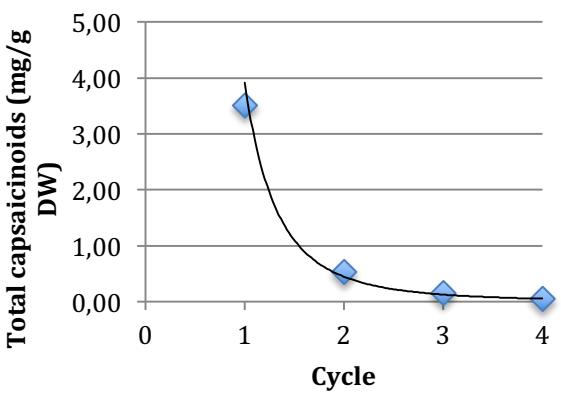


Figure 9: Amount of capsaicinoids obtained in four consecutive experiments using the same fruit and fresh ethanol in each one.

The amount capsaicinoids obtained after 4 cycles (n=2) using the same fruit and fresh solvent each time gave a total value of 4.21mg/g DW (See figure 9).

83% of this total amount was obtained with the first cycle, reaching 95% in the second. There was no dihydrocapsaicin detected in the fourth cycle. From the results, can be assumed that 2 extractions could be recommended to make the most of the raw material.

10.5.2 Solvent

The same solvent was used for 2 consecutive extractions (n=2). In the first and second extraction, the total capsaicinoids obtained were 6.09 ± 2.89 and 15.17 ± 1.61 respectively. The results suggested that there was no saturation after two extractions, since not only more capsaicinoids were obtained using the same solvent but also the final value is around 2 times more than in the first extraction.

The use of the same solvent twice will represent an advantage in terms of efficiency since less solvent will be needed to extract more solute and, even more it will diminish costs.

10.6 Distribution of capsaicinoids in different parts of chilli peppers and classification of the raw material

Capsaicinoids are originated in the placenta of *Capsicum* fruits (Suzuki et al, 1984; Appendino, 2008). For this reason, they are found in higher amounts in this part than in the rest of the pepper (Mokhtar et al., 2016). The results of the present experiment can be seen in Table 10.

Table 10: Amount of capsaicinoids (mg/g DW) obtained from different parts of chilli peppers and total amount found in the whole chilli.

Part	Capsaicin (mg/g DW)	Dihydrocapsaicin (mg/g DW)	Total Capsaicinoids (mg/g DW)	Average DW (g)	Capsaicinoids in each part (mg)
Pericarp	1.4 ± 1.21	0.23 ± 0.09	1.47 ± 1.27	0.96 ± 0.04	1.41
Placenta	42.0 ± 7.86	13.47 ± 5.07	56.49 ± 3.18	0.07 ± 0.01	3.95
Seeds	0.2 ± 0.22	0.05 ± 0.05	0.26 ± 0.26	0.23 ± 0.10	0.06
			Total per chilli	1.26 ± 0.08	5.42

Capsaicin represents more than 70% of the total value of capsaicinoids. These were found mainly in the placenta, being the amount 37 times higher than in the pericarp.

This is in agreement with Mokhtar et al. (2016), who found a difference of 32 times. However, during the current experiments, seeds and pericarp had the highest variability. The presence of small amount of placenta left in pericarp or seeds that can represent a big difference in the

results. Since seeds are attached to the placenta and this one to the pericarp, it may be difficult to remove all the tissue from them.

The total amount of capsaicinoids found in one chilli was 5,42mg, which corresponded to 0,43% of the total DW of one chilli pepper. This result agreed with <1% proposed by Barceloux, (2008). Additionally, it can be said that the habanero chilli peppers used during these experiments were classified as highly pungent (Weiss, 2002), since they had 4.3 mg/g DW of capsaicinoids or 68 800 SHU.

10.7 Optimization of extraction solvent

10.7.1 Variation of ethanol composition

The total amount of capsaicinoids extracted with different ethanol percentages can be seen in Figure 10.

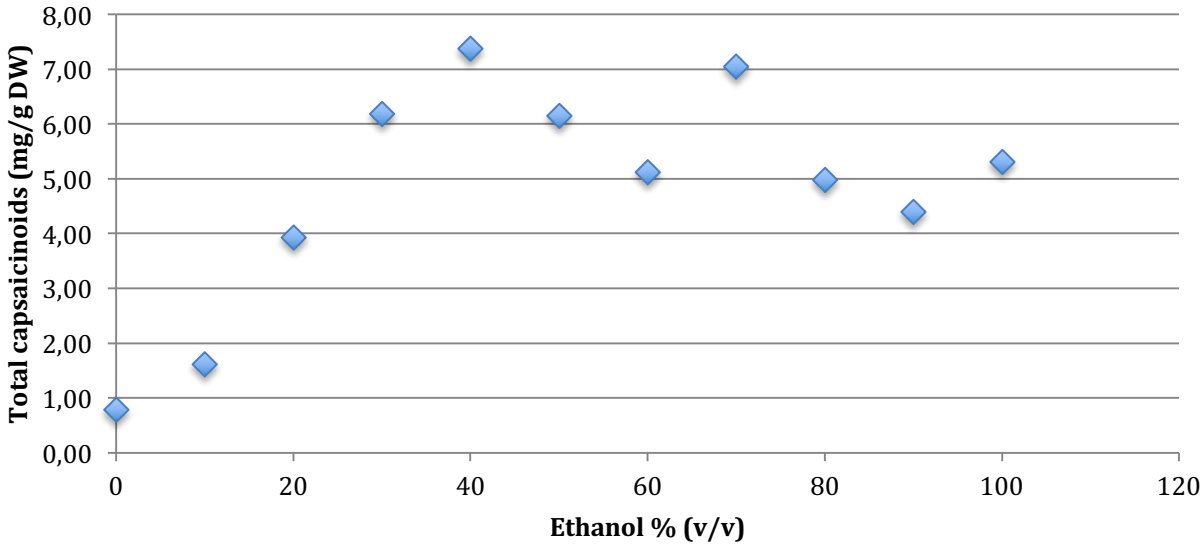


Figure 10: Amount of capsaicinoids (mg/g DW) obtained after extraction with different ethanol composition

According to the data obtained the amount of capsaicinoids extracted was higher between 40 to 70%. However, no significant difference was found between 20 to 100% (v/v) ethanol. On the contrary, extraction with 100% (v/v) water gave the lowest amount of capsaicinoids extracted and the highest significant difference was found between this solvent composition and 40% ethanol (P=9e-6). Nonetheless, the data obtained scattered and one assumption could be that the fruit sample was non-homogeneous, since both placenta and pericarp were in the sample.

Fruits were cut into squares, which make it more difficult to homogeneize than if they were in powder. Furthermore, 0,46grams were used for each extraction and this could have been a small amount to have a representative sample. The amount of placenta in the fruit is much less in terms of weight than the pericarp but it has higher capsaicinoid content. As a consequence, the presence of more amount of placenta in the sample could have given higher values of capsaicinoids in the oleoresin.

10.7.2 Water extraction at different temperatures

The amount of capsaicinoids obtained with water in the previous section was the lowest compared to the extraction with different ethanol percentages. This was expected since these

alkaloids are soluble in organic solvents and slightly soluble in hot water (Suzuki et al, 1984). However, could be that more capsaicinoids could be extracted by rising the temperature over 65 °C, such as 80 °C or even 120 °C.

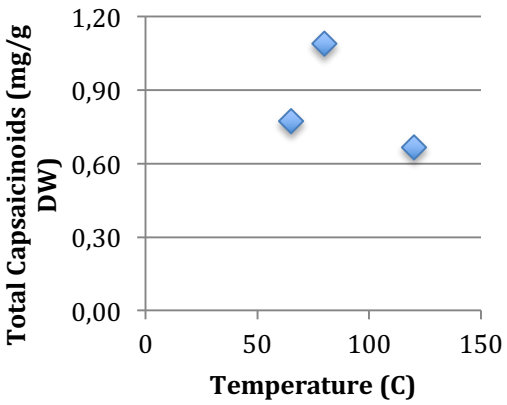


Figure 11: Total capsaicinoids extracted with deionized water at different temperatures.

The results obtained can be seen in Figure 11. The extraction was favoured at temperatures in the range of 80 to 100°C but the amount obtained at 120°C was lower than at 65°C. Mokhtar et al. (2016) suggested that over 120°C there could be some degradation. However, there is an inconsistency with this argument, since Chantai et al. (2012) extracted capsaicinoids with ASE at 200°C with better results than at lower temperatures using organic solvents and also mixing them with water. Could be that the time expended for this experiment was more than needed and this could affect the capsaicinoids.

This assumption can be supported by comparing total capsaicinoids obtained at 80°C (1.09 ± 0.12) with the blanching water (0.98 ± 0.6), where it could be assumed that at the beginning of the extraction most capsaicinoids are solubilised. After all, the statistical analysis did not show any significant difference between the applied temperatures employing 100% (v/v) water as a solvent. If these extractions with water at different temperatures are compared to 40% ethanol at 65°C, there was still a significant difference ($p<0,05$), making the latter more efficient

The selection of the extraction conditions should not only rely on the yield obtained, but also on the stability of the solute. On one hand, extraction with water could lead to further crystallization of the capsaicinoids when the extract is cooled down, being difficult to resuspend them since they are insoluble in cold water (Suzuki et al, 1984). On the other hand, when using ethanol as a solvent it must be considered that its elimination could be a challenge, since capsaicinoids could be degraded not only because of high temperatures, but also due to the time that it has to be exposed to the heat source.

10.8 Consequences of ethanol evaporation

Solvent mixture with 40% ethanol showed good results in terms of yield compared to 100% water. Nevertheless, the objective of this study was to develop an extract that could also be considered as halal. Hence, if ethanol was used it needed to be evaporated, but the effect that the duration of the heating process on the capsaicinoid stability could have, was not known. For this reason, this was tested.

The boiling point of a mixture of 40:60% (v/v) ethanol: water is 83°C (see Figure 4). However, the extract was not boiling at this temperature, probably due to the amount of sugars present in the samples, which represents around 60% DW of the fruit (Grubben et al., 2014) and are soluble in water. Therefore, the temperature during this trial was set at 95°C, which could have been higher than the boiling point of the oleoresin, but it was also desired to see the effect of higher temperatures in the capsaicinoid content. Oleoresin has a density of 0.935–0.945 g/ml (Zachariah et al., 2008) but during this experiment it was assumed as 1mg/ml, since the difference in weight was measured to see the volume loss and it would make the calculations easier.

The equation used to calculate the preservation was:

$$Preservation = \frac{CxV\ (Final)}{CxV\ (Initial)} \qquad (5)$$

Where:
C= Concentration
V=Volume

The preservation of capsaicinoids was of 100% after 1,5h (See Table 11), suggesting that the temperature applied for that time will not damage the molecules.

Table 11: Preservation of capsaicin and dihydrocapsaicin after 1,5h of ethanol evaporation and average volume loss (n=3).

Capsaicin Concentration (ppm)		Dihydrocapsaicin Concentration (ppm)		Volume (ml)		Preservation	
Initial	Final	Initial	Final	Initial	Final	Capsaicin	Dihydrocapsaicin
347 ± 81	666 ± 59	74 ± 19	138 ± 10	10	5.7 ± 0.7	1,1 ± 0,15	1,1 ± 0,14

After 90 minutes almost 43% of the volume was lost but the process should continue until only 30% of the volume of the original solution remains to have 0,03% (w/v) of ethanol in the oleoresin (Bergenståhl, 2017). Both types of capsaicinoids were equally preserved over time. Even if the total evaporation of ethanol to reach the target level was not completed, more than 50% was already evaporated with optimistic results.

The main limitation during this project in all the trials was the scattering of the data. This could have been because 10 different batches of chilli peppers were analysed. There is some evidence that harvesting at the right time and controlling the conditions during the cultivation can affect the amount of capsaicinoids in the fruit. Thus, the first step is to control the fruit from the source to standardize the process. Additionally, the fruit should be homogenised for the extraction, with regards to the amount of placenta and pericarp to have a more representative sample. Since the total amount considered during the lab experiments was 0,46g, this could have been insignificant.

11. Conclusions

Organic chilli peppers are a natural source used for the extraction of capsaicinoids, which are responsible for the pungency of the fruits and they are synthesised in the placenta. These capsaicinoids are mainly capsaicin and dihydrocapsaicin and to have an organic extract that can be used for food applications they should be extracted with water and/or ethanol.

The extraction method developed using cut and dried fruits with magnetic stirring for one hour at 65 °C gave a good yield. The least amount of ethanol that can be used is 20% and the evaporation process to further eliminate it did not affect the capsaicinoids, since 100% were preserved. Nonetheless, water is an alternative solvent, which gave lower yield than ethanol mixtures but a halal extract can be directly obtained without further processing.

HPLC is the most accurate instrumental method to detect and quantify capsaicinoids. The method developed during this study gave a good chromatogram resolution, since capsaicinoids were clearly separated, hence favouring their identification.

12. Further perspectives

-In order to establish the best option between using 100% water or 20% ethanol, a cost comparison of the two methods would have to be done.

-The stability of capsaicinoids over time in both solvent alternatives could be investigated.

13. References

Agilent Technologies: "HPLC Basics. Fundamentals of Liquid Chromatography (HPLC)". No date. Electronic source. Accesed on the 19/02/17. Available at: http://polymer.ustc.edu.cn/xwxx_20/xw/201109/P020110906263097048536.pdf

Al Othman, Z.A; Hadj Ahmed, Y.B; Habila, M.A; Ghafar, A.A.: "Determination of Capsaicin and Dihydrocapsaicin in Capsicum Fruit Samples using High Performance Liquid Chromatography". Molecules. 2011.

Amruthraj, N.J; Raj, J.P.P; Lebel, L.A.: "Effect Of Vegetable Oil In The Solubility Of Capsaicinoids Extracted From Capsicum Chinense Bhut Jolokia". Asian Journal Of Pharmaceutical and Clinical Research. Volume 7. Pages 48-51. 2014a.

Amruthraj, N.J; Raj, J.P.P; Lebel, L.A.:" Comparative Study on the Extraction of Capsaicinoids from Capsicum chinese and their Analysis by Phosphomolybdic Acid Reduction and HPLC". International Journal of Pharmaceutical Sciences Review and Research. 28(2). Article 44. Pages 247-252. 2014b.

Amruthraj, N.J; Raj, J.P.P; Lebel, L.A : "Impact of Organic Solvents in the Extraction Efficiency of Therapeutic Analogue Capsaicin from Capsicum chinense Bhut Jolokia Fruits". International Journal of Pharmaceutical and Clinical Research Volume 6(2). Pages 159-164. 2014c.

Angioi, S; Gennaro, M.C.; Gianotti, V.; Marengo, E.; Robotti, E. *Organic Bases*, Chapter 18 in "Handbook of Food Analysis: Physical Characterization and Nutrient Analysis". Page 636. Volume 1. Second Edition. Edited by Nollet, L.M.L. Marcel Dekker Inc. New York. USA. 2004

Appendino, G. *Capsaicin and Capsaicinoids*. Chapter 4 in "Modern Alkaloids: Structure, Isolation, Synthesis, and Biology". Pages 73-104. Edited by Fattorusso, E.; Taglialatela-Scafati, O. Wiley-VCH. Weinheim. Germany. 2008

Arnnok, P.; Ruangviriyachai, C.; Mahachai, R.; Techawongstien, S.; Chanthai, S.: "Optimization and determination of polyphenol oxidase and peroxidase activities in hot pepper (Capsicum annuum L.) pericarb". International Food Research Journal. Volume 17. Pages 385-392. 2010.

Aten, W.C. *Solvent action and measurement*. Chapter 2 in "Directory of Solvents". Pages 22-28. Edited by Whim, B.P.; Johnson, P.G. Blackie Academic and Professional. London. UK. 1996.

Attuquayefio, V.K and Buckle, K.A.: "Rapid Sample Preparation Method for HPLC Analysis of Capsaicinoids in Capsicum Fruits and Oleoresins". Journal of Agricultural Food Chemistry. Volume 35. 5. 1987.

Barceloux, D.G. *Pepper and Capsaicin*. Chapter 10 in "Medical Toxicology of Natural Substances: Foods, Fungi, Medicinal Herbs, Plants and Venomous Animals". Part 1: Foodborne and microbial toxins. Pages 72-75. Wiley. New Yersey. USA. 2008

Bergenståhl, B.: "The chemical origin of taste and odour". Lecture in Food Chemistry. Lund University. 2015

Bergenståhl, B. Personal Communication. Lund University. 2017

Berke, T.G.; Shieh, S.C. *Capsicum Cultivars*. Chapter 7 in "Handbook of Herbs and Spices". Volume 1. Pages 116-129. Edited by Peter K. V. Second Edition. Woodhead Publishing Limited. Cambridge. UK. 2012

Bernal, M.A.; Calderon, A.A.; Pedreño, M.A.; Muñoz,R.; Barceló, A.R.; Merino de Cáceres, F.: "Capsaicin oxidation by peroxidase from Capsicum annuum (variety Annuum) fruits". Journal of Agricultural and Food Chemistry. Volume 41 (7). Pages 1041-1044. 1993.

Bobba, M.; Gianotti, V.; Gosetti, F.; Manfredi, M.; Marengo, E.; Mazzucco, E.; Robotti, E. *Organic Bases*. Chapter 29 in "Handbook of Food Analysis". Edited by Nollet, L.M.L; Toldra, F. Third Edition. Volume I. Pages 674-680. CRC Press. Florida. USA. 2015

Burtness, C. : "Blanching Vegetables". University of Minnesota Extension: USA. 2015. Electronic source. Accesed on the 10/02/17. Available at:
http://www.extension.umn.edu/food/food-safety/preserving/vegetables-herbs/blanching-vegetables/

Chantai, S.; Juangsamoot, J.; Ruangviriyachai, C.; Techawongstien, S.: "Determination of Capsaicin and Dihydrocapsaicin in Some Chilli Varieties using Accelerated Solvent Extraction Associated with Solid-Phase Extraction Methods and RP-HPLC-Fluorescence". E-Journal of Chemistry. Volume 9(3). Pages 1550-1561. 2012.

Chinn, M.S.; Sharma-Shivappa, R.R.; Cotter, J.L.: "Solvent extraction and quantification of capsaicinoids from *Capsicum chinense*". Food and Bioproducts Processing. Volume 89. Pages 340-345. 2011.

Clark, J.: "High Performance Liquid Cromatography-HPLC". 2007. Modified in June 2016. Electronic Source. Accessed on the 19/02/17. Available at: http://www.chemguide.co.uk/analysis/chromatography/hplc.html

Coultate, T. *Colours*. Chapter 6 in "Food. The chemistry of its components". The Royal Society of Chemistry (RSC Publishing). Page 238. Fifth Edition. London. UK. 2009a.

Coultate, T. *Flavours*. Chapter 7 in "Food. The chemistry of its components". The Royal Society of Chemistry (RSC Publishing). Page 285. Fifth Edition. London. UK. 2009b.

(a)Crawford Scientific: "Speeding up your HPLC". No date. Electronic Source. Accesed on the 19/02/17. Available at:
http://www.chromacademy.com/chromatography-speeding-up-your-HPLC.html

Davis, C.B.; Markey, C.E.; Busch, M.A.; Busch, K.W.: "Determination of Capsaicinoids in Habanero Peppers by Chemometric Analysis of UV Spectral Data". Journal of Agricultural and Food Chemistry. Volume 55. Pages 5925-5933. 2007.

Dolan, J.W.: "When Should an Internal Standard be Used?". LCGC magazine North America. Volume 30. Issue 6. Pages 474–480. USA. 2012.

European Comission: "Commission Regulation (EC) No 889/2008 of *5 September 2008*. Laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and control". Official Journal of the European Comission. Annexe VIII. Pages 250/48-250/51. 2008a

European Comission: "Regulation (Ec) No 1272/2008 Of The European Parliament and of The Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006". Official Journal of the European Union. Table 3.1. Page L353/60. 2008c

European Comission: "Regulation (Ec) No 1334/2008 Of The European Parliament and of The Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC". Official Journal of the European Union. L354/34-L354/50. 2008b

Grubben, G.J.H.; Denton, O.A.: "Plant Resources of Tropical Africa 2. Vegetables". PROTA Foundation. Wageningen. Netherlands. Page 157. 2004

Guzzeta, A.: "Calculating the Appropriate Flow Rates for Columns of Differing Diameters or Maintaining Linear Velocity". 2016. Electronic Source. Accessed on the 19/02/17. Available at: http://www.ionsource.com/Card/linvelocity/linvol.htm

IFOAM EU Group: "European Organic Regulations (EC) No 834/2007, 889/2008 and 1235/2008. An Evaluation of the First Three Years. Looking for Further Development". Page 46. Brussels. 2012.

Islamguidens Halalcertifiering AB: "Halal Standard: Guidelines for Halal Certification". Stockholm. Page 15. 2013. Electronic source. Accessed on the 26/10/16. Available at: http://www.halalcertification.se/Islamguidens_halalcertifiering_Halal_Standard.pdf

Ismail, N.; Revathi, R.: "Studies on the effects of blanching time, evaporation time,temperature and hydrocolloid on physical properties of chili (Capsicum annum var kulai) puree". LWT-Food Science and Technology. Volume 39. Issue 1. Pages 91-97. 2006.

Jincheng, W.; Xiaoyu, Z.; Sihao, C.: "Preparation and Properties of Nanocapsulated Capsaicin by Complex Coacervation Method". Chemical Engineering Communications. Volume 197. Pages 919-933. 2010.

Juangsamoot, J.; Ruangviriyachai, C.; Techawongstien, S.; Chanthai, S.: "Determination of capsaicin and dihydrocapsaicin in some hot chilli varieties by RP-HPLC-PDA after magnetic stirring extraction and clean up with C_{18} cartridge".
International Food Research Journal. Volume 19 (3). Pages 1217-1226. 2012.

Kemsley, S. “Plant cell (color)”. ABCTeach. 2008. Electronic source. Accessed on the 31/04/17. Available at: http://www.abcteach.com/documents/poster-plant-cell-color-34644

Mokhtar, M.; Russo, M.; Cacciola, F.; Donato, P.; Giuffrida, D.; Riazi, A.; Farnetti, S.; Dugo, P.; Mondello, L.: “Capsaicinoids and Carotenoids in Capsicum annuum L.: Optimization of the Extraction Method, Analytical Characterization, and Evaluation of its Biological Properties”.Food Anal. Methods. Pages 1381–1390. 2016.

National Institute for Occupational Safety and Health (NIOSH): “International Chemical Safety Cards. Ethanol Anhydrous”. CDC. U.S. Department of Health & Human Services. 2014. Electronic source. Accesed on the 26/04/17. Available at: https://www.cdc.gov/niosh/ipcsneng/neng0044.html.

Orak, H.H.; Demirci, M.: “Effect of different blanching methods and period of frozen storage on enzyme activities and some quality criteria of hot and sweet red peppers (*Capsicum annuum* L.)”. Pakistan Journal of Biological Sciences. Volume 8. Pages 641–648. 2005.

Perucka, I.; Oleszek, W.: “Extraction and determination of capsaicinoids in fruit of hot pepper *Capsicum annuum L*. by spectrophotometry and high-performance liquid chromatography”. Food Chemistry. Vol 71. Pages 287-291. 2000.

Pew Research Center. “Religious Composition by Country, 2010-2050”. USA. 2015. Electronic source. Accessed on the 5/01/17. Available at: http://www.pewforum.org/2015/04/02/religious-projection-table/2010/percent/all/

Pruthi, J.S. *Advances in post-harvest processing of Capsicum*. Chapter 10 in “Capsicum: The Genus Capsicum”. Pages 197-198. Edited by Krishna De, A. Taylor and Francis Group. London. UK. 2003.

Schweiggert, U.; Schieber, A.; Carle, R.: “Inactivation of peroxidase, polyphenoloxidase, and lipoxygenase in paprika and chili powder after immediate thermal treatment of the plant material”. Innovative Food Science & Emerging Technologies. Volume 6, Issue 4. Pages 403–411. 2005

Serva Electrophoresis GmbH: “Cellulases”. Germany. No date. Electronic source. Accessed on the 10/02/2017. Available at: http://www.serva.de/www_root/documents/16419_26.pdf

Standard, J.M.: “The Van't Hoff Equation”. Illinois State University. Department of Chemistry. 2015. Electronic source. Accessed on the 27/04/17. Available at http://chemistry.illinoisstate.edu/standard/che360/handouts/360vanthoff.pdf

Suzuki, T.; Iwai, K. *Constituents of Red Pepper Species: Chemistry, Biochemistry, Pharmacology, and food Science of the Pungent Principle of Capsicum Species*. Chapter 4 in “The alkaloids. Chemistry and pharmacology”. Volume 23. Pages 227-299. Edited by Arnold Brossi. Academic Press Inc. California. USA. 1984.

Taylor, T.: “Important Aspects of UV Detection for HPLC”. LCGC magazine North America. Volume 33. Issue 11. Page 870. 2015.

Tucker, S.P.: “Determination of Capsaicin and Dihydrocapsaicin in Air in a Pickle and Pepper Processing Plant”. AIHAJ - American Industrial Hygiene Association. Volume 62:1. Pages 45-48. 2001.

USDA: Natural Resources Conservation Service. “Classification for Kingdom Plantae Down to Genus *Capsicum* L.”. No date. Electronic source. Accessed on the 12/11/16. Available at: https://plants.usda.gov/java/ClassificationServlet?source=display&classid=CAPSI.

Vineyard, L.; “National Tactical Training Association Of Bail Enforcement Agents. Fugitive Recovery Manual Level 2”. Page 173. 2012.

Waite, M.S.; Aubin, A.J.: “A Modular HPLC System for routine Analysis of Capsaicin from Hot Sauces”. Waters Corporation. USA. 2008. Electronic source. Accessed on the 28/04/17. Available at: http://www.waters.com/webassets/cms/library/docs/720002753en.pdf

Weiss E.A. : *Solanaceae*. Chapter 9 in “Spice Crops”. Edited by Weiss, E.A. CAB International. New York. USA. 2002

Wink, M.: “The Plant Vacuole: A Multifunctional Compartment”. Journal of Experimental Botany. Volume 44. Pages 231-246. 1993.

Zachariah, T.J.; Gobinath, P.. *Paprika and Chilli*. Chapter 14 in “Chemistry of Spices”. Edited by Parthasarathy, V. A.; Chempakam, B.; Zachariah, T. J. CAB International. Wallingford. UK. 2008