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Chemical Composition and Flavour Development of Cocoa Products by Thermal and Enzymatic Technologies

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Table of Content

List of Figures

List of Tables

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It has been a long and complex journey to arrive finally to this moment and even now, I cannot believe that I am writing the last pages of my PhD thesis. Within this thesis there is much more than results, hypotheses and conclusions. Inside these pages, apart from many hours of work there are love, pain, passion, happiness, sadness, disappointments, obsessions, friendships, loneliness... a personal adventure that started many years ago when an enthusiastic young dreamer with a eager mind decided to calm his fascination for research and chemistry.

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Declaration

I certify that this dissertation consists of my own original work. All quotations from published and unpublished sources are acknowledged as such in the text. No portion of the work referred to in this dissertation has been submitted in support of an application for another degree or qualification of this or any other university or other institution of learning.

Signature: ..... Date:...15-02-2008...

Abbreviations

CCP	Conched chocolate powder
CW	Carbowax
DCM	Dichloromethane
DHS	Dynamic headspace
DVB	Divinylbenzene
FAG	Free amino group(s)
GC	Gas chromatography
HMF	Hydroxymethyl furfural
HPLC	High performance liquid chromatography
HS	Headspace
IS	Internal standard
min	minute(s)
MS	Mass spectrometry
NCP	Natural cocoa powder
NIST	National Institute of Standards and Technology
OPA	O*rtho*-phthaldialdehyde
PA	Polyacrylate
PDMS	Polydimethylsiloxane
RI	Retention index
SFE	Supercritical Fluid Extraction
SHS	Static Headspace
SPME	Solid phase microextraction
TPC	Total polyphenol content
TPR	Templated resin

Abstract

A novel technology to process traditional chocolate-making ingredients has been investigated with the main objective to promote the generation of key chocolate odorant volatiles as a part of the commercial interest of the industrial partner to create a new line of natural products with intense chocolate flavour.

The novel technology is based on the thermal generation of flavours (non-enzymatic browning) using a closed-batch reactor that is able to hold six bars of over-pressures, speeding up the aromatic reactions, and the use of enzymes to increase the flavours precursors.

Pilot scale experiments were carried using sugars, cocoa powder and water in various ratios and were submitted to temperatures from 70°C to 130°C during various reaction times (1 to 10 hours) reaching over pressures from 0.2 to 3.5 bars.

The assessment of the evolution of the products obtained was carried out chemically and sensorially. Chemically, using a laboratory-developed headspace solid phase microextration gas chromatography mass spectrometry (HS-SPME-GC-MS) methodology and sensorially with a trained panel of tasters.

Temperatures of 130°C with enzymatic pre-treatment of the cocoa resulted with the highest generation of volatiles scoring the maximum in the aromatically but on the other hand 90°C, without enzymatic pre-treatment, had the best taste evaluation.

Publications

Sylvie Ducki, Javier Miralles-Garcia, Albert Zumbé, Antonio Tornero, David M Storey "Evaluation of Solid Phase Microextraction Coupled to Gas Chromatography - Mass Spectrometry for the Headspace Analysis of Volatile Compounds in Cocoa Products", *Talanta*, Volume 74, *Issue 5*, 15 February 2008, *Pages 1166-1174*.

Sylvie Ducki, Javier Miralles-Garcia, David M. Storey "Evaluation of HS-SPME coupled to GC-MS for the analysis of volatile and semi-volatile compounds in cocoa products" Presented at the Division of Agricultural & Food Chemistry for the 231st ACS National Meeting, Atlanta, GA, 26-30 March **2006**.

Javier Miralles-Garcia, Sylvie Ducki, David M. Storey "Evaluation of HS-SPME coupled to GC-MS for the analysis of volatiles and semi-volatiles compounds in cocoa powders and conched chocolate powders" Presented at the RSC Analytical Research Forum, University of Plymouth, Plymouth, UK, 18-20 July **2005**.

INTRODUCTION

Chapter 1

1 History and industrial manufacture of chocolate

1.1 History of chocolate

The origin of this solid substance, sensual and addictive for a lot of people that we call *chocolate* has its roots in the prehistory of the New World, in the kingdom of Olmecas and Mayas. They lived in the heart of the equatorial Central America and were the first ones to cultivate the tree where the chocolate comes from, *Theobroma cacao*. [1-5]

Three thousand years ago, the Olmecas, one of the eldest Mesoamerican civilisations, lived in the tropical rainforest next to the Mexican gulf. Experts in the Olmecas civilisation have found many references to cocoa in pictures or sculptures (Figure 1.1).

Some centuries after the end of the Olmecas civilisation, the Mayas settled in a large region in the south of Mexico. The wet weather of this region was perfect for the cocoa tree, which flowered easily in the deeper areas of the tropical forest. The Mayas called this tree "*cacahuaqucht*". In fact, for them this tree came from the gods, and the pods that grow on the trunk were a present that the gods gave to men. Many references about the cocoa and cocoa tree have been found in ancient temples. To the Mayas, cocoa was a symbol of vitality and fertility. There are a lot of records where the Mayas have illustrations of gods performing religious ceremonies where cocoa pod appears, and many texts spoke about cocoa as *food of the gods* (Figure 1.1).

The Mayas prepared a bitter concoction made with the beans of the cocoa for the exclusive consumption of the kings and the members of the royalty and for some religious holy rituals. In their books, the Mayas described many ways to make and

aromatise this concoction. Different recipes have been found, from mixing the milled cocoa beans with corn flour to obtain a very thick and viscous preparation to a beverage very well shaken to obtain a good froth. Some spices were used as additives to perfume this beverage, red-hot chilli as the preferred one, and sometimes honey was added to sweeten the shake, to make *Xocolatl*.

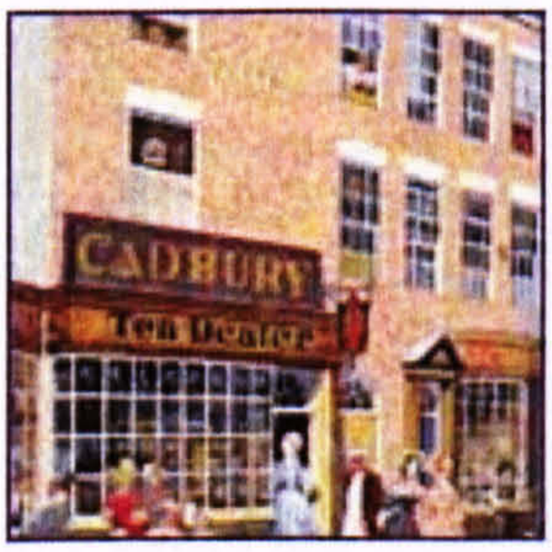
Figure 1.1 Cocoa and the Mayas [5, 6]

After the disappearance of the Maya Empire around 900 A.D., the Toltecas and subsequently the Aztecs of Mexico settled in the old Maya territory and inherited all the knowledge about cocoa. Due to their great value and esteem, the cocoa beans were used as a local currency in the Aztec civilisation.

The history of chocolate is linked to the conquest of America by the Spaniards. The Spaniards were very impressed with the beverage, but they made some changes to the recipe, for example, they added boiling water. This is how cocoa and *Xocolatl* arrived in Spain. It very quickly expanded to the rest of the old Europe. At the beginning, cocoa was very expensive and became a very exclusive food for kings and

very rich nobility. But its fast distribution increased its trade very quickly making it accessible to everybody. It was in Europe where cocoa started to be mixed with milk and sugar and where it acquired its current soft and sweet taste that we know today. The Spaniards could not do without cocoa so they broadened their growing areas. This is how cocoa cultivation was exported to Trinidad and afterwards to the whole tropical region of America, and with time, and trade to other tropical colonies of Spain in Africa and Asia.

According to history, in 1728 the first chocolate factory was opened in England. Other chocolate factories opened in the rest of the old continent, Germany [7], France (1781) to give a few examples. But the Swiss soon became the most renowned because they developed the industrial process to manufacture the cocoa mass in the chocolate that everybody knows today. Among the most famous chocolate masters, we will mention Cadbury, Heinrich Nestlé and Philip Suchard. Their factories are still amongst the most important in the world (Figure 1.2).



(a) Cadbury [8]

(c) Nestlé [9]

(b) Suchard [10]

Figure 1.2. Chocolate Masters.

1.2 Processes in Cocoa and Chocolate Making

The conventional cocoa and chocolate-making procedures are described in books like "Industrial Chocolate Manufacture and Use" edited by S. T. Beckett [11] or "Chocolate, cocoa, and confectionery: Science and technology" edited by B. W. Minifie [12] the contents of which are incorporated by reference.

Figure 1.3 shows a flowchart of the traditional cocoa and chocolate manufacturing steps. In summary cocoa and chocolate manufacturing start in the country where the cocoa tree grows. [1, 11, 13] There, the freshly harvested cocoa seeds are fermented and dried. Then the cocoa seeds are transported to the factory where they are cleaned, winnowed, roasted and ground. The cocoa mass obtained after this, is utilized to produce cocoa powder and cocoa butter after being pressed or goes to the chocolate factory where it is mixed with the other ingredients (sugar, milk powder, flavourings, etc) to produce chocolate after being refined and conched. These steps will be described in more detail in sections 1.2.2, 1.2.3 and 1.2.4.

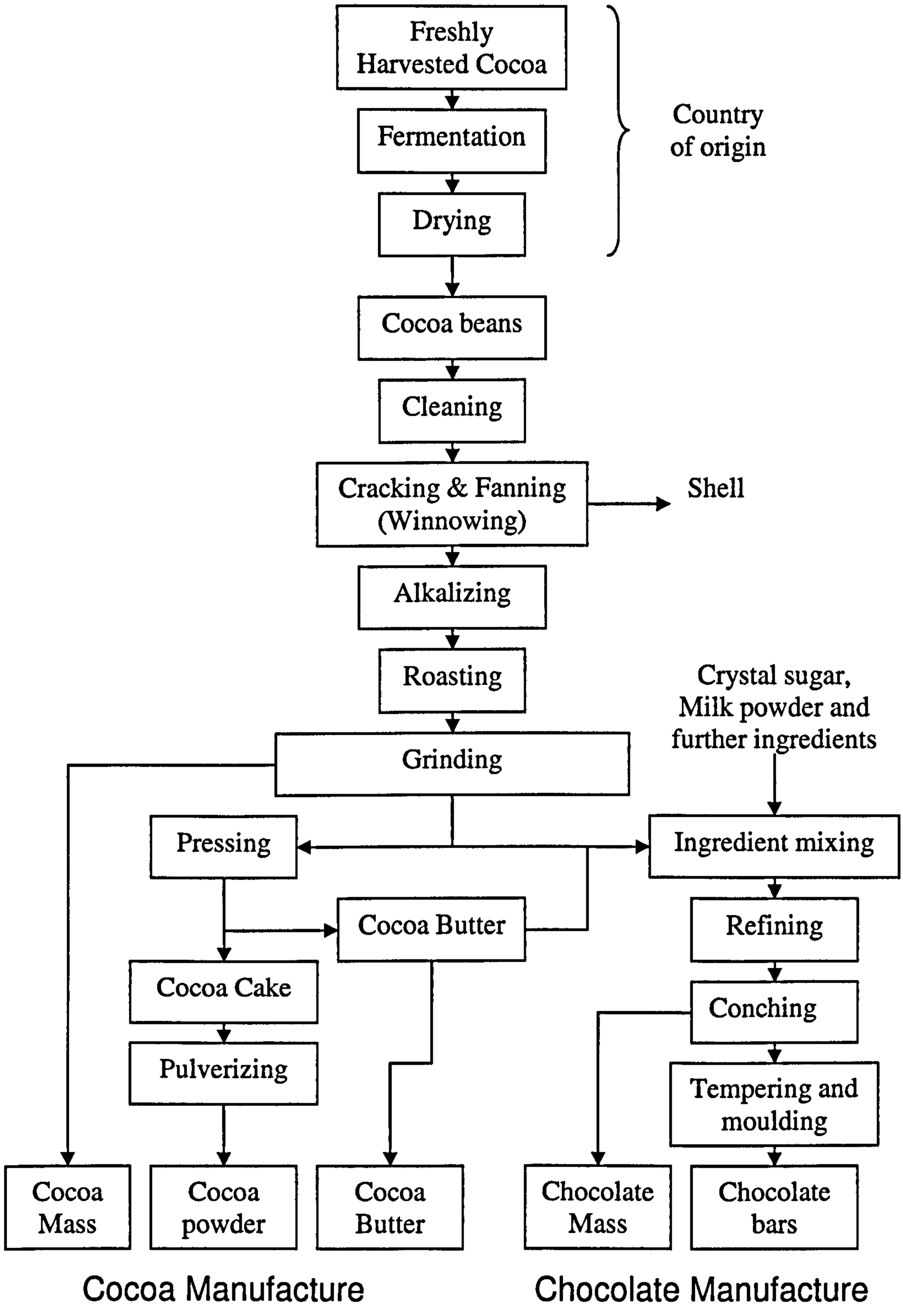


Figure 1.3 Cocoa and Chocolate manufacturing process.

1.2.1 Botany of the cocoa plant

The cocoa tree, *Theobroma cacao,* only grows in equatorial regions; therefore all the producers' countries are from these areas (Figure 1.4). The West Africa is the most important producing area. Other countried include South Americ, India and South-East Asia.

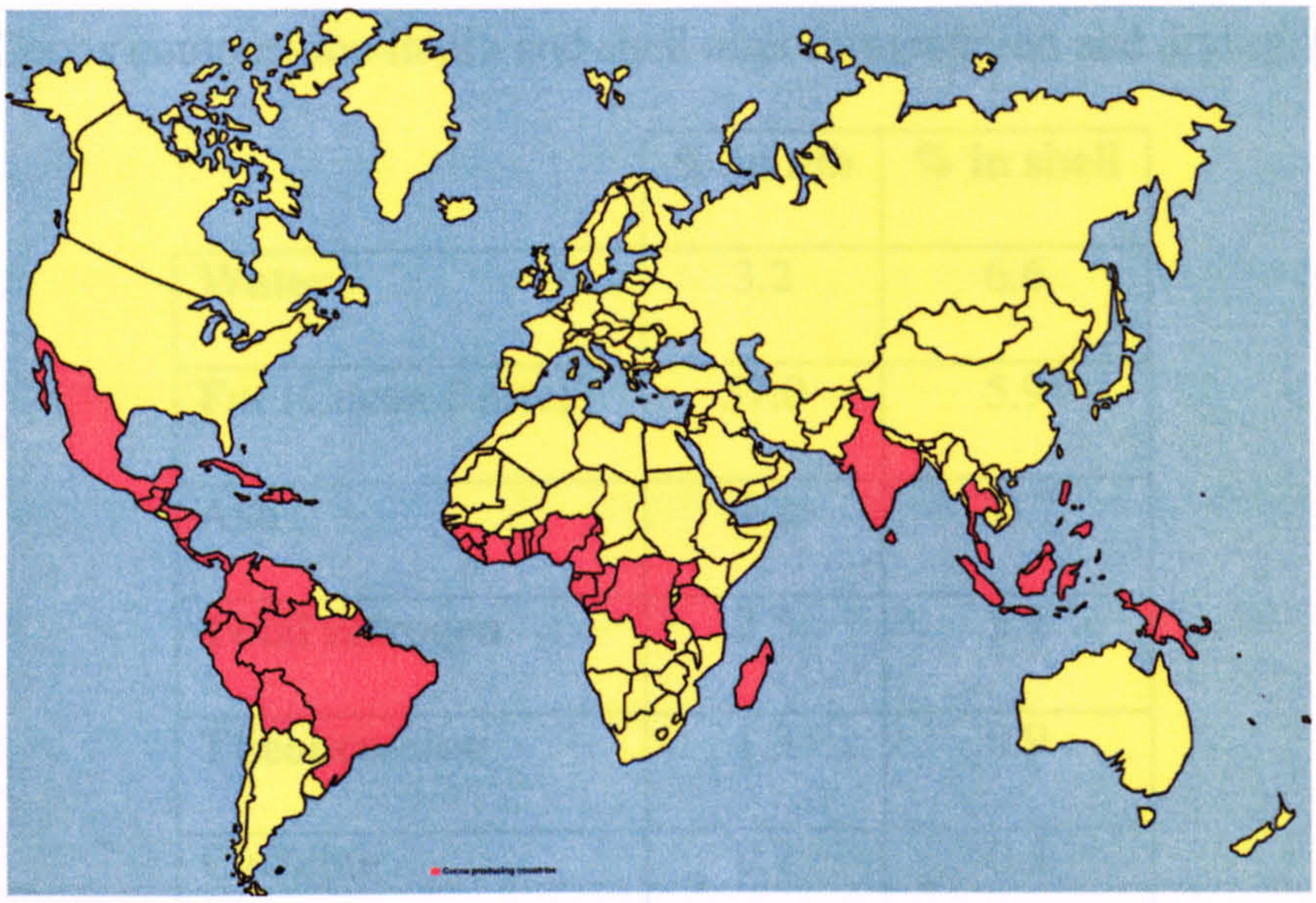


Figure 1.4 World map showing the area growing cocoa in pink [14]

There have been at least three botanical groups of *Theobroma cacao* identified; Criollo, Trinitario and Forastero. Criollo is also called fine or flavour cocoa, it presents a mild chocolate flavour, very appreciated by the finest *chocolatiers*, and it is considered a delicatessen ingredient. Its production represents only 1-2% of the total cocoa production. The Criollo tree is weak, very vulnerable to diseases and in comparison with a Forastero tree, has a low productivity. Forastero or bulk cocoa represents 93–95% of the worldwide production. The Forastero tree is vigorous and has a good resistance against pests and diseases. Trinitario cocoa is a hybrid between the Criollo and Forastero or National, and has intermediate characteristics. It accounts for 3-4 % of the world's cocoa production.

Analysis of the chemical composition of beans after fermentation and drying is presented in Table 1.1. These figures are indicative as the composition often varies depending on the type of bean, the quality of the fermentation and drying and the subsequent processing of the bean.

Table 1.1 Cocoa composition in nib and shell after fermentation and drying. [12]

	% in nib	**% in shell**
Water	3.2	6.6
Fat (Cocoa Butter)	57.0	5.9
Ash	4.2	20.7
Total nitrogen	2.5	3.2
Theobromine	1.3	0.9
Caffeine	0.7	0.3
Starch	9.0	5.2
Fibre	3.2	19.2

1.2.2 On-farm processing of cocoa

1.2.2.1 Fermentation

The cocoa pod is the fruit of the cocoa tree (Figure 1.5). The pod, that contains the cocoa seed, is manually harvested by the local farmers. The pod is opened and emptied. Its sweet pulp and seeds are mixed for fermentation (Figure 1.6.a). Normally the fermentation takes five to eight days for forastero cocoa types, but fine cocoas, such as criollo require much shorter fermentation time (one or two days).

Figure 1.5 Open pod with the seed surrounded by pulp. [15]

There are two traditional techniques for carrying out the fermentation, the heap and box fermentation (Figure 1.6):

- Heap fermentation (Figure 1.6.b) : Fresh beans with a small amount of white pulp are placed in a heap and covered with banana leaves. About 5 or 6 days are needed to achieve proper fermentation.

- Box fermentation (Figure 1.6.c): The beans are tipped from one box to another each day to increase aeration and give more uniform treatment. The fermentation time is similar to the heap method or sometimes a bit longer.

(a) Seeds (b) Heap fermentation (c) Box fermentation

Figure 1.6 Fermentation [15]

Proper fermentation is essential to produce a good flavour in the final chocolate. It is a process that causes the death of the bean, so that it cannot be spoiled by

germination. Certain chemicals are formed; these are known as flavour precursors as they react to afford the flavour in future steps.

1.2.2.2 Drying

After fermentation, it is necessary to decrease the moisture of the beans to 5 – 7.5% to prevent mould formation during the transportation from the growing countries to the factories and during its storage before being manufactured. The drying can be achieved by natural sunlight or using drying machines, dependent on the weather of the growing country (Figure 1.7). A good drying contributes to a good final flavour, good brown colour and low astringency and bitterness. Poor drying contributes to off-flavours formation.

Figure 1.7 Drying. [15]

1.2.3 Cocoa bean processing

1.2.3.1 Cleaning

The first step when the seeds arrive in the factory is the cleaning. Sand, stones, iron, plant materials are often found mixed with the beans. These foreign materials must be removed to preserve the integrity of the processing machinery and the quality of the final product.

1.2.3.2 Winnowing

Winnowing is the process by which the shell (hull) and meat of the bean (nib) are separated. It is a process driven by economics and product integrity and in many countries, government regulations that specify the maximum content of shell in nib. There is no regulation of maximum nib in shell, but if the percentage is high, the manufacturer will lose out on the final product. Other reasons to keep low amount of the shell in the nib are that the shell is very abrasive for the equipment and it could influence negatively the final flavour.

1.2.3.3 Alkalising

The alkalising process is applied to modify the flavour and colour of the cocoa liqueur. This process was developed in the Netherlands in the 19th century and is known as *Dutch processing*. Normally it can be applied to the beans and to the cocoa mass as well but always before roasting. The alkalising agent used is potassium carbonate. The final alkalized product can be in the range pH 6.5 to 9.0 depending on the recipe used. Normally the alkalised cocoa is mainly used to produce cocoa powder.

1.2.3.4 Roasting

This is one of the most important stages in the flavour development. The process involves heating the beans to 110-220°C depending on the bean type and the recipe. The normal times are between 15 – 90 min. There are three roasting methods:

- *Whole bean* roasting is the traditional roasting method and is suitable to produce high quality chocolates. Different roasting grades are available if the beans are not the same size. The beans are easily deshelled after the roasting.

- *Nib* roasting is economically more viable and also more suitable for alkalised cocoa production. The beans must be broken and deshelled prior to roasting

- *Liquor* roasting requires the beans to be deshelled and ground. This releases the fat and the cocoa becomes a paste, this is called liquor. With this method, a very homogeneous roasting is achieved.

The high temperature during the roasting contributes to the reduction of the remaining moisture and to the removal or reduction of the volatile acids. The possible microbiological contaminants are removed and help to release the shell in the case of the whole bean roasting. Overall, the roasting develops the specific flavour of the cocoa produced by the **Maillard reactions**.

1.2.3.5 Grinding

There are two main reasons for grinding the cocoa. Firstly, it is necessary to reach a small particle size to make the cocoa suitable for chocolate production as this is important for organoleptic properties. Secondly, the cocoa nib contains about 55% fat, and to release the cocoa butter that is inside of the cell, the cellular structure must be broken. In a regular grinding the maximum particle size needs to be less than 30 μm. This roasted ground cocoa or cocoa mass (cocoa liquor in USA) is the first manufactured product obtained in the industrial cocoa process.

1.2.3.6 Pressing

After the grinding of the cocoa, the cocoa butter is released and the product looks like a viscous black and highly aromatic fluid. In the pressing step, the cocoa butter is separated from the cocoa solid particles. The hot cocoa mass is introduced into a hydraulic press where the cocoa butter passes through a metallic mesh and the solid

particles are retained. After this pressing, a hard round disc of material known as cocoa press cake is pulverized to make the popular cocoa powder. Both products, **cocoa butter** and **cocoa powder** are final products.

Cocoa butter has many applications and not only in the food industry. The melting temperature of cocoa butter is around 37°C, the physiological temperature, this makes it very interesting for the cosmetics industry for example.

Cocoa powder is used in many confectionery products such as the popular cocoa milkshakes, the chocolate breakfast powder or cookies.

1.2.4 Chocolate factory processing

1.2.4.1 Batching

Although continuous production lines of chocolate exist (normally for milk chocolate that needs soft conch), the chocolate factories traditionally use a batch system. Batching is the combination of all initial ingredients; cocoa mass, sweeteners, milk powders (if applicable), cocoa butter and non-volatile flavouring material as specified by the recipe. The main objective of this step is to obtain a homogeneous mixture.

1.2.4.2 Particle size reduction

The particle size reduction in chocolate production is a key step. The ingredients need to be ground into very fine particles to avoid a gritty taste when the chocolate melts in the mouth. To reach this objective a separate grinding or a combined milling can be used.

In the separate grinder the ingredients added to the cocoa mass (sweeteners, milk powder) are milled together until the right particle size is achieved. Afterwards, these ingredients are mixed with the cocoa mass that comes with the right finesse from the cocoa factory.

In the combined milling all the ingredients are mixed and then milled using roll refiners. With this refining the ingredients obtain an effective size particle reduction as well as a micro mixed this helps to reduce the times in the conching process.

1.2.4.3 Conching

The main objective of conching is the physico-chemical improvement of chocolate to promote its sensorial properties and texture. The conching process consists in an intense kneading to mix the ingredients while controlling the temperature. The temperature in the conching can vary between 70 to 90ºC for dark chocolates and between 55 to 70ºC for milk chocolates. The kneader especially designed for that purpose and is only used in the chocolate industry, is called **conche** (Figure 1.8). This name comes from the form of the firsts kneader that ever composed by a granite rolls that were moved by steam engines over conch form granite beds .

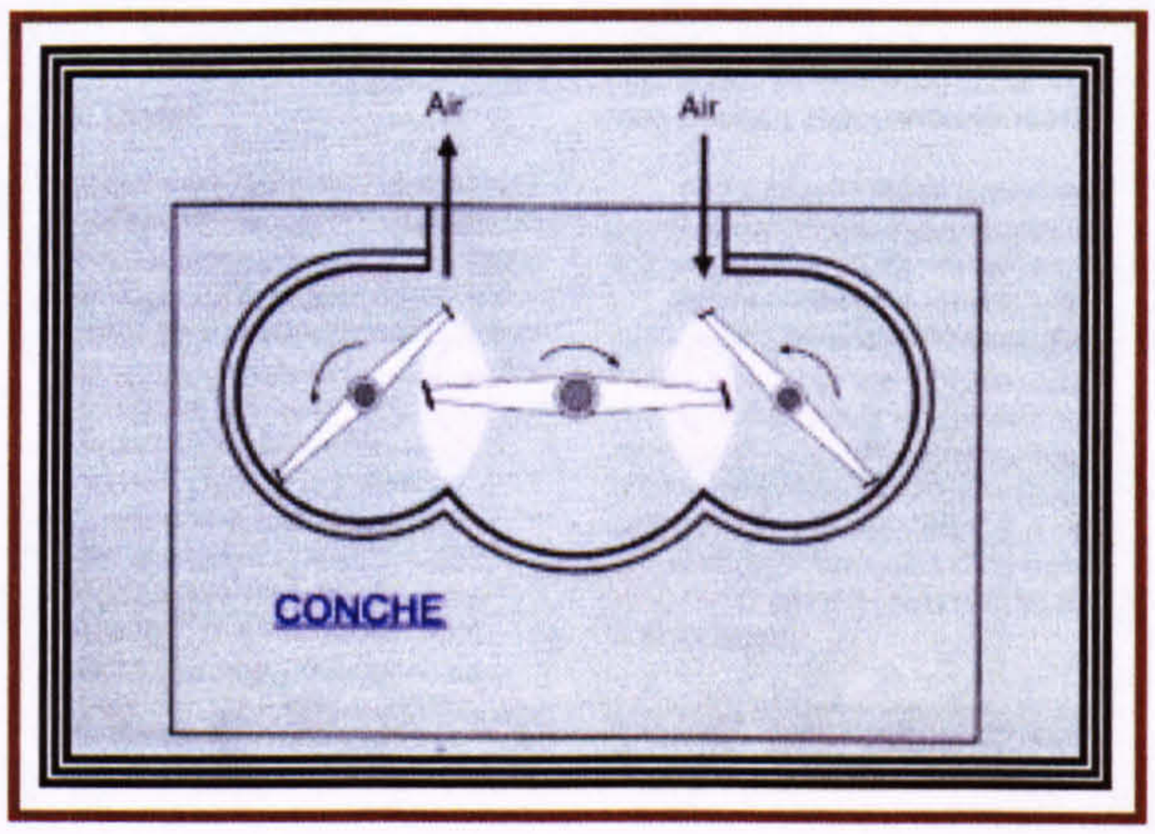


Figure 1.8 Conche [16]

During conching, the flavour is improved by elimination of off-flavours (volatiles) and generation of pleasant ones. During the grinding step, more surface areas are generated (mainly from sugar) which are not coated with fat. These give the

chocolate mixture poor flow properties in its liquid state. During the conching, these surfaces get coated with fat, improving the chocolate's flow properties.

There are three phases in the conching process (Figure 1.9). At the beginning there is a *dry-paste phase* where shearing forces are very strong and the mixture suffers moisture evaporation plus removal of other volatiles. The *pasty or plastic phase* is where the flavour development occur by means of shearing and heating, moisture removal, homogenizing. The last step of the process is the *liquid phase* where some emulsifiers are added to liquefy the mixture. The speed of the paddles is increased to obtain an intense homogenisation. The product of conching is called **chocolate mass** and can be commercialised under this form.

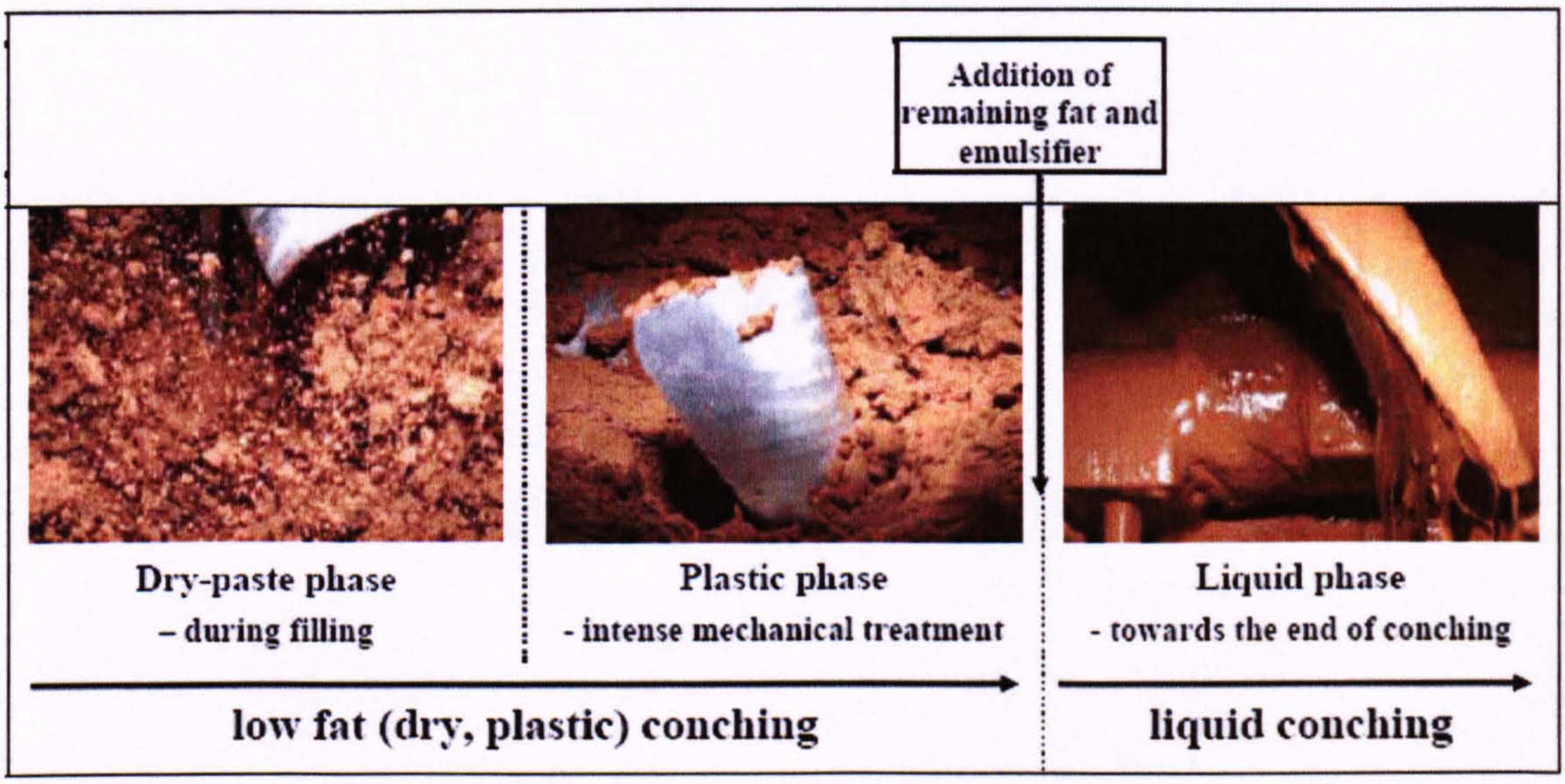


Figure 1.9 Conching [17]

1.2.4.4 Tempering

The tempering is the last step in the chocolate process and is necessary to obtain the well-liked chocolate bars. It is all about the visual aspect of the final product. Commercially it is very important to attract the customer, the chocolate needs to be

shiny and attractive. To obtain a nice and shiny chocolate, the cooling process from liquid chocolate to solid chocolate needs to be controlled carefully. The fat is a critical factor as it has at least five forms of crystallisation, some of them more stable than others. It is necessary to control its solidification to obtain adequate fat crystals. The chocolate mass is cooled down in moulds to obtain chocolate bars or other commercial forms.

Chapter 2

2 Chocolate flavour

The flavour of chocolate is the most popular flavour in the world. The worldwide production of chocolate is some millions of metric tons, and is progressively increasing. Chocolate is usually directly consumed but it is possible to find it manufactured in beverages, yogurts, spreads or bakery products, to give a few examples. The variability of the flavour is widespread. It is only necessary to think about the different flavours of the different important trademarks, Cadbury, Nestlé, Lindt, Hershey. All these chocolate companies have different standards for, at first sight, very similar chocolates. However, after a simple tasting and without being expert, clear differences can be found. The variability of chocolate flavour perhaps is what makes chocolate so universally popular.

The flavour of chocolate consists of a mixture of 500 to 600 compounds [11, 18-29] and new research constantly increases this number. [24, 30] These aromatic compounds belong to several kinds of chemical classes such as hydrocarbons, ethers, ketones, alcohols, aldehydes, acids, furans, lactones esters, amines, pyrroles, phenols, sulphur or pyrazines (Figure 2.1).

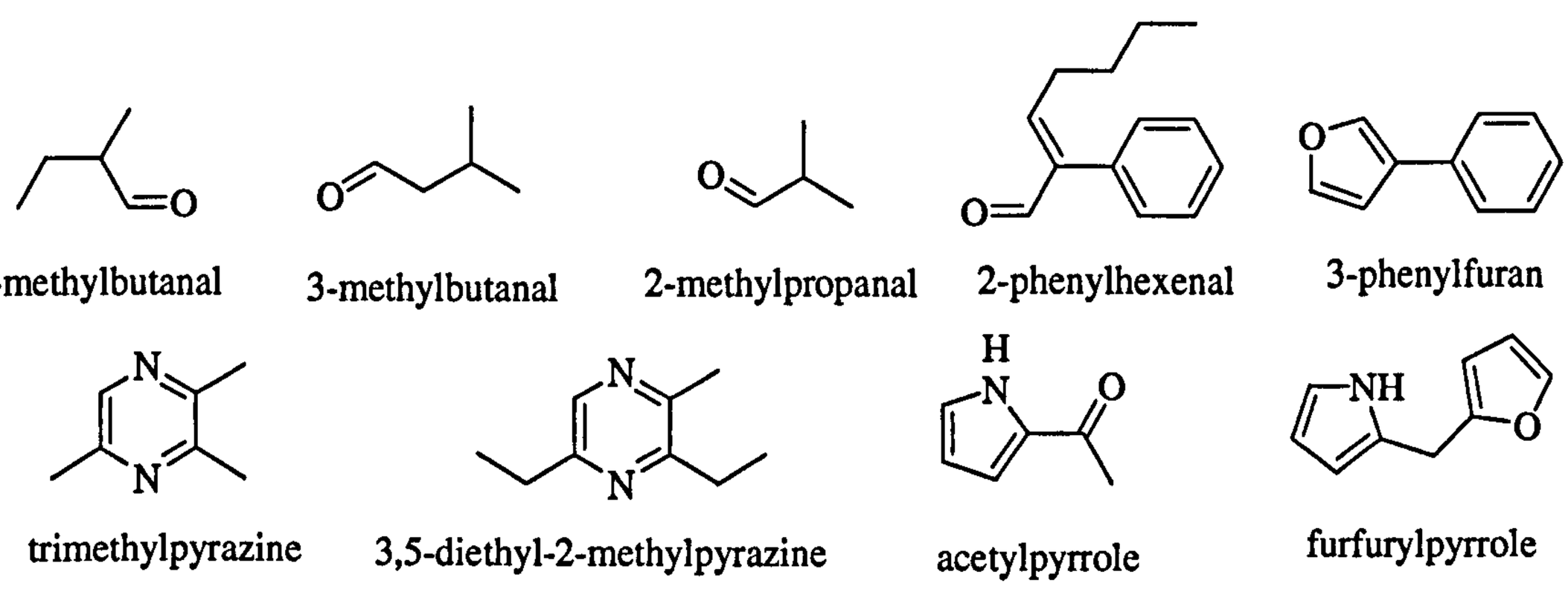


Figure 2.1 Some important cocoa and chocolate flavour molecules [11, 18-29]

Recent works [31, 32] have reported the quantitative determinations of odour-active compounds in natural roasted cocoa powder finding acetic acid as the largest odour-active compound in cocoa powder. Other main compounds found were 2- and 3-methylbutanal, 2,3,5,6-tetramethylpyrazine, benzaldehyde, 2-phenylacetaldehyde, acetophenone, 3-methylbutyric acid, 5-methyl-2-phenyl-2-hexenal, ethyl phenylacetate, and 3-hydroxy-2-methyl-4-pyrone (found in quantities greater than 1 mg kg^{-1}).

The chemical complexity of the chocolate flavour is evident taking into account the parameters that can affect its development. This is clearly obvious when considering that, even today, this popular flavour has not been duplicated by the flavour chemist.

Frauendorfer and Schieberle [32] identified the 24 highest odour-active compounds and added them to a deodorised cocoa powder. The result was compared to a regular cocoa powder and no relevant difference was found.

New investigations using avant-garde biotechnology will help to clarify this area.[33-37]

The final composition of the flavour of a chocolate depends on various factors:

- Origin of cocoa bean. [38, 39]
- Cocoa bean variety; forastero, criollo and trinitario or nacional (genotype). [40]
- Differences in the processes (fermentation, drying or roasting). [40-42]

The chocolate flavour is usually developed in three important steps: fermentation, roasting and conching (described briefly in section 1.2). During fermentation the primary amines and amino acids increase due to a proteolysis creating smaller peptides and free amino acids. Their quantities come back to the initial after

roasting with an important sugar reduction that react together developing the typical cocoa flavour.[43] We will now explore the chemical aspects of these steps.

2.1 Fermentation

There are many microbial and chemical changes during fermentation. Natural yeasts and bacteria multiply in the pulp, causing the breakdown of the sugars and mucilage. Much of the pulp then drains away as a liquid as a consequence of the micro-organism activity which is responsible for the death of the bean and the creation of the cocoa flavour precursors. The pulp is an excellent medium for the micro-biological proliferation; it contains 82 – 87 % of water, 10 – 15 % of sugar, 2 – 3 % of pentosans, 1 – 3% of citric acid, and 1 – 1.5 % pectin. Proteins, amino acids, vitamins and minerals are also present. Before opening, the pod is microbiologically sterile. When the pod is opened and the pulp comes into contact with the outside environment, it becomes contaminated with a variety of micro-organisms that may contribute to the subsequent fermentation. These microorganisms come from unwashed knives, hands of the workers, baskets, and dried mucilage left on the walls of boxes from previous fermentations.

2.1.1 Anaerobic yeasts

In the first 24-36 h, anaerobic yeasts convert sugar of the pulp into alcohol under conditions of low oxygen and pH below four (i.e. quite acidic). Bean death usually occurs on the second day and is caused by acetic acid and alcohol (the rise in temperature is relatively unimportant). There are many different types of yeast responsible for this step. Studies in several countries demonstrate that the yeasts involved can vary with geographical or fermentation practices. [44]

2.1.2 Lactic acid bacteria

Lactic acid bacteria are present from the start of the fermentation, but only become dominant after the pulp and the "sweating" have largely drained away, between 48 and 96 hours. Lactic acid bacteria convert sugars and some organic acids into lactic acid.

2.1.3 Acetic acid bacteria

Acetic acid bacteria are also present throughout the fermentation, but become more significant towards the end when aeration increases. They are responsible for converting alcohols to acetic acid and further oxidation to carbon dioxide and water. This is a strongly exothermic reaction that is mainly responsible for the rise in temperature (50 °C or higher in some cases), which causes diffusion and hydrolysis of proteins in the cotyledons. Thus, the acetic acid bacterium plays a key role in the formation of the precursors of chocolate flavour. [7]

It is not easy to establish an endpoint for each stage as they overlap. The different environments (regions) and fermentation techniques can vary the duration of the stages.

The major role of the micro-organisms is to produce acids and alcohols that will penetrate the shell and start the chemical reactions that will form the precursors of chocolate flavour. There is no evidence that enzymes from the micro-organisms penetrate the shell and create flavour compounds but hydrolytic enzymes inside the beans are activated by microbial metabolites such as acetic acid. [44, 45]

There are several compound groups responsible for the flavours that are affected directly by the fermentation process:

- *Methylxantines* (mainly theobromine and caffeine) are responsible for the bitter taste. Their concentrations decrease during the fermentation by about 30% probably by diffusion from the cotyledons.
- *Polyphenols,* responsible for the astringent taste, their levels drop down through the fermentation. Anthocyanins are hydrolyzed to cyanidins by glycosidase enzymes. Polyphenols ((-)epicatechin mainly) are converted to quinines by oxidase enzymes. Proteins react as well with polyphenols to produce complex compounds responsible for the typical brown of the fermented and dry cocoa seeds.
- *Volatiles production.* Yeasts produce a large quantity of aroma compounds, principally fused alcohols, fatty acids, and fatty acid esters. [46]
- Another important group is the *Maillard reaction precursors.* Invertase enzymes convert sucrose to fructose and glucose (reducing sugars). The storage proteins are hydrolysed by peptidase enzymes into oligopeptides and free amino acids. These precursors will be involved in the flavour reactions during roasting and conching

Many studies have been carried out to identify the species of yeast and bacteria responsible for natural cocoa fermentation. Four studies from different countries [44] (Brazil, Ghana, Malaysia and Belize) demonstrated that there are different micro-organisms involved depending on the geographical area. Schwan [47] reported a study where the biomass (beans and pulp) were kept uncontaminated and the fermentation was carried out using a cocktail of yeasts and bacterial. The manufactured chocolate from these cocoa beans was compared with a regular one by trained tasters but no significant differences in taste were found. Such controlled fermentation is a step

forward to ensure the quality of the final product since natural fermentations are subject to random fluctuations in the inoculums and the fermentation does not always proceed correctly. New research can improve these cocktails to enhance the flavour development. The quality of the fermented beans has not been a priority for the farmers because there is not a financial incentive to produce high quality beans. It is clear that the research done by major manufacturers on roasting and processing has enabled them to produce good quality products from inferior sources.

Looking at other traditional naturally fermented products such as wine, beers and cider, their quality have been improved over the last decades due to better control of the fermentation. The cocoa industry is in the first stages of this evolution and a lot more research and investment is needed in the countries where the plants are grown.

2.2 Roasting

Roasting is probably the most important step in the flavour and aroma development. Physically, due to the heating, many volatiles are lost. Acetic acid is one of the most important compounds lost during this process causing significant decrease in acidity. Chemically the roasting stage is where the precursors generated during the previous steps are converted into flavour molecules. The heat during roasting induces **non-enzymatic browning** reactions. These complex reactions usually start between amino compounds (amino acid, peptides or proteins) and reducing sugars, and have a key role in the flavour development. Figure 2.2 shows a general flow diagram for the Maillard reaction. There are three main phases in the non-enzymatic browning:

- Phase 1: The initial reaction is the condensation of an amino compound with a simple sugar, which loses a molecule of water to form *N*-substituted aldosylamine (Figure 2.2, Step A). This is unstable and undergoes the famous "Amadori

rearrangement" to form 1-amino-1-deoxy-2-ketoses, known as ketosamines, which can undergo complex subsequent dehydration, fission and polymerization reactions (Figure 2.2, Step B).

- Phase 2: The ketosamine products of the Amadori rearrangement can then react in three ways:
  - Further dehydration (loss of two water molecules) into reductones and dehydro-reductones. These are essentially caramel products and in their reduced state are powerful antioxidants (Figure 2.2, Step C)
  - Production of short chain hydrolytic fission products such as diacetyl, acetol, pyruvaldehyde etc. These then undergo the famous "Strecker degradation" with amino acids to aldehydes and by condensation to aldols (Figure 2.2, Steps D, E & F).
  - Schiff's base/furfural path. This involves the loss of three water molecules, then a reaction with amino acids and water. These also undergo aldol condensation and polymerise further into true melanoids (Figure 2.2, Steps C & G).
- Phase 3: All these products react further with amino acids to form the brown pigments and flavour active compounds collectively called "Melanoids". These can be off-flavours (bitter, burnt), off-aromas (burnt, onion, solvent, rancid, sweaty, cabbage) or positive flavours (malty, bread crust-like, caramel, coffee, roasted) and positive aromas (bready, cracker, fine malt) (Figure 2.2, Step G).

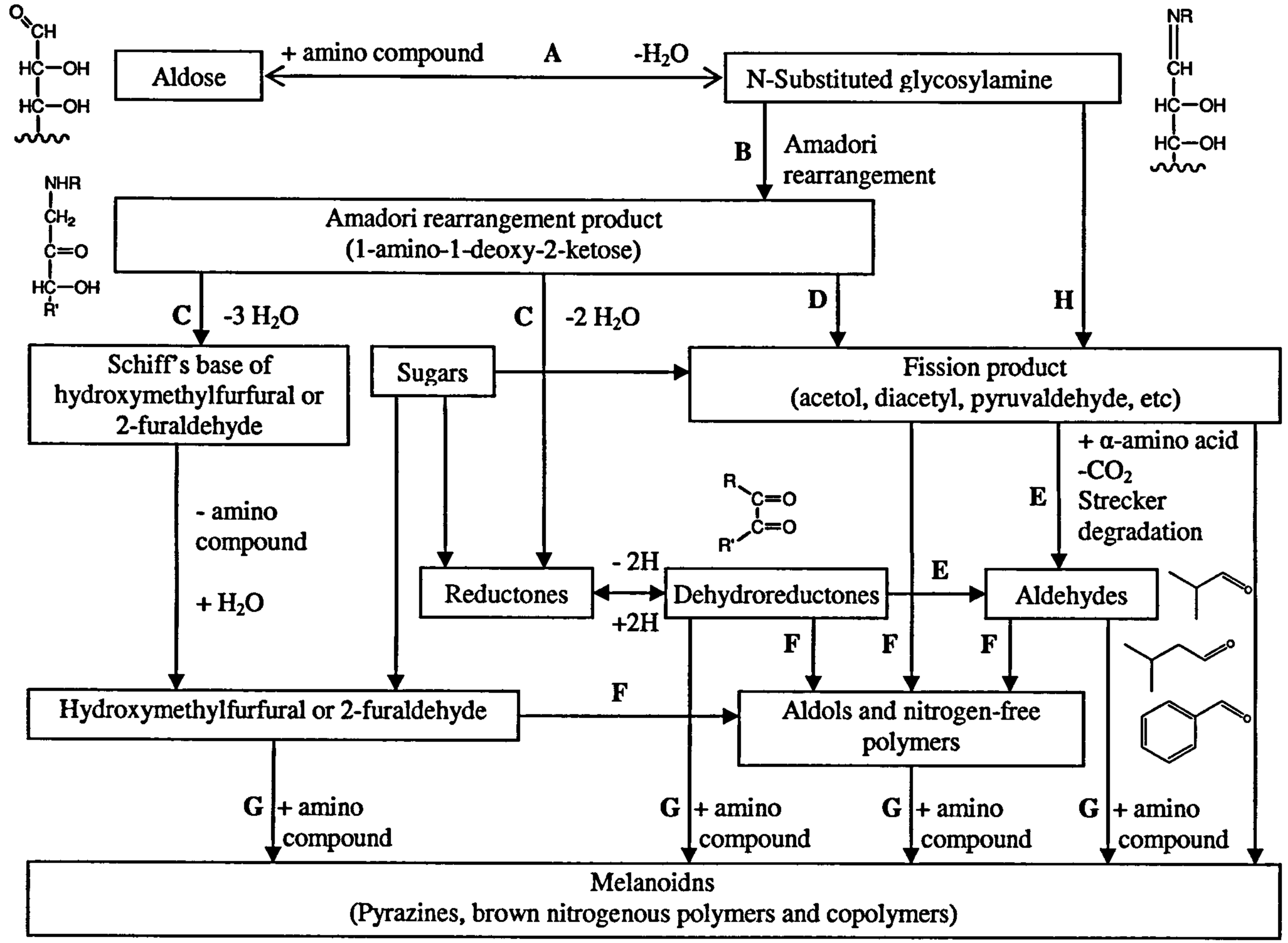


Figure 2.2 Outline of Maillard reaction. Adapted from [48].

The Strecker degradation needs special attention as it is one of the main pathways to produce two of the more important families of compounds responsible for good chocolate flavour, aldehydes and pyrazines.

The amino acids (e.g. Valine) react with dioxocompounds (e.g. Pyruvaldehyde) that come from sugars or dehydration reactions. The resulting nitrile compound is decarboxylated and deaminated, producing two aldehydes. The pyrazines are formed by intermolecular cyclisation and oxidation of two amino carbonyl compounds formed in the previous step (Figure 2.3).

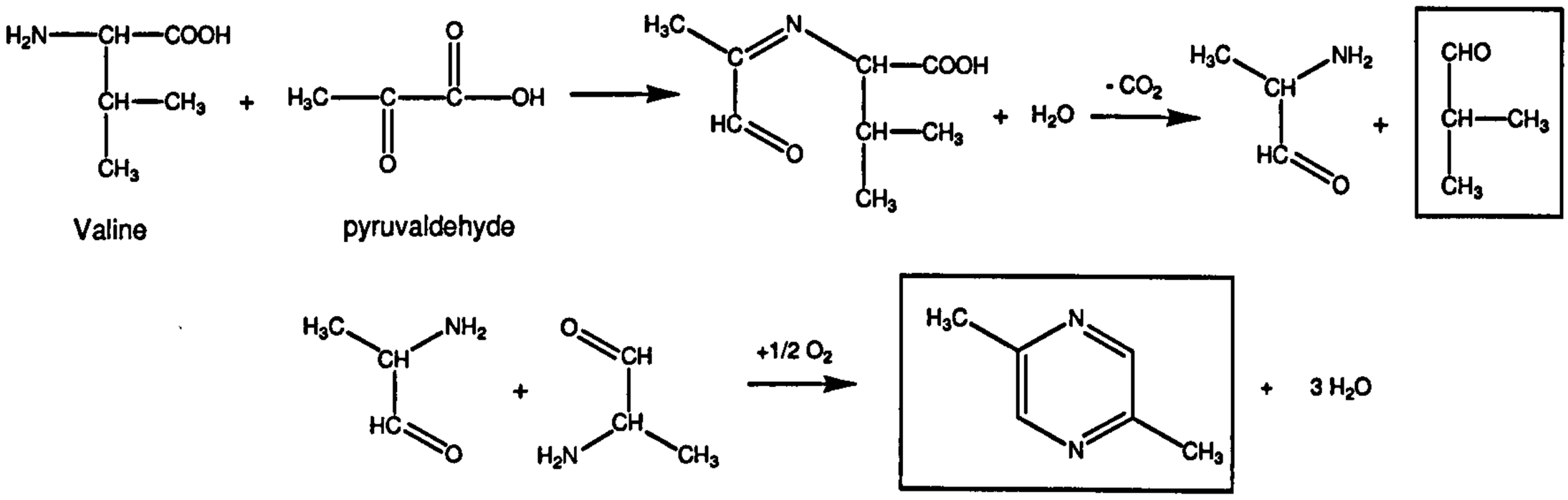


Figure 2.3 Major pathway for alkyl pyrazine formation via Strecker degradation.

It is necessary to mention that pyrazines are very characteristic of the Maillard reactions. They are formed in cocoa during the roasting and are of great importance in the chocolate flavour profile. They confer desirable aromas at very low thresholds. Most of them have roasted, chocolate, cocoa or hazelnut notes. Thus, the pyrazine levels have been considered like a roasting indicator, in many scientific studies.[41, 42, 49, 50]

A number of research studies can be found in the literature about the chemical changes during cocoa roasting and how the temperature and time affect these changes.

Jinap and collaborators [50] reported how the roasting time and temperature affect the volatile component profile. They found that increasing the temperature and time favours the formation of pyrazines. Esters increased with the roasting time. At short roasting times, the carbonyls linearly decreased with increasing roasting temperature. Phenols presented a big reduction at the highest roasting temperature (160-170 °C) with longest roasting time (45-65 min).

Bonvehí and Ventura [41] noted that the amount of pyrazines during roasting also increases with the temperature. Samples roasted under alkaline conditions (pH 7.20 – 7.92) exhibited darker brown colour formation, but the alkylpyrazines were adversely affected. These effects were explained by the alkalisation of the nibs favouring the oxidative deamination of free amino acids by some polyphenols to produce brown products (polymerization).

These results were corroborated by Misnawi and collaborators [51, 52]. They reported that the amount of polyphenols affects the pyrazine formation during the roasting. They claimed that polyphenols react with Maillard reaction precursors (alpha-amino compounds and sugars); the phenolic hydroxyl group is an excellent hydrogen bond donor and forms strong hydrogen bonds with the amide carbonyls of the peptide backbone. Moreover, polyphenols produce quinines which are very reactive agents through enzymatic oxidation. They may react with amino acids and proteins, or polymerize with them to form large molecules, the so-called "condensed tannins". In this research work an extra amount of polyphenols extracted from cocoa were added prior to roasting. The presence of this additional quantity of polyphenols during roasting decreased cocoa flavour formation and increased the astringency and bitterness.

De Brito and collaborators have presented three interesting papers where the main objective was to enhance aroma and flavour quality of the roasted cocoa. [49] In

2001 they reported a study where the cocoa nibs were autoclaved before roasting. This pre-treatment had a significant influence on the level of compounds which contribute to the cocoa flavour. The autoclaved sample presented a considerable decrease in reducing sugars and α-nitrogen compounds suggesting that this treatment favoured the Maillard reaction; pyrazine levels were increased as well. Its sensory evaluation showed that the chocolate made with this cocoa was significantly different from the regular one. This modification in the flavour was attributed to the increase of the water activity in the autoclaved samples. It has been previously reported that a moist-thermal treatment of the cocoa could increase the level of Amadori-compounds and the roasted aroma intensified. [53] In a second paper [54], prior to roasting, the cocoa mass was enriched with Maillard reaction precursors: glucose and glycine. These samples did not show higher levels of pyrazine, and no increase in the aroma formation was observed. In 2004 de Brito and collaborators [55] reported a new study where the fermented cocoa was treated with a protease to increase the protein hydrolysis prior to roasting. The results showed an increased perception of chocolate flavour and bitter taste in the product formulated with the protease treated cocoa.

As important as the carbonyl-amine reactions are, it is unlikely that a food system will consist of only amino acids and sugars. Other compounds such as peptides, proteins, vitamins, fats and their oxidation products and other derivatives can enter into the reaction and influence the final product. With the many compounds found in chocolate, it is virtually impossible to identify all the reactions and pathways involved in the chocolate flavour production.

2.3 Conching

Conching has been described for many years as the working of chocolate flake and crumb into a fluid liquid. This was the main aim of the conching before the invention of the roller refiners where conching reduced the particle size of the sugar and the cocoa liquor after pounding for days in a conch. Nowadays, this duty is perfectly carried out in a previous step (refining).

The objective of the chocolate conching at present time is different; conching improves the texture and the final flavour of chocolate. The modification of the flavour is important and conched chocolates are described as mellow compared to non-conched. Bitterness and astringency is reduced, allowing other flavour notes to be more pronounced. Since the conches are open during the heating and the intense kneading, some volatiles are lost; short-chain fatty acids, such as acetic acid present since fermentation as well as short chain aldehydes and short chain ketones. The humidity is reduced; regularly during conching the water decreases from 2% to around 0.5%.

The literature provides little and conflicting information on conching chemistry. According to Mohr [56], during conching atmospheric oxygen leads to more refined chocolate flavours. Investigations into cocoa bean roasting revealed that the Strecker degradation was not complete at the end of the roasting process and suggested that it continued during conching [57]. However according *to* Dimick and Hoskin [58], the concentration of amino acids and reducing sugars exhibited no change as a result of conching. On the other hand, Ziegleder and Sandmeier [59] found a 15% reduction of free amino acid. Also they reported the production of isovaleraldehyde (Strecker degradation product) during the conching of semi-sweet chocolate masses.

In a recent study Counet [24] described that most of the key odorant compounds in dark chocolate are already in the roasted cocoa but during conching their levels vary.

Short Strecker aldehydes were partially lost due to evaporation or/and chemical reactions. They reported a significant increase in alkyl pyrazines, furans and acetylpyrroles. These last findings may tilt the balance to the side of the people that believe that some Maillard reactions still occur during the conching.

The amount of the polyphenols is reduced, may be by combination with proteins in the conching step according to Hagerman [60]. This would give the chocolate a less astringent and mellower flavour.

Chapter 3

3 Chemical analysis of volatiles

In the context of this work, volatiles are any organic chemicals that produce vapours readily at room temperature and normal atmospheric pressure. The characterisation of volatile compounds responsible for the aroma and flavour of natural products or foods is very important to several branches of industry (quality of the final product) and science. [61, 62] The development of analytical methods for the determination of chemical composition of the aroma is not an easy task. Usually, the concentration of these compounds is very low. The minimum concentration for odour perception of some molecules can be as low as 0.1 ng/L. This requires very sensitive analytical techniques. The matrix may be very complex and can obstruct the isolation. The aroma profile of some natural products involve a large amount of odour-active molecules; for cocoa more than 600 volatiles have been identified, and around 100 have been found odour active.

3.1 Sample preparation

Most of the analytical equipment cannot handle the sample matrices directly. Therefore, the samples need preparations to make them suitable for analysis. Depending on the objective of the analysis (volatiles, proteins, metals, etc) the same matrix can be treated in different ways. The preparation will depend on the desired result. This preparation can involve extraction, concentration, separation, fractionation, etc. It is not an exaggeration to say that it is crucial to choose an appropriate sampling method to have a reliable and accurate analysis.

In volatiles analysis, the sample preparation will determine the analytes to be analysed. The different sample preparation methods generate extracts of different compositions. In the aroma analysis, the profile of the extracted sample should be as close as possible to the original. Therefore, manipulation of the sample must be as mild as possible, elevated temperatures and other strong conditions may destroy some volatiles and/or generate artefacts that must be avoided.

3.1.1 Solvent Extraction

This technique is probably the most widely used in chemistry to isolate organic molecules. The concept is to transfer the aroma molecules from the matrix, usually aqueous, into an organic solvent. The extraction can be done manually, automatically, in batches or in continuous flow. The selection of the extraction solvent needs to fulfil certain criteria. It must have a low boiling point so that it can be easily removed from the extract without significant loss of the extracted volatiles. It must be able to recover both polar and non-polar molecules to obtain a representative aroma profile of samples.

Concentration of the extract is usually needed. After the extraction, the concentration of the volatiles for analysis is very low. The concentration may be accomplished by techniques such as Vigreux column distillation, rotary evaporation or by use of a Kuderna-Danish concentrator.

Advantages	*Disadvantages*
- Good recovery of aroma compounds - Quantitative recovery. - Relatively easy to perform.	- The solvent removal may cause loss, degradation or modification of the extracted volatiles. - The organic solvents used may be flammables and/or toxics can cause safety and environmental problems. - A large sample is required to produce enough extract for analysis. - Emulsification may cause problem in the separation of the phases. This problem can be partially solved by increasing the ionic strength of the aqueous phase and using centrifugation. - The solvent peak in the chromatogram may mask analytes peaks.

3.1.1.1 Soxhlet Extraction

The Soxhlet extraction is a continuous liquid-solid extraction technique (Figure 3.1). The Soxhlet extractor was originally designed for the extraction of lipids from a solid test material, but can be used whenever it is difficult to extract any compound from a solid, semi-solid or viscous sample.

Typically, the sample is placed inside a porous thimble (Figure 3.1). The solvent is heated, causing it to evaporate. The hot solvent vapour travels up to the condenser, where it cools and drips down onto the sample. The chamber containing the sample slowly fills up with warm solvent until, when it is almost full, it is emptied by siphon action, back down to the flask. This cycle is allowed to repeat as often as required.

This extraction method is used when the solubility of the analytes in the solvent is very low. Only clean and warm solvents are used in each extraction cycle.

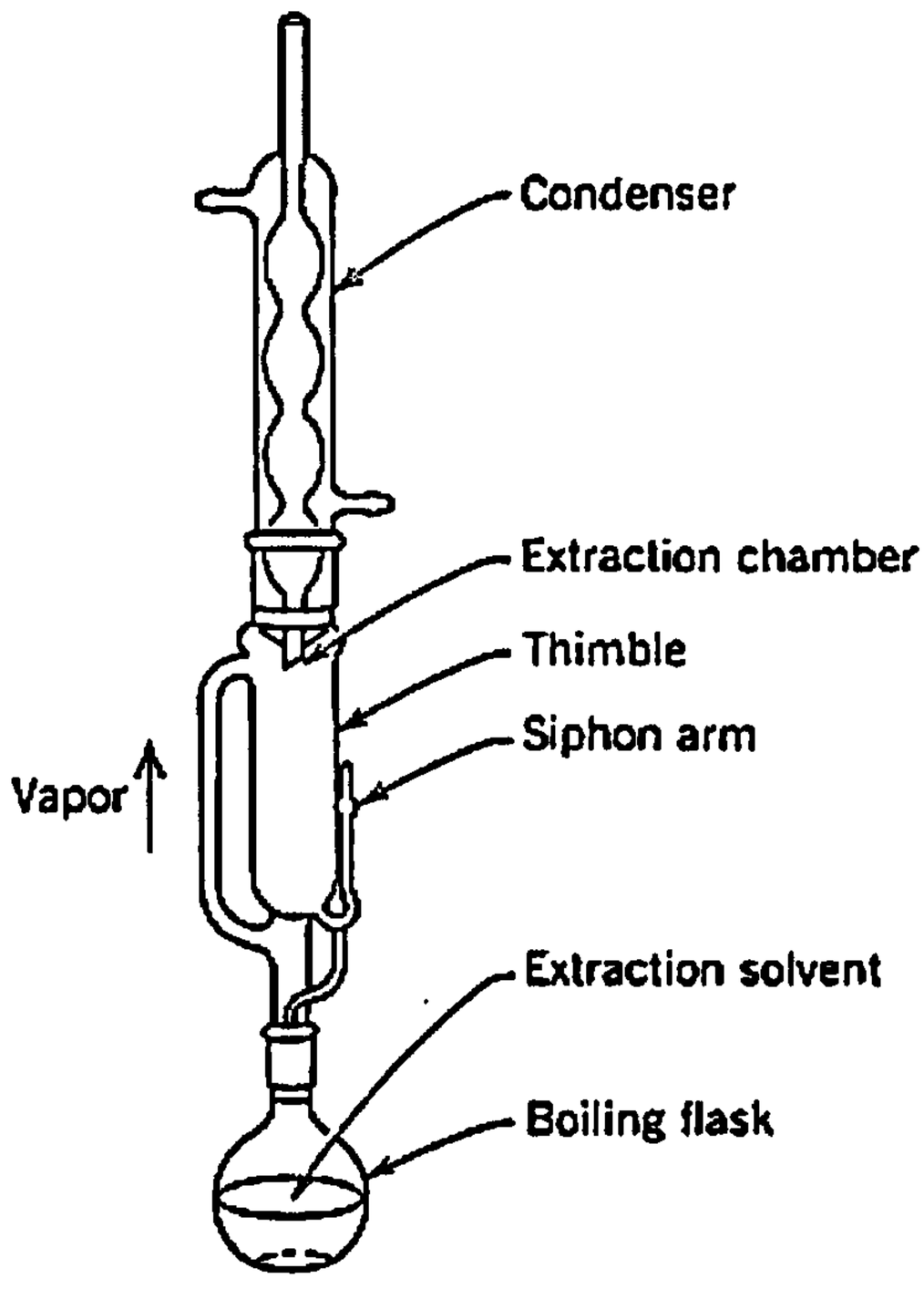


Figure 3.1 Soxhlet Extraction [63]

3.1.1.2 Supercritical Fluid Extraction (SFE)

Supercritical fluids can be used to extract analytes from samples. The properties of supercritical fluids also provide some advantages for analytical extractions. Supercritical fluids can have solvating powers similar to organic solvents, but with higher diffusion, lower viscosity, and lower surface tension. The solvating power can be

adjusted by changing the pressure or temperature, or adding modifiers to the supercritical fluid. For example, methanol is used as modifier (typically 1-10%) to increase the polarity of supercritical CO_2. Controlling these parameters can allow the extraction of the desired analytes.

SFE is used in the food [64] and flavouring [65] industries because the residual solvent is easily removed from the product. Since, supercritical CO_2 (the most widely used) has a critical point of 31.1°C and 73.8 Bar, extractions can be carried out under milder temperatures conditions and without needing further aggressive procedures. The biggest application is the decaffeination of tea and coffee. Other important areas are the extraction of essential oils and aroma materials from spices. The brewery industry uses SFE for the extraction of hops. This method is also used in extracting some edible oils and producing cholesterol-free egg powder.

On the other hand, the initial capital investment in equipment is high, moreover SFE as is a high-pressure process and needs strong safety precautions like any high-pressure facility.

3.1.2 Steam Distillation

Many complex organic compounds tend to decompose at sustained high temperatures. Separation by normal distillation would then not be an option, so water or steam is introduced into the distillation apparatus. By adding water or steam, the boiling point of the compounds is decreased, allowing them to evaporate at lower temperatures, preferably below the temperatures at which the deterioration of the material becomes appreciable (Figure 3.2). If the substances to be distilled are very sensitive to heat, steam distillation can also be combined with vacuum distillation. This may lead to some volatile losses due to the vacuum. After distillation the vapours are condensed, and in

the best of the cases yielding a two-phase system of water and the organic compounds, allowing for simple separation. If this does not happen, a further liquid-liquid extraction with an organic solvent is required.

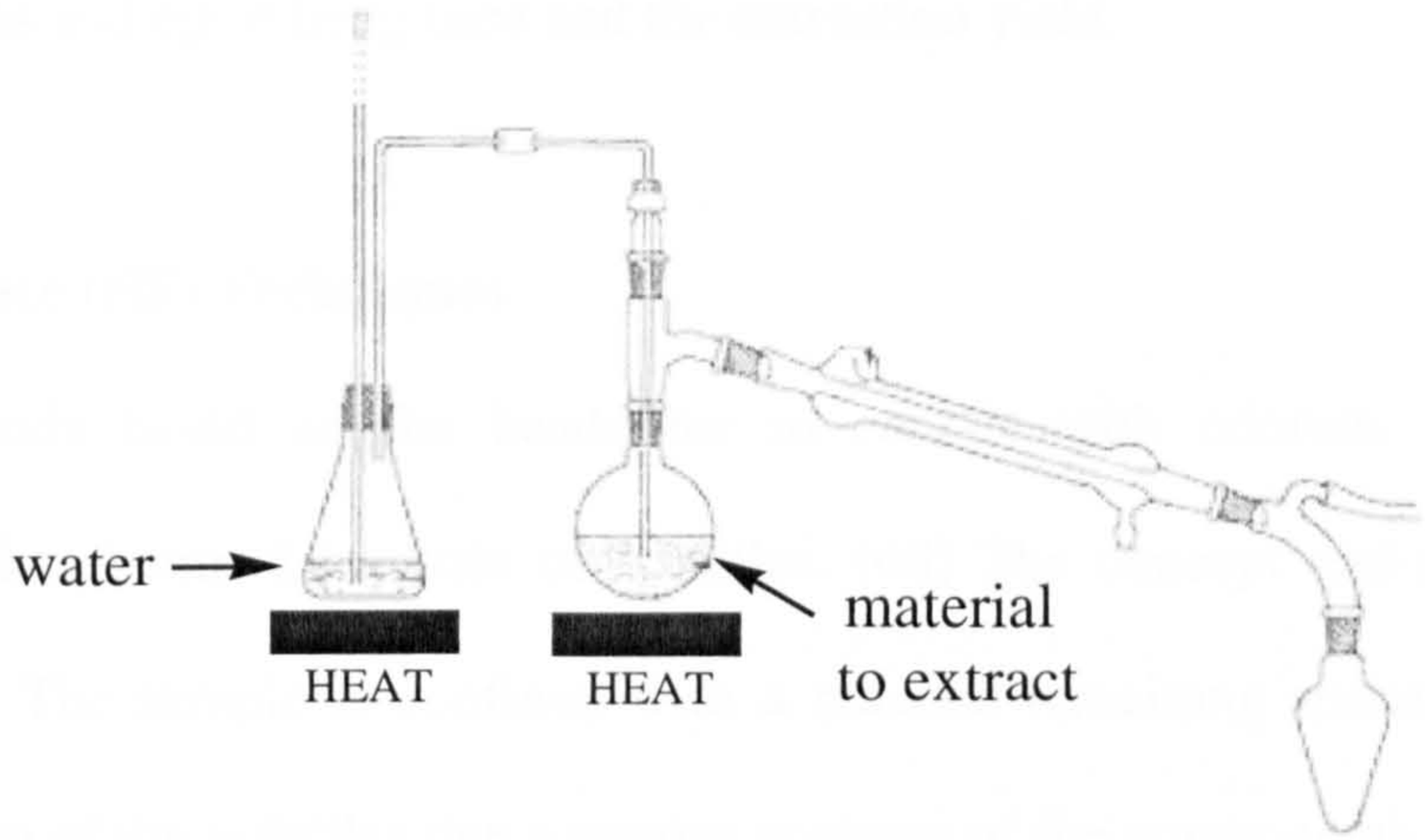


Figure 3.2 Steam Distillation [66]

Advantages	*Disadvantages*
- The extracts produced do not contain any high-blowing or non-volatiles materials. - It is possible to concentrate the extracts, enabling trace component detection.	- Highly polar or hydrophilic compounds may not be extracted from water. - Not suitable for fresh materials like fruit or vegetable, the extracts is more akin to a cooked aroma, rather than fresh. - Thermal degradation of some molecules may occur, introducing artefacts in the extract. - Loss of volatiles due to the vacuum.

Simultaneous distillation-extraction apparatus called Likens-Nickerson extractor, is usually used [67] for extraction of fragrances and aroma. In this apparatus the steam distillation and the organic solvent extraction takes place at the same time, reducing the losses of volatiles and optimizing time and the extraction yield.

3.1.3 Headspace (HS) Techniques

The methods based on the headspace in contact with odorous samples are widespread in the chemical analysis of volatiles. [68] The concept and procedure is straightforward. The sample is confined with a reduced remaining space on the top. After equilibrium of the volatiles due a vapour pressure of the components, a sample of this gas phase is taken/extracted and analysed. Two main techniques have being developed using HS, static headspace (SHS) and dynamic headspace (DHS).

SHS is the classical HS technique; the sample is placed inside a sealed vial and left to equilibrate, the atmosphere above the sample is taken with a gas tight syringe (usually 0.1-2.0 mL) and injected into a gas chromatograph (GC). The main concern of this technique is that it has not a concentrating sample treatment. Taking into account the typical trace and ultra-trace levels in the matrix of the odorant components, make this technique unsuitable for aroma analysis.

DHS uses a carried gas, normally nitrogen or helium to avoid unwanted reactions, to force the volatiles into a sorbent or a cold trap where they accumulate; the carrier gas goes out without the volatiles. The constant depletion of the analytes from the sample or from the adjacent atmosphere improves the sensitivity when compared to SHS. The

desorption of volatiles can be done in a small quantity of an appropriate solvent or suitable thermal desorption devices.

Advantages	*Disadvantages*
- Simple and quick - Solventless technique - No artefacts are formed, and if properly used, no contaminants introduced.	- Aroma profile is dependent upon the sampling temperature. - The relative concentration of components in the headspace does not reflect the concentration in the sample due to the differences in volatility of aroma compounds.

3.1.4 Solid Phase MicroExtraction (SPME)

3.1.4.1 Introduction

This sorptive technique was officially presented in public in 1990 by Pawliszyn and co-workers. [69] This relatively new technique is becoming more widely used and the number of papers published using this sampling method is growing exponentially. Its main merits are: that it is reasonably cheap, solventless, with a good reproducibility, qualitative, quantitative, fast, with none or very small sample preparation and easy to use.

The key component of the SMPE device is a piece of fused silica fibre (1 cm or 2 cm of length) coated with a thin film of several polymeric stationary phases. This film acts like a sponge, concentrating the organic analytes onto its surface. There are commercially available several kinds of films. Their chemical properties make them suitable to extract diverse types of molecules from liquid or gas phases.

SPME also presents some problems; the aroma profile collected is dependent upon the type, thickness and length of the used fibre as on the sampling time and temperature.

When the fibre is exposed to a sample, a partitioning of the compounds between the matrix or/and headspace and the fibre surface occurs. This redistribution reaches the equilibrium in a short time (normally between 5 and 30 min depending on the family of molecules and the sampling temperature). Once the extraction have been done, the extracted molecules are thermally desorbed in the injector port of a GC or in the case of a LC, the desorption takes place in a special chamber where the fibre is exposed to the mobile phase or a desorbing liquid mixture. Thus, the extract is separated and analyzed as usual.

With these properties, the fields where the SPME can be used are very wide. The inventors of the SPME used it for the analysis of substituted benzene compounds in groundwater as a first reported application. [70, 71] Since their commercialization researchers have used the fibres for applications in many areas; plastics and coatings [72, 73], contaminants in water as pesticides [74, 75], drugs analysis [76], food [77-83], flavour and fragrances [84-88], etc. SPME has rapidly become popular. The most significant piece of information is that after less than 15 years on the market, there are enough works published to find reviews about SMPE in food analysis [77, 89], environmental analysis [90], pesticide analysis [91, 92] and drug analysis. [89, 93]

Regarding the use of SPME methods in cocoa analysis, two recent works [94, 95] came out where the technique was used to ensure the cocoa quality by controlling the content of pyrazines.

3.1.4.2 Sampling with an SPME fibre

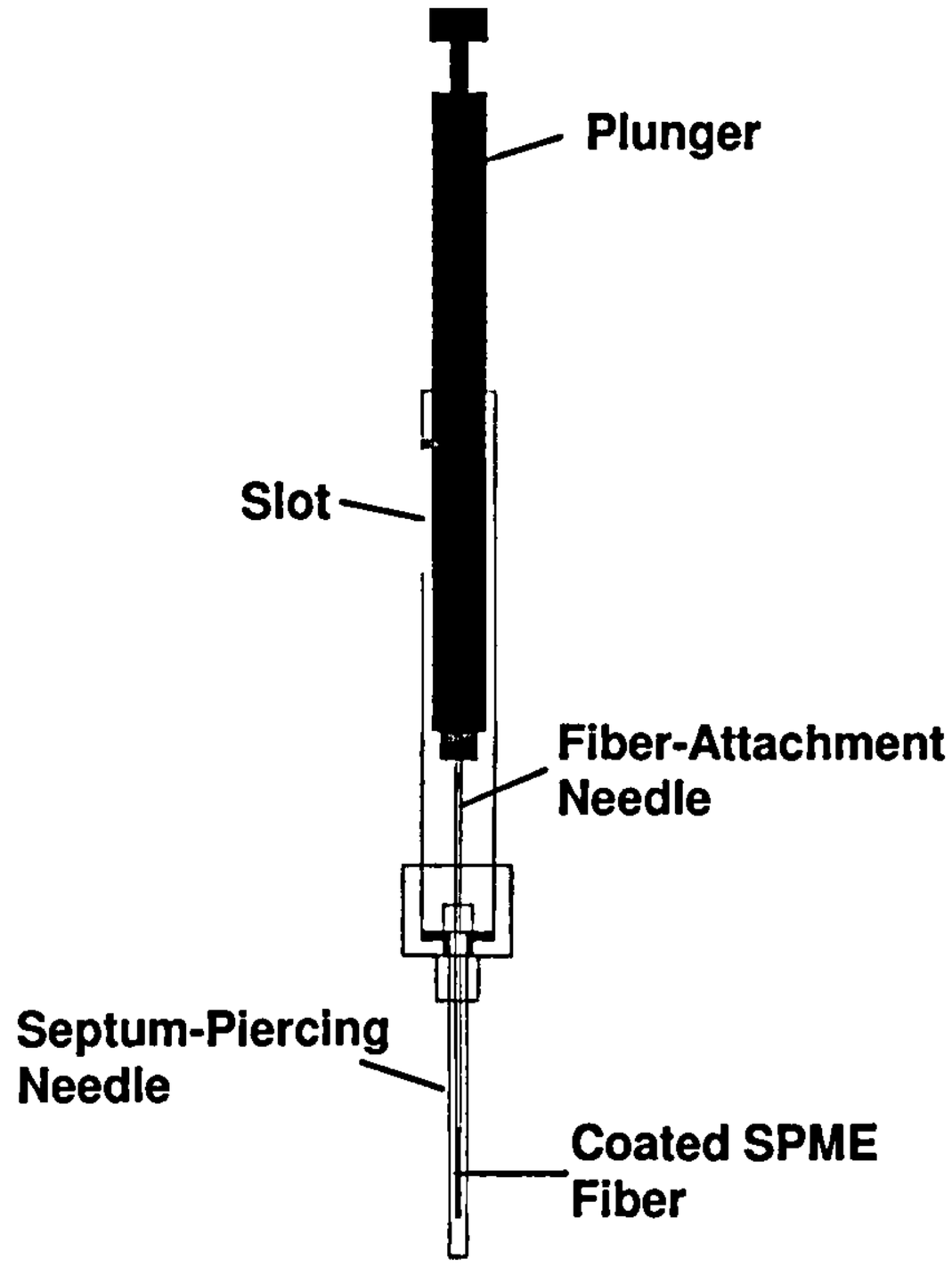


Figure 3.3 SPME holder (Sigma-Aldrich ©)

The fibre holder looks like a modified syringe (Figure 3.3) where the fibre is attached at the end of a fine metallic bar. The SPME fibre is inserted into the needle when no sampling is carried out. The fibres are easily damaged and their contact with anything may result in malfunction. Typically, the samples to be analysed are placed inside a sealed vial with a septum. During the perforation of the septum the fibre is retracted inside the metallic needle which passes through the septum. The fibre is then exposed to the sample for extraction. After the extraction the fibre is retracted and the needle is removed (Figure 3.4).

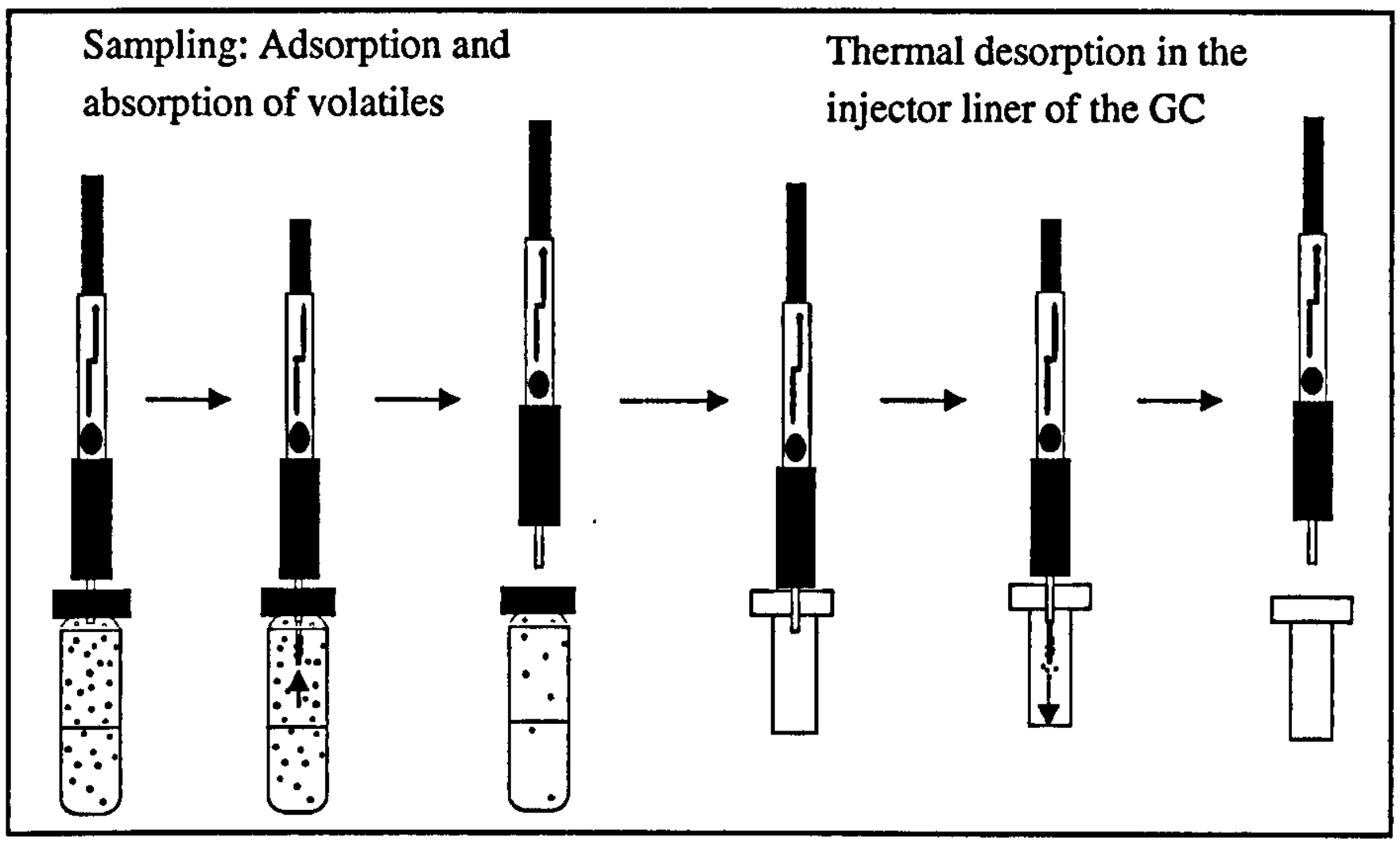


Figure 3.4 Extraction and thermal desorption in a GC injector liner. (Sigma-Aldrich ©)

There are two ways to use the SPME fibres:

- Direct immersion (DI). In the DI, the fibre is immersed in a solution where the analytes are dissolved. There is a two-phase diffusion process and a partition coefficient solution/coating. The analytes diffuse from the solution to the fibre until the equilibrium is reached. The time to reach this equilibrium establishes the extraction time.[96]
- Headspace (HS). In the HS, the fibre is exposed to the vapour phase of the samples (liquid, solid or gas) without direct contact (lifetime of the fibres is increased). There is a three-phase diffusion process, with two partition coefficients matrix/gas and gas/coating. The analytes diffuse from the matrix to the gas phase and from the gas phase to the coating of the fibre until the equilibrium is reached.[97]

Theoretical models of the distribution of molecules for both cases, DI and HS have been described [96, 97] allowing to predict the equilibrium times and how the molecules will behave.

3.1.4.3 SPME fibre coating

Table 3.1 shows the different SPME coatings commercially available (Sigma-Aldrich ©). The first column describes the chemical name and the thickness of the fibres. The second column describes the physical process that takes place in the extraction (Absorbent or Adsorbent). The last column states the polarity properties of the fibre.

Table 3.1 Types and properties of the commercial SPME fibres (Sigma-Aldrich ©)

Commercial name – Composition	Physic effect	Polarity
7µm Polydimethylsiloxane (PDMS)	Absorbent	Nonpolar
30µm PDMS	Absorbent	Nonpolar
100µm PDMS	Absorbent	Nonpolar
85µm Polyacrylate (PA)	Absorbent	Polar
65µm PDMS-DVB,	Adsorbent	Bipolar
65µm Carbowax-DVB	Adsorbent	Polar
55µm/30µm DVB/Carboxen™-PDMS,	Adsorbent	Bipolar
85µm Carboxen-PDMS, StableFlex	Adsorbent	Bipolar
Carbowax-TPR, for HPLC use	Adsorbent	Polar

There are two absorbent fibres: polydimethylsiloxane (PDMS) and polyacrylate (PA). The non-polar PDMS fibres come in three different coating thicknesses (7, 30 and 100 µm) and the polar PA fibre is only available in one (85 µm). The thickness of the fibres is directly proportional to the amount of extract. On the other hand, thicker fibres need longer times to reach the equilibrium.

The adsorbent type fibres contain porous particles such as divinylbenzene (DVB), Carboxen, and/or templated resin (TPR), as synthetic silica particle. Depending upon desired polarity (Figure 3.5), the DVB fibres are available with either PDMS or Carbowax (CW) as a binder (Figure 3.6).

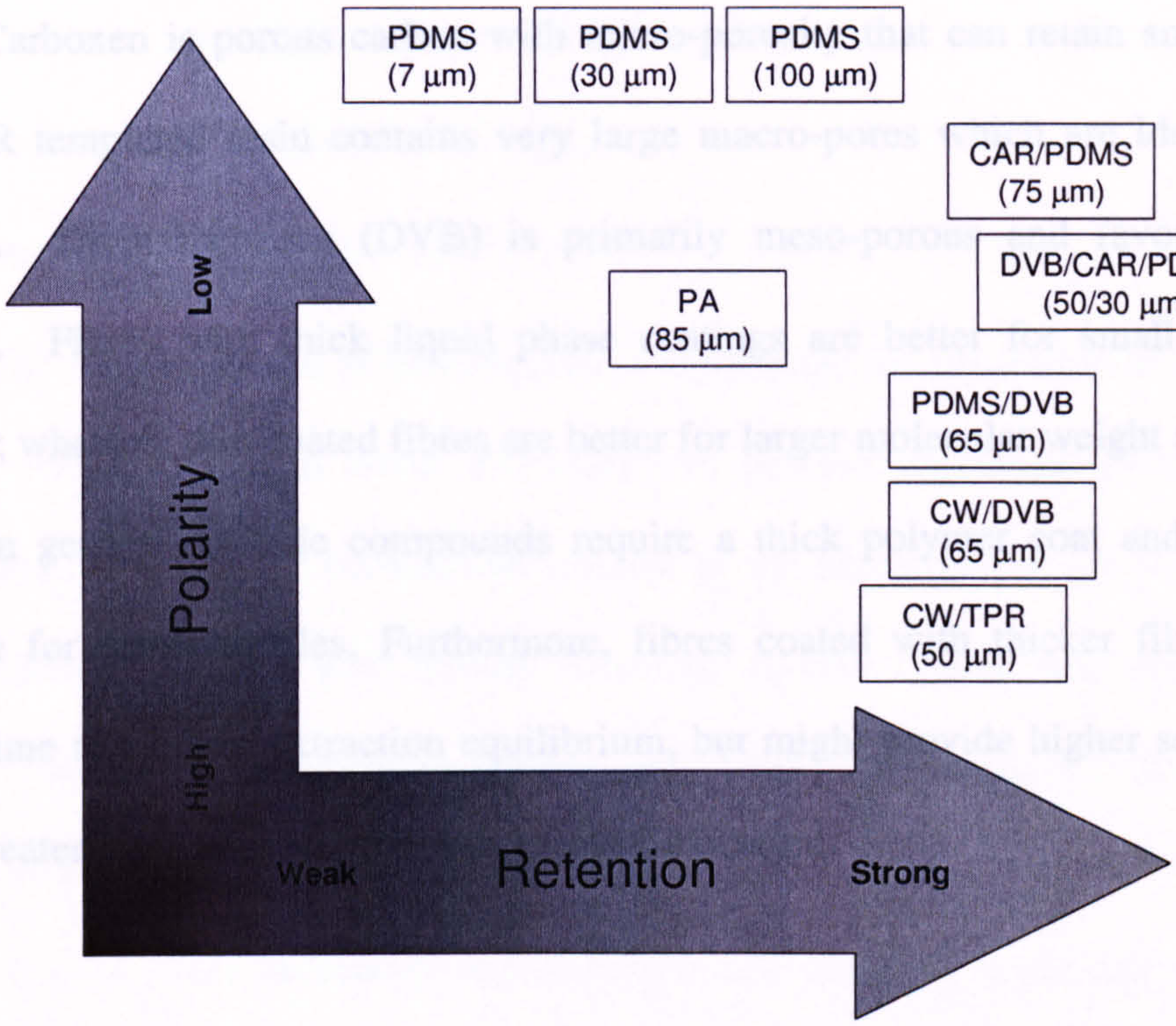


Figure 3.5 Distribution of the coating for polarity and retention [89]

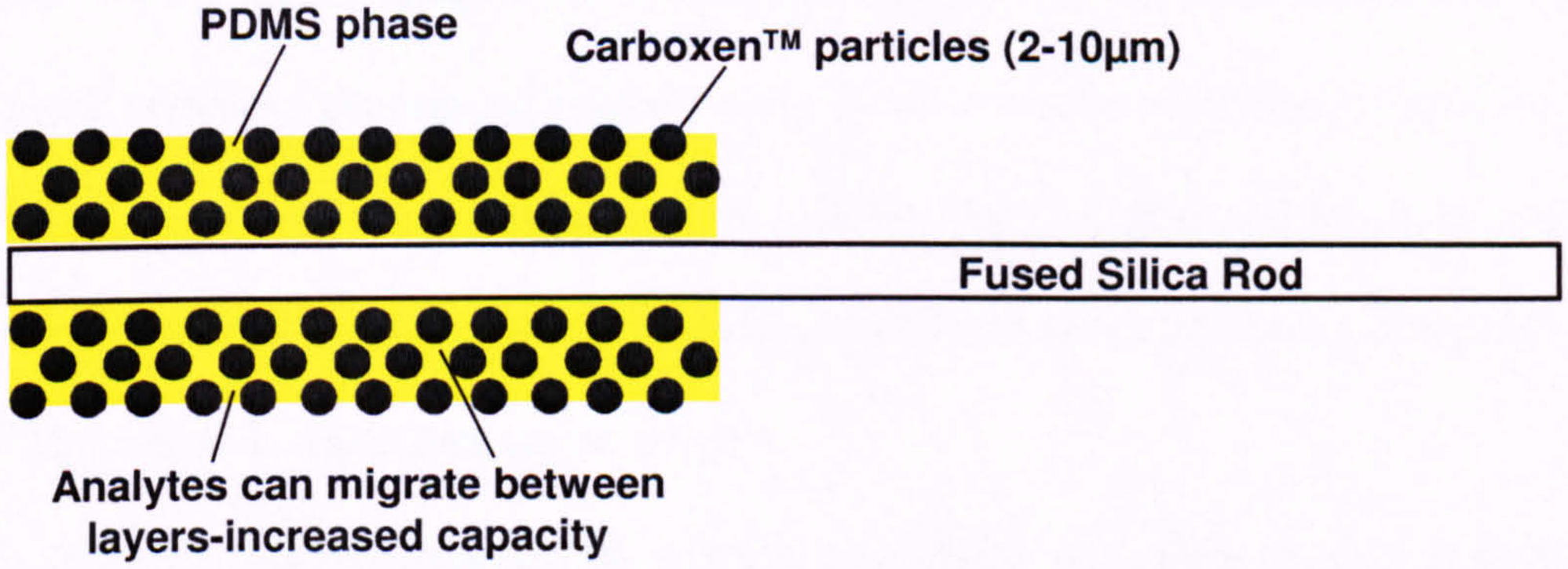


Figure 3.6 Schematic draw of a Carboxen/PDMS fibre.

The affinity of the fibre for an analyte depends on the principle of 'like dissolve like', and coating fibres having different properties or thickness are selected in accordance with different compounds. For example, the non-polar fibre PDMS is the best to extract non-polar molecules like alkanes. The thicker the better for low molecular weight (and thinner for high molecular weight).

Carboxen is porous carbon with micro-porosity that can retain small analytes. The TPR templated resin contains very large macro-pores which are ideal for larger analytes. Divinylbenzene (DVB) is primarily meso-porous and favours midsized analytes. Fibres with thick liquid phase coatings are better for small to midsized analytes; whereas, thin coated fibres are better for larger molecular weight analytes.

In general, volatile compounds require a thick polymer coat and thin coat is effective for semi-volatiles. Furthermore, fibres coated with thicker films require a longer time to achieve extraction equilibrium, but might provide higher sensitivity due to the greater mass of the analytes that can be extracted.

Chapter 4

4 Aims of the Project

The main objective of this research is to promote the generation of key chocolate odorant volatiles using a novel technology to process traditional chocolate-making ingredients as part of the commercial interest of the industrial partner to create a new line of natural products with intense chocolate flavour. These new products should display a very intense chocolate flavour and should be suitable for use in the food industry as an aromatic/functional additive.

Cocoa, or more accurately reduced fat cocoa powders, sugar and water will be used as the principal ingredients. The project will aim to develop the aromatic flavours by reacting these ingredients in a closed pressured reactor, thus simulating the conching process. Using higher temperatures, pressure and longer reaction times than in the traditional conching process, an improvement of the aromatic reactions is expected.

The progress of the reactions will be followed by chemical and sensorial analyses of the products and compared with standards, traditional chocolate and cocoa products.

The research objectives are as follows:

- Identify suitable ingredients (natural/traditional chocolate-making ingredients) including cocoa types, sugars, proteins, enzymes, to be reacted together;
- Develop the reaction technology;
- Develop suitable analytical methods to analyse the chemical content of the products;
- Develop suitable procedures for the sensorial evaluation of the products (use of trained panel);

- Optimise reaction conditions to promote the formation of volatile precursors by hydrolysis of cocoa powders using enzyme technologies;
- Optimise the reaction conditions in order to increase the generation of volatiles and the intensity of the flavour.

RESULTS AND DISCUSSION

Chapter 5

5 Development of the analytical methodologies

The development of appropriate analytical methodologies to control the evolution of the thermally processed cocoa products is essential for the project. The reaction of chocolate-making ingredients will generate volatile and non-volatile compounds. To assess the optimum reaction conditions, we will need to analyse the samples both chemically and sensorially.

From the chemical point of view, several analyses were carried out to characterise the cocoa products such as determination of methylxantines (bitterness) and total polyphenol content (astringency) but most of the analytical work relied on the development and validation of a methodology to analyse volatile and semi-volatile compounds. Gas Chromatography Mass Spectrometry (GC-MS) was used to study the volatile fraction. We evaluated three sampling techniques, which we describe in this chapter. In addition, the development and optimization of the solid phase micro-extraction (SPME) method is presented. To validate this methodology, a study of the evolution of volatiles during the industrial chocolate process is reported.

The second part of this chapter will describe the characterisation of different kinds of commercial cocoa powders that were acquired from Natra Cacao S.L. (Valencia, Spain) at the beginning of the project. Special emphasis is drawn to the differences found between the natural and the alkalised cocoas.

To finish, the procedure to setup the sensory evaluation of the products generated is presented.

5.1 Chemical analysis of volatiles

The analyses of the volatiles were carried out using a Varian GC-MS instrument with a (5%-phenyl)-methylpolysiloxane 30 m, 0.25 mm ID 0.25 μm IF capillary column. The tentative assignment of the peaks registered in the chromatograms was performed using the NIST (National Institute of Standards and Technology) Mass Spectral database software, by comparison with the retention index (RI) published in other publications or by straight comparison with standards. The NIST Mass Spectral database has an extensive library with more than 120,000 mass spectra of compounds registered.[98] The software performs an intensive search looking for the closest match for each peak. The peaks were tentatively assigned as the closest match when the coincidence of the mass spectrum of the peak and the library was at least 90 % or higher.

5.1.1 Screening for suitable sampling methodology

In section 3.1, several sampling methodologies for volatile analysis were presented. We were able to assess three of these techniques: steam distillation, static head space (SHS) and solid phase microextraction (SPME).

The screening was carried out for two cocoa products, obtained from Natra Cacao S.L (Valencia, Spain): natural cocoa powder (NCP) and conched chocolate powder (CCP). NCP is a product obtained from the cocoa liquor which has been pressed hydraulically to reduce the fat content and milled into a powder. CCP is a new product obtained by a similar process but using chocolate. [99] The chocolate is prepared following the traditional process (mixing, refining and conching), then pressed hydraulically to reduce the fat content and ground into a fine powder. The fat content of

the NCP was 10-12%. The fat content of the CCP was 10-12% and the cocoa content was 69%.

Several compounds present in cocoa and chocolate with known flavour activity were purchased as standards (Table 5.1). These standards, suitable to be used in the method development, were selected based on their importance in the chocolate aroma, their amount in cocoa/chocolate and commercial availability [30, 31, 57, 100, 101].

Table 5.1 Selected Standards

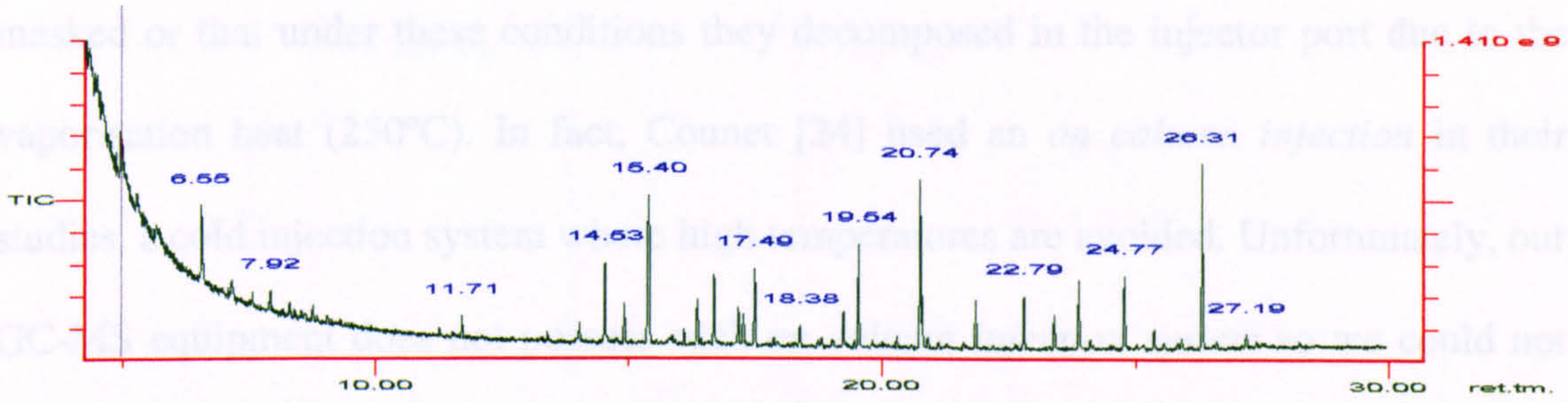
#	**Compounds**	**Odour description [24]**
	Aldehydes	
1	2-Methylpropanal (isobutanal)	Chocolate
2	3-Methylbutanal	Chocolate
3	2-Methylbutanal	Chocolate
4	2-phenyl-5-methyl-2-hexanal	
	Pyrazines	
5	2,3-dimethylpyrazine	Hazelnut, roasted
6	Trimethylpyrazine	Cocoa, roasted, green
7	Tetramethylpyrazine	Milk coffee, mocha, roasted
	Pyrroles	
8	1-(2-furanylmethyl)-1H-pyrrole	Roasted, chocolate, green

5.1.1.1 Steam distillation

The steam distillation (section 3.1.2) was carried out under reduced pressure following the method described by Van Praag [23] and more recently by Counet. [24] The distillate was extracted with dichloromethane (DCM) under basic and acid conditions. By controlling the pH, molecules that are likely to be protonated (e.g. amines) or deprotonated (e.g. carboxylic acids) can be quantitatively recovered.

Although the DCM extracts had a chocolaty aroma, the GC-MS analysis of the crude extract gave a flat chromatogram. These extracts required an intensive

concentration step, the use a Snyder-Kuderna concentrator system was necessary. Only when extracts were concentrated at least 10 times were some peaks recorded in the chromatogram (Figure 5.1). Eleven peaks were tentatively assigned (Table 5.2) with the MS data using the NIST software [98] or by MS and retention time straight comparison with the standards.



Conditions: 1.5 µL injected in 30m x 0.25mm ID 0.25 µm IF CP-SIL 8 CB Low Bleed/MS. Temp program 40°C (2 min) – 10°C/min – 280°C (5 min). Injector Temp 250°C. Mobile phase He 1 mL/min.

Figure 5.1 Chromatogram of the basic extract from steam distillation of CCP.

Table 5.2 Compounds identified in the basic extract from steam distillation of CCP.

Compounds	Retention time (min)
3-Methyl Pyridine[a]	4.97
2,6-Dimethyl Pyridine[a]	5.27
2,5-Dimethyl Pyrazine[a]	5.67
Benzaldehyde[b]	5.88
Benzene Acetaldehyde[a]	7.92
Acetophenone[b]	8.30
3-Ethyl-2,5-Dimethyl Pyrazine[a]	8.40
Phenylethyl Alcohol[a]	9.05
2-Phenylethyl Ester Acetic Acid[a]	11.27
3-Hydroxy-4-Methoxy Benzaldehyde[a]	13.40
3,5-Dihydroxy-6-Dimethylamino-1,2,4-Triazine[a]	23.87

[a] Compound tentatively assigned using NIST98 database.
[b] Compound identified by GC-MS and RI using authentic compounds.
Conditions: 1.5 µL injected in 30m x 0.25mm ID 0.25 µm IF CP-SIL 8 CB Low Bleed/MS. Temp program 40°C (2 min) – 10°C/min – 280°C (5 min). Injector Temp 250°C. Mobile phase He 1 mL/min.

The standards (Table 5.1) were dissolved in DCM (10 ppm) to emulate the extraction conditions and analysed by GC-MS. Sharp peaks and good mass spectra for all standards except for short aldehydes (Compounds 1, 2 and 3 in Table 5.1) were obtained. Some weak peaks were observed when the concentration of these aldehydes was increased, but their mass spectra did not correspond. The probable reason for this phenomenon was that these short aldehydes co-elute with the solvent peak and were masked or that under these conditions they decomposed in the injector port due to the vaporisation heat (250°C). In fact, Counet [24] used an *on column injection* in their studies, a cold injection system where high temperatures are avoided. Unfortunately, our GC-MS equipment does not possess such *on column injection system* so we could not confirm what the problem was. The same problems were encountered in the cocoa extracts where none of the short aldehydes were found.

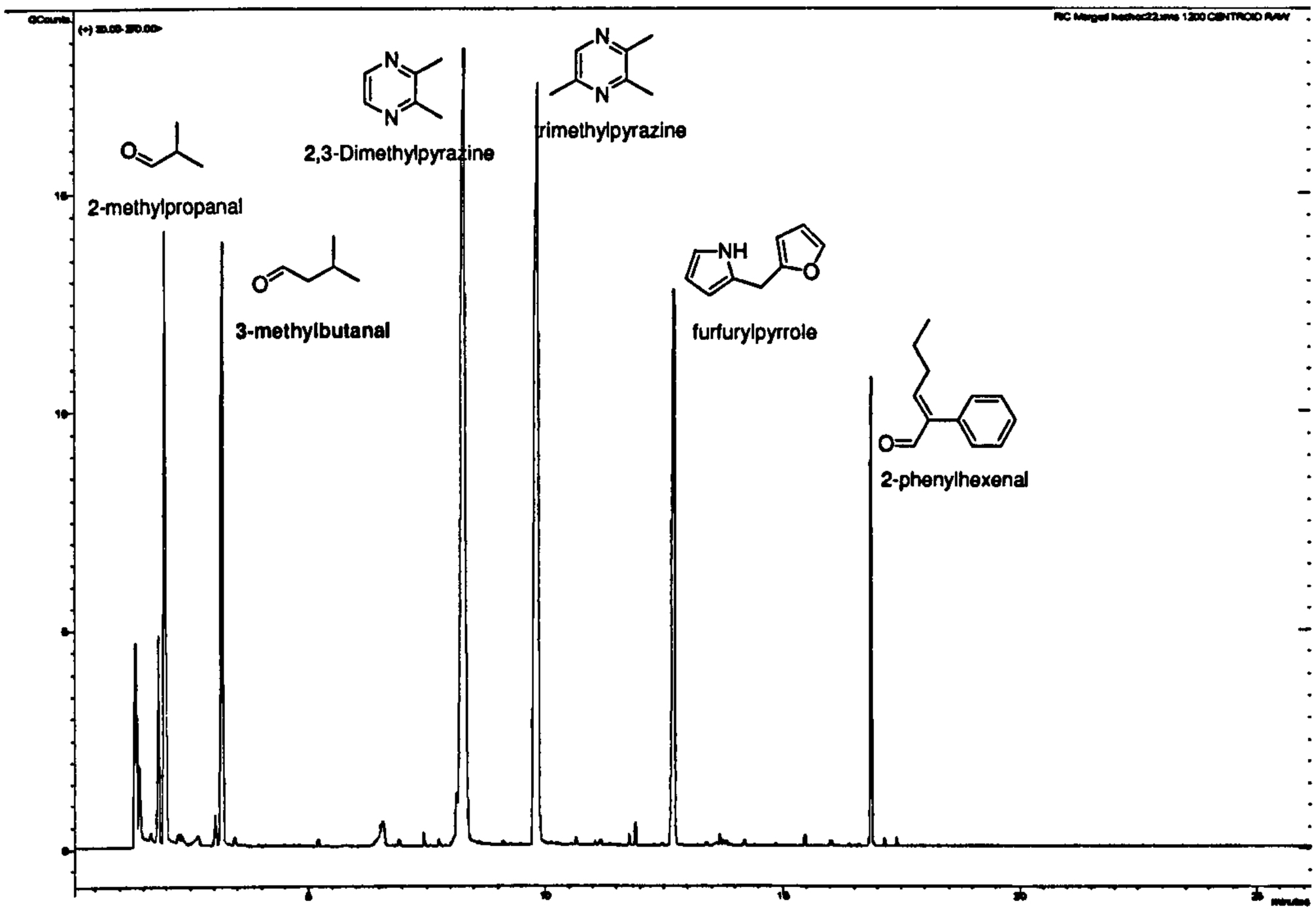
5.1.1.2 Static head space (SHS)

Static headspace (Section 0) is based on the injection of large volumes of gas phase from the headspace of a confined sample in a regular gas chromatograph.

In our case, one gram of sample was placed in a 10 mL vial. The vial, hermetically closed, was incubated at a given temperature for a given time. One millilitre of the head space gas was then injected in the GC-MS using a thermostated 1 mL syringe.

One microlitre of each standard was placed in an empty vial which was incubated at 120°C for 10 min. The injection of one millilitre of the headspace gas gave a clean chromatogram with peaks corresponding to all the standards introduced in the vial (Figure 5.2). The short aldehydes were recorded without problem and presented short retention times. Under the conditions used, the injector port was at 200°C. Although the injector temperature is 50°C cooler than the one used in the liquid injection, these

results pointed to a problem of peak masking due to the solvent front as most probable reason for not detecting the short aldehydes in the liquid injection (Section 5.1.1.1).

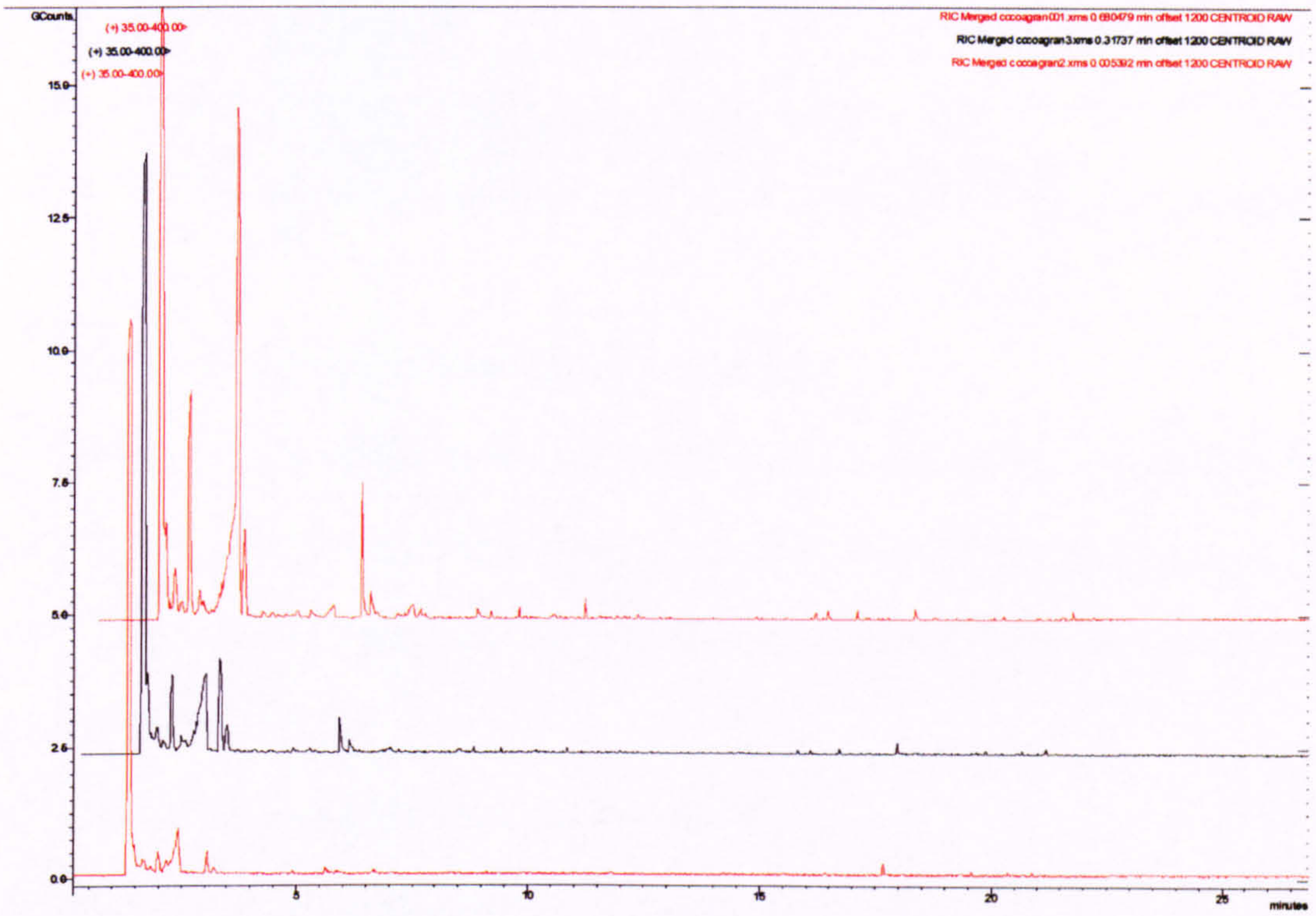


Conditions : Incubation at 100°C for 10 min. 15 µL injected in Varian Column 30m x 0.25mm ID 0.25 µm IF CP-SIL 8 CB Low Bleed/MS.

Figure 5.2 Chromatogram of the standards using SHS.

The cocoa samples were then analysed under the same sampling conditions. The chromatograms were recorded under increasing incubation temperatures (Figure 5.3). It clearly shows that the incubation temperature is a key factor. Looking at incubation temperature versus the total area integrated shows a linear increase (Figure 5.4). The total area is equivalent to the amount of volatiles recovered. Only at high incubation temperatures did the chromatograms become interesting for study. Unfortunately at such high temperatures (>90°C), artefacts may be generated due to thermal reactions or concentrations may be falsified due to the heating. At workable incubation temperatures (less than 80°C to minimize changes and generation of artefacts) only a few compounds

with high volatility and concentration were detected. All recorded peaks have short retention times at the beginning of the chromatogram.



Conditions : Incubation time 10 min, Varian Column 30m x 0.25mm ID 0.25 µm IF CP-SIL 8 CB Low Bleed/MS

Figure 5.3 Superposition of three SHS chromatograms of cocoa samples using three different incubation temperatures; 80 ºC (bottom), 100ºC (middle), 120ºC (top).

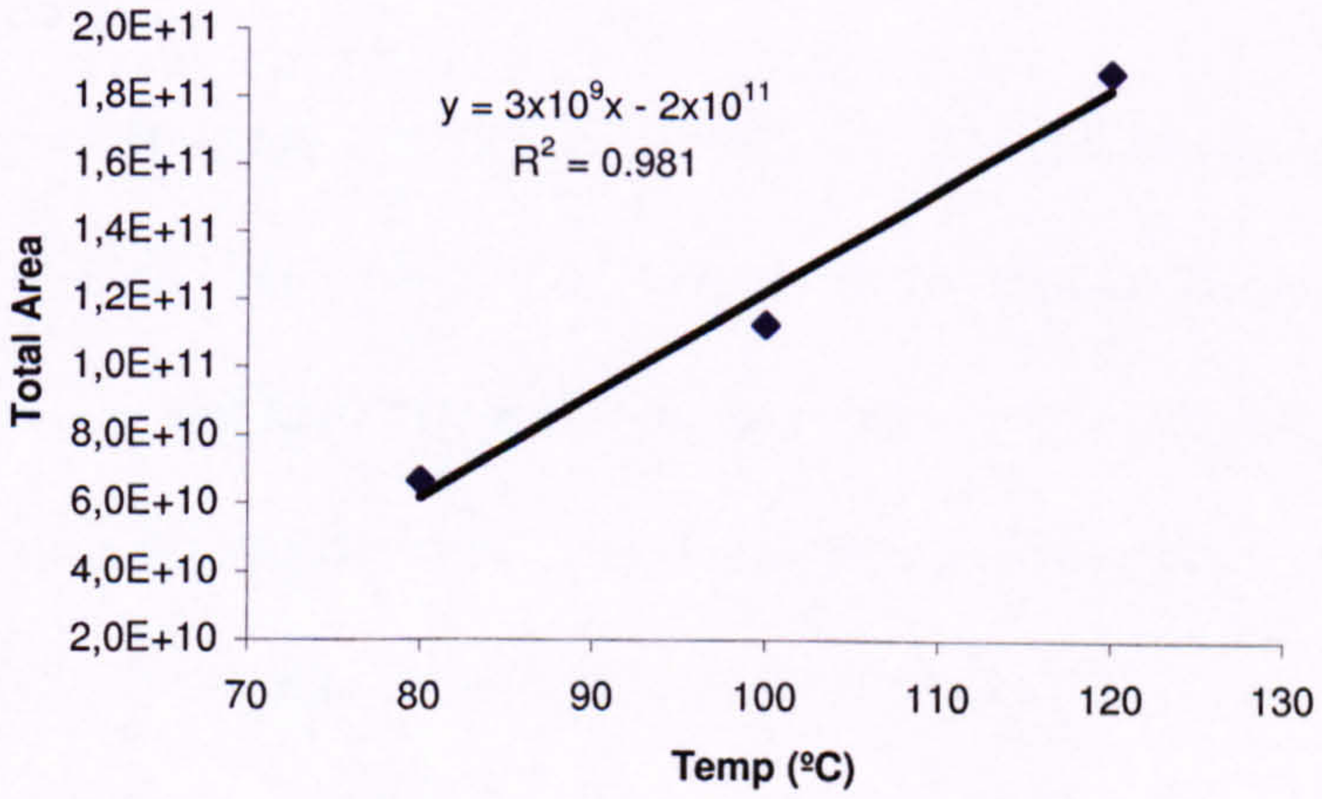


Figure 5.4 Total area versus incubated temperature for SHS sampling.

Table 5.3 Peaks recorded using SHS of a cocoa sample.

Products	Retention time (min)
2-Methyl propanal[b]	1.97
Acetic acid[b]	2.70
3-Methyl butanal[b]	3.02
2-Methyl butanal[b]	3.17
2,3 butanediol[a]	5.61

[a] Compound tentatively MS assignation using NIST98 database.
[b] Compound identified by GC-MS and RI using authentic compounds.
Conditions: Varian Column 30m x 0.25mm ID 0.25 µm IF CP-SIL 8 CB Low Bleed/MS 1mL/min He.

5.1.1.3 Solid Phase MicroExtraction (SPME)

There is a large portfolio of different SPME fibres commercially available (Section 3.1.4). To evaluate this sampling technique, a pack of three SPME fibres indicated by the supplier for the flavour and odour analysis was purchased. The pack contained the following SPME fibres:

- 100 µm polydimethylsiloxane coating (PDMS)
- 65 µm polydimethylsiloxane/divinylbenzene coating (PDMS/DVB)
- 75 µm Carboxen/polydimethylsiloxane coating (CAR/PDMS)

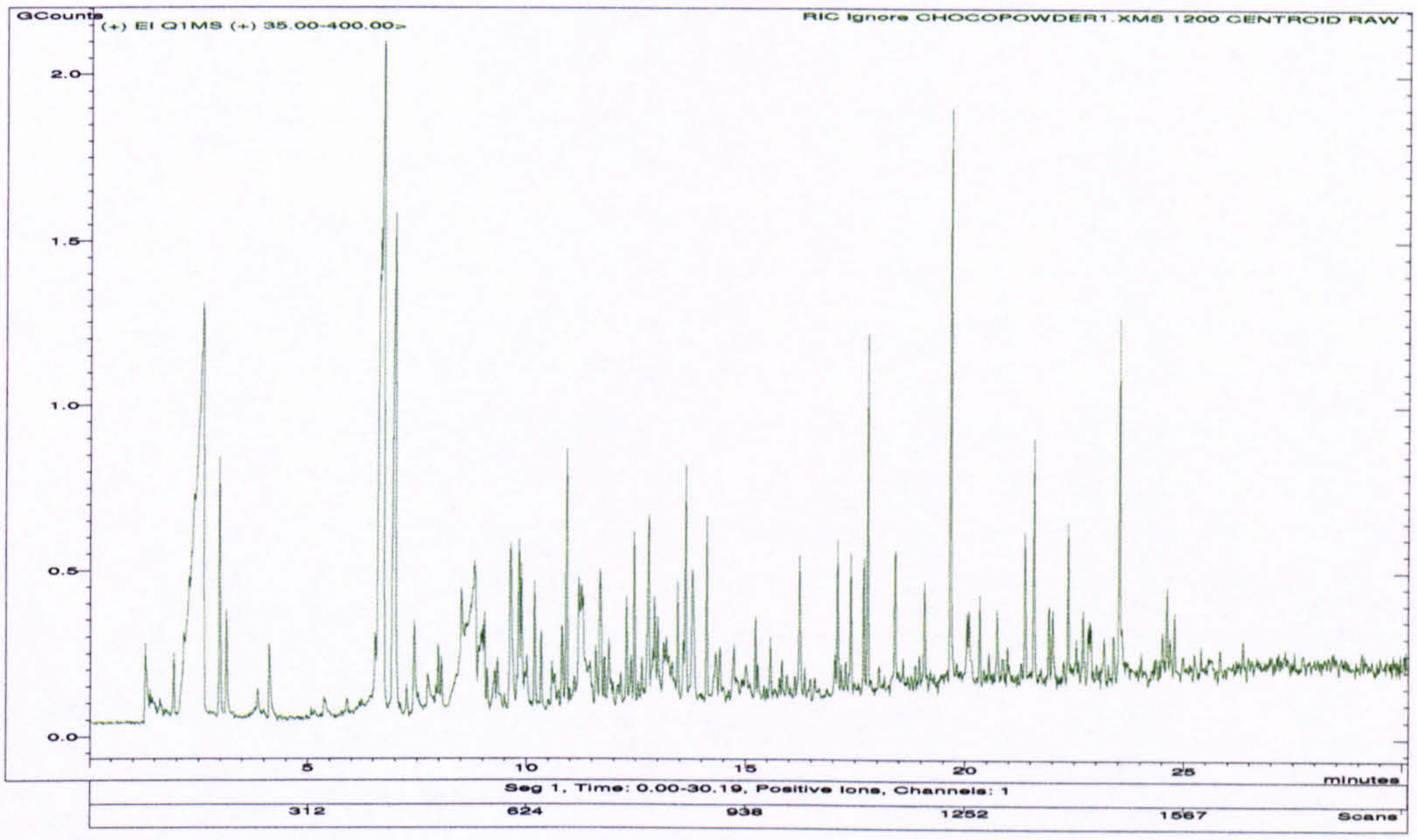
An additional fibre was purchased,

- 50/30 µm divinylbenzene/carboxen on polydimethylsiloxane on a StableFlex fibre (DVB/CAR-PDMS)

This latter fibre was a new product that came out from the supplier when we started developing our method. This fibre was designated for flavour analysis, suitable for molecules from C-3 to C-20 and molecular weights between 40 and 280 $g.mol^{-1}$.

Since our interest lies on the volatile profile, the presented work was done using the headspace sampling. Four grams of chocolate powder were added to a 10 mL headspace vial with a pre-drilled septum. The sample was incubated for 15 min at 60°C and the SPME fibre was allowed to absorb/adsorb for 15 min at the same temperature.

The results from the first injection were promising, the chromatogram contained peaks all along the time range recorded (30 minutes). The results showed a good performance for volatiles and semi-volatiles (Figure 5.5). The recovery for many of the peaks was satisfactory to allow tentative mass spectra identification (Table 5.4).



Conditions : Incubation for 10 min at 60°C, Extraction for 10 min, Varian Column 30m x 0.25mm ID 0.25 µm IF CP-SIL 8 CB Low Bleed/MS

Figure 5.5 Chromatogram of CCP sample using a PDMS/DVB SPME fibre.

Table 5.4 gathers the compounds tentatively assigned and identified by direct comparison with standards. Each compound has its retention index, its retention time and its ion selected for their quantification. The retention index (RI) were calculated by injecting, under the same conditions, several n-alkanes (from C-5 to C-18) and assigning the time of each n-alkanes to a RI equal to the number of carbons per 100 (e. C-5 → RI = 500). The RI of the other molecules were calculated by extrapolation. The mode of acquisition of the mass detector was set on full scan in all cases, but for each molecule, an ion was selected for its quantification. The ion selected was the main ion or in the case of major interference another significant ion without noise.

Table 5.4 Compounds identified from SPME analysis of CCP with PDMS/DVB fibre[a].

Peaks	Compounds	Retention index	Retention time (min)[a]	Quant Ion
1	Acetone [b]	495	3.660	43.0
2	Methyl acetate [c]	521	4.610	43.0
3	2-Methyl-propanal [b]	550	5.638	43.0
4	2,3-Butanedione [c]	581	6.632	43.0
5	2-Butanone [c]	586	6.805	43.0
6	Acetic acid [b]	630	8.140	60.0
7	3-Methyl-butanal [b]	653	8.805	41.1
8	2-Methyl-butanal [b]	662	9.082	41.0
9	Pentanal [c]	696	9.991	44.0
10	3-Hydroxy-2-butanone [c]	707	10.281	45.0
11	Dimethylpropanedioic acid [c]	755	11.514	43.0
12	35-Dimethyl-dihydro-furan-2-one [c]	766	11.778	42.0
13	2,3-Butanediol [c,d]	782	12.161	45.0
14	2,3-Butanediol [c,d]	792	12.390	45.0
15	Hexanal [c]	802	12.633	56.0
16	Methylpyrazine [c]	832	13.299	94.0
17	3-Methyl-butanoic acid [c]	837	13.428	60.0
18	2-Methyl-butanoic acid [c]	847	13.636	74.0
19	2-Furanmethanol [c]	854	13.802	98.1
20	2,5-Dimethyl-pyrazine [c]	921	15.194	42.0
21	2,3-Dimethyl-pyrazine [b]	928	15.338	108.1
22	Ethylpyrazine [c]	926	15.296	107.1
23	α-Pinene [c]	949	15.748	93.1
24	Benzaldehyde [b]	981	16.365	77.1
25	β-Pinene [c]	999	16.694	93.1
26	35-Dimethyl-octane [c]	1007	16.844	43.0
27	Trimethylpyrazine [b]	1011	16.930	42.0
28	2-Ethyl-6-methyl-pyrazine [c]	1013	16.963	121.2
29	1H-Pyrrole-2-carboxaldehyde [c]	1018	17.050	95.0
30	Benzyl alcohol [b]	1048	17.591	79.0
31	Benzeneacetaldehyde [c]	1062	17.838	91.0
32	2-Acetylpyrrole [c]	1072	18.018	94.0
33	3-Ethyl-2,5-dimethyl-pyrazine [c]	1086	18.245	135.2
34	Tetramethylpyrazine [b]	1094	18.392	54.0
35	Phenylethyl alcohol [c]	1133	19.039	91.0
36	Benzoic acid [c]	1157	19.434	105.1
37	3,5-Dihydroxy-6-methyl-2,3-dihydro-pyran-4-one [c]	1164	19.542	42.9
38	3,5-Dimethyl-benzaldehyde [c]	1250	20.856	133.1
39	Isopentyl benzoate [c]	1404	23.021	105.1
40	Vanillin [b]	1419	23.215	151.1
41	8-Hydroxy-3-methyl-iso-chroman-1-one [c]	1565	24.722	134.1
42	Caffeine [b]	1783	26.897	194.2

[a] Conditions: Varian Column 30m x 0.25mm ID 0.25 µm IF CP-SIL 8 CB Low Bleed/MS. 1mL/min He.
[b] Compound identified by GC-MS and RI using authentic compounds.
[c] Compound tentatively MS assignation using NIST98 database.
[d] Diastereoisomers

5.1.1.4 Conclusion

The steam distillation followed by acid/base extraction afforded good recovery, number of peaks and performance along the chromatogram. There are many disadvantages to this methodology: long preparation times (around 2 days), many steps (distillation, acid and basic extractions, drying, and concentration), the use of thermal processes for distillation and concentration affect volatile profile and generate artefacts.

The static headspace (SHS) technique is quick and easy to execute with practically no sample preparation. On the other hand, incubation temperatures over 100°C were needed to obtain suitable chromatograms, making this method not appropriate for our purpose.

The SPME sampling methodology compiles the best properties of the two previous methodologies. It is quick and easy to use, solvent free, with very small sample preparation and fully automatable. It presented a good performance over the whole chromatogram. Volatiles and semi-volatiles were extracted, and the number of molecules recorded was important.

After screening three possible sampling methods indicated for chemical analysis of volatiles, the SPME sampling technique presented itself as the best one. The next section presents the optimization of the conditions as well as the comparison between the performances of the different fibres.

5.1.2 Development and optimization of the SPME sampling methodology

5.1.2.1 Selection of the fibre coating

Fibre selection is very important to define the optimum extraction conditions from the headspace. Several types of coating fibres, with a range of polarities and mechanisms, have been reported as suitable for the analysis of volatiles in food samples [89]. PDMS-supported fibres are the best as they result in best long-term stability and overall performance, including chromatography, extraction characteristics and analyte recovery. These fibres are also very durable and usually retain their performance for up to 100 analytical cycles.

The non-polar PDMS fibre is recommended by the supplier for the extraction of non-polar analytes, such as volatile flavour compounds but can also be applied to more-polar compounds. Mixed fibre coatings, containing DVB or CAR, increase retention capacity due to mutually potential effect of extraction and distribution of the stationary phase. PDMS/DVB and CAR/PDMS have been used for the extraction of volatile low-molecular-mass and polar analytes. The dual-coated fibre DVB/CAR-PDMS comprises a layer of DVB-PDMS over a layer of CAR-PDMS and is recommended for flavour and odour extraction (volatiles and semi-volatiles). The natural cocoa powder (NCP) and conched chocolate powder (CCP) were sampled using these four SPME fibres and analyzed by GC-MS. The objective at this point was to establish which fibres have the best performance to analyse cocoa products.

Altogether 42 compounds were identified some tentatively on the basis of their mass spectra [102] and retention indexes (RI) by comparison with the NIST98 database and some by pure standards analysed under the same conditions (Table 5.5). In general, the four fibres extracted compounds with RI from less than 500 (RI 495 for acetone) to almost 1,800 (RI 1,783 for caffeine). These included 9 aldehydes, 14 heterocyclic

compounds, 5 ketones, 4 alcohols, 2 esters, 5 acids and 3 terpenes. Table 5.5 presents the compounds classified in five groups; volatiles aldehydes and ketones, acids and alcohols, pyrazines, semi-volatiles aldehydes and ketones and terpenes and others. This classification groups the components by functional group families and in the case of the aldehydes and ketones have been divided into volatiles and semi-volatiles. Although there is a no official definition of volatiles and semi-volatiles, we will consider as volatiles all the components with a retention index RI $\leq$ 800 and semi-volatiles RI > 800. These retention indexed have been calculated for a Varian Column 30m x 0.25mm ID 1 µm IF VF-4 Factor 4.

Inspection of the total peak area for each fibre clearly showed that the most efficient fibre was CAR-PDMS, extracting around five times more than the DVB-PDMS and DVB/CAR-PDMS fibres and 25 times more than the PDMS fibre. Extraction with the PDMS fibre was very weak compared to the other three fibres. Indeed very few peaks were observed on the chromatogram, with some undetected by the MS, making this fibre unsuitable for our study. The CAR-PDMS fibre presented the highest total peak area (Table 5.5) and proved to be very efficient at extracting low molecular weight volatiles, affording wide peaks at the beginning of the chromatogram (RI < 700). A split injection would be needed to obtain sharper peaks. However even under splitless conditions, most of the semi-volatiles were not extracted, making this fibre undesirable for our study. Finally, the DVB/PDMS and DVB/CAR-PDMS fibres resulted in similar total peak areas; the peaks were sharp and well distributed throughout the chromatogram. Both fibres would be suitable for our study.

Table 5.5 Effect of fibre type on the peak area (area units x10^8) of natural cocoa powder (NCP) and conched chocolate powder (CCP) using HS-SPME-GC-MS.[a]

	Compound [b]	RI	PDMS		DVB-PDMS	
			NCP	CCP	NCP	CCP
	Volatile Aldehydes and Ketones					
1	Acetone [b]	495	65 ± 11	22 ± 8	1246 ± 11	243 ± 61
2	Methyl acetate [c]	521	18 ± 2	ND	456 ± 2	49 ± 23
3	2-Methyl-propanal [b]	550	69 ± 12	66 ± 14	916 ± 12	661 ± 128
4	2,3-Butanedione [c]	581	1783 ± 251	1292 ± 185	757 ± 251	562 ± 152
5	2-Butanone [c]	586	ND	ND	813 ± 145	224 ± 49
7	3-Methyl-butanal [b]	653	139 ± 32	169 ± 29	3401 ± 242	3091 ± 423
8	2-Methyl-butanal [b]	662	69 ± 14	97 ± 21	1361 ± 142	1476 ± 252
9	Pentanal [c]	696	ND	ND	304 ± 64	86 ± 26
10	3-Hydroxy-2-butanone [c]	707	ND	63 ± 10	1550 ± 194	1610 ± 210
12	35-Dimethyl-dihydro-furan-2-one [c]	766	11 ± 4	ND	610 ± 154	59 ± 24
15	Hexanal [c]	802	ND	ND	403 ± 94	57 ± 21
	Acids and Alcohols					
6	Acetic acid [b]	630	1612 ± 211	1519 ± 261	29290 ± 3250	21460 ± 2854
11	Dimethylpropanedioic acid [c]	755	N.D.	N.D.	4875 ± 954	3125 ± 487
13	2,3-Butanediol [c,d]	782	560 ± 102	670 ± 123	55860 ± 5821	60960 ± 6221
14	2,3-Butanediol [c,d]	792	306 ± 51	314 ± 62	32670 ± 4151	28590 ± 3562
17	3-Methyl-butanoic acid [c]	837	185 ± 42	N.D.	16180 ± 1822	15420 ± 2511
18	2-Methyl-butanoic acid [c]	847	N.D.	N.D.	3491 ± 385	3.091 ± 520
19	2-Furanmethanol [c]	854	6 ± 3	N.D.	439 ± 84	203 ± 38
30	Benzyl alcohol [b]	1048	N.D.	N.D.	256 ± 62	586 ± 154
35	Phenylethyl alcohol [c]	1133	78 ± 44	44 ± 9	6248 ± 1114	11850 ± 1514
36	Benzoic acid [c]	1157	17 ± 5	12 ± 4	157 ± 34	456 ± 86
39	Isopentyl benzoate [c]	1404	45 ± 11	N.D.	831 ± 214	166 ± 53
	Pyrazines					
16	Methylpyrazine [c]	832	30 ± 13	18 ± 6	1117 ± 224	551 ± 94
20	2,5-Dimethyl-pyrazine [c]	921	146 ± 44	116 ± 34	8254 ± 1244	5817 ± 854
21	2,3-Dimethyl-pyrazine [b]	928	14 ± 6	N.D.	396 ± 85	764 ± 152
22	Ethylpyrazine [c]	926	N.D.	N.D.	N.D.	N.D.
27	Trimethylpyrazine [b]	1011	75 ± 21	123 ± 42	3517 ± 655	4936 ± 866
28	2-Ethyl-6-methyl-pyrazine [c]	1013	2 ± 1	N.D.	143 ± 44	243 ± 62
33	3-Ethyl-2,5-dimethyl-pyrazine [c]	1086	3 ± 1	N.D.	202 ± 57	824 ± 122
34	Tetramethylpyrazine [b]	1094	169 ± 54	105 ± 37	2648 ± 454	2484 ± 482
	Semi-volatile Aldehydes and Ketones					
24	Benzaldehyde [b]	981	75 ± 34	54 ± 25	6138 ± 1032	6617 ± 1102
29	1H-Pyrrole-2-carboxaldehyde [c]	1018	7 ± 4	N.D.	424 ± 82	654 ± 148
31	Benzeneacetaldehyde [c]	1062	33 ± 15	58 ± 34	1567 ± 284	13780 ± 2152
32	2-Acetylpyrrole [c]	1072	19 ± 6	19 ± 8	1479 ± 314	4235 ± 651
37	3,5-Dihydroxy-6-methyl-2,3-dihydro-pyran-4-one [c]	1164	39 ± 21	N.D.	1341 ± 253	2628 ± 541
38	3,5-Dimethyl-benzaldehyde [c]	1250	N.D.	N.D.	391 ± 132	147 ± 25
40	Vanillin [b]	1419	7 ± 4	451 ± 148	699 ± 157	11180 ± 2104
	Terpenes and others					
23	α-Pinene [c]	949	66 ± 28	N.D.	7478 ± 1612	N.D.
25	β-Pinene [c]	999	N.D.	N.D.	544 ± 126	N.D.
26	35-Dimethyl-octane [c]	1007	N.D.	N.D.	4701 ± 745	N.D.
41	8-Hydroxy-3-methyl-iso-chroman-1-one [c]	1565	N.D.	N.D.	106 ± 34	112 ± 35
42	Caffeine [b]	1783	170 ± 46	101 ± 54	348 ± 72	594 ± 142
	TOTAL		5,816	5,311	203,606	209,591

N.D. Compound not detected. [a] All runs preformed with extraction at 60°C for 15 min under dry conditions and results reported as average and errors as the standard deviation of 3 analyses.

Table 5.5 (Continued)

No.	Compounds	RI	CAR-PDMS		DVB/CAR-PDMS	
			NCP	CCP	NCP	CCP
	Volatile Aldehydes and Ketones					
1	Acetone [b]	495	33050 ± 3275	6311 ± 945	3073 ± 458	1660 ± 355
2	Methyl acetate [c]	521	14080 ± 1648	982 ± 145	1416 ± 342	332 ± 82
3	2-Methyl-propanal [b]	550	40910 ± 5224	15550 ± 2513	914 ± 245	1719 ± 320
4	2,3-Butanedione [c]	581	13760 ± 2360	15970 ± 2458	1908 ± 265	2925 ± 236
5	2-Butanone [c]	586	37620 ± 5124	10060 ± 1420	3470 ± 521	1567 ± 251
7	3-Methyl-butanal [b]	653	77690 ± 9423	86430 ± 11045	4439 ± 623	7179 ± 1366
8	2-Methyl-butanal [b]	662	41520 ± 6210	59890 ± 8541	2241 ± 252	4231 ± 651
9	Pentanal [c]	696	7509 ± 845	3940 ± 687	906 ± 260	544 ± 124
10	3-Hydroxy-2-butanone [c]	707	19490 ± 2180	49900 ± 8511	1560 ± 310	4926 ± 845
12	35-Dimethyl-dihydro-furan-2-one [c]	766	5700 ± 945	2344 ± 322	583 ± 240	240 ± 62
15	Hexanal [c]	802	1221 ± 182	N.D.	607 ± 124	322 ± 58
	Acids and Alcohols					
6	Acetic acid [c]	630	389700 ± 40211	509000 ± 61525	78310 ± 11254	134000 ± 21804
11	Dimethylpropanedioic acid [c]	755	33710 ± 4127	N.D.	2400 ± 325	3256 ± 812
13	2,3-Butanediol [c,d]	782	170000 ± 22943	276500 ± 36102	33990 ± 5442	61060 ± 12209
14	2,3-Butanediol [c,d]	792	89220 ± 12452	123300 ± 19450	11990 ± 2112	22210 ± 3251
17	3-Methyl-butanoic acid [c]	837	35600 ± 3254	92010 ± 14520	9667 ± 1225	14860 ± 2524
18	2-Methyl-butanoic acid [c]	847	7457 ± 1346	15680 ± 2364	2146 ± 363	2666 ± 326
19	2-Furanmethanol [c]	854	3288 ± 651	3245 ± 458	996 ± 251	860 ± 152
30	Benzyl alcohol [b]	1048	234 ± 74	871 ± 210	118 ± 22	344 ± 54
35	Phenylethyl alcohol [c]	1133	3158 ± 845	6628 ± 1125	5645 ± 945	7342 ± 945
36	Benzoic acid [c]	1157	106 ± 32	796 ± 124	189 ± 34	464 ± 214
39	Isopentyl benzoate [c]	1404	30 ± 15	N.D.	226 ± 14	61 ± 24
	Pyrazines					
16	Methylpyrazine [c]	832	17480 ± 3254	19690 ± 3458	3243 ± 432	4249 ± 832
20	2,5-Dimethyl-pyrazine [c]	921	30760 ± 1346	40710 ± 8245	6987 ± 732	12760 ± 1532
21	2,3-Dimethyl-pyrazine [b]	928	705 ± 251	N.D.	351 ± 122	1175 ± 232
22	Ethylpyrazine [c]	926	603 ± 124	259 ± 51	428 ± 74	1429 ± 645
27	Trimethylpyrazine [b]	1011	2578 ± 345	8396 ± 1257	1693 ± 645	4206 ± 532
28	2-Ethyl-6-methyl-pyrazine [c]	1013	106 ± 31	850 ± 53	101 ± 32	1489 ± 445
33	3-Ethyl-2,5-dimethyl-pyrazine [c]	1086	136 ± 42	651 ± 132	160 ± 35	690 ± 232
34	Tetramethylpyrazine [b]	1094	1150 ± 185	1846 ± 324	1714 ± 382	1829 ± 352
	Semi-volatile Aldehydes and Ketones					
24	Benzaldehyde [b]	981	19790 ± 3232	34290 ± 4251	11790 ± 1845	17010 ± 2842
29	1H-Pyrrole-2-carboxaldehyde [c]	1018	777 ± 151	2653 ± 450	255 ± 82	624 ± 255
31	Benzeneacetaldehyde [c]	1062	164 ± 21	626 ± 122	1192 ± 355	5198 ± 1252
32	2-Acetylpyrrole [c]	1072	1829 ± 251	6870 ± 853	925 ± 132	2561 ± 512
37	3,5-Dihydroxy-6-methyl-2,3-dihydro-pyran-4-one [c]	1164	N.D.	N.D.	339 ± 82	734 ± 252
38	3,5-Dimethylbenzaldehyde [c]	1250	47 ± 25	N.D.	161 ± 25	69 ± 18
40	Vanillin [b]	1419	22 ± 9	777 ± 178	135 ± 34	1661 ± 258
	Terpenes and others					
23	α-Pinene [c]	949	20440 ± 3512	69 ± 22	6259 ± 845	N.D.
25	β-Pinene [c]	999	1273 ± 210	N.D.	287 ± 42	N.D.
26	35-Dimethyl-octane [c]	1007	N.D.	N.D.	1340 ± 281	N.D.
41	8-Hydroxy-3-methyl-iso-chroman-1-one [c]	1565	N.D.	N.D.	22 ± 12	24 ± 18
42	Caffeine [b]	1783	N.D.	N.D.	107 ± 32	109 ± 34
	TOTAL		1,122,912	139,7093	204,283	328,585

[b] Compound identified by GC-MS and RI using authentic compounds.
[c] Compound tentatively identified by GC-MS and RI using NIST98 database.
[d] Diastereoisomers.

A number of compounds have been identified by gas chromatography-olfactometry (GC-O) as key odorant compounds in dark chocolate [24]. 2-Methylpropanal, 3-methylpropanal and 2-methylbutanal have been related to chocolate odours while trimethylpyrazine, 2,5-dimethylpyrazine and acetylpyrrole have been attributed cocoa odours. Methylpyrazine, 2,3-dimethylpyrazine, 2-ethyl-6-methylpyrazine and benzaldehyde added a hazelnut/almond note to the aroma profile of chocolate; ethylpyrazine, 3-ethyl-2,5-dimethylpyrazine and tetramethylpyrazine a roasty note. These compounds were extracted by all four fibres (Figure 5.6 & Figure 5.7).

In general, the PDMS fibre proved very inefficient at extracting key odorant compounds. The CAR-PDMS fibre was found to be the best fibre for the extraction of volatiles (RI < 950) while the DVB-PDMS was best at extracting semi-volatiles (RI > 950). Interestingly, the DVB/CAR-PDMS seemed to extract both volatiles and semi-volatiles with the similar good efficiency. This dual-coated fibre proved to be the best fibre for the analysis of odorant compounds for both NCP and CCP because it combined the characteristics of the CAR-PDMS fibre with the addition of the properties of DVB-PDMS. The CAR layer allowed the fibre to extract the low molecular weight analytes (RI > 950), but with less efficiency than the CAR-PDMS, presumably because the layer of CAR is thinner in the dual-coated fibre (75 μm in CAR-PDMS versus 30 μm in DVB/CAR-PDMS). Furthermore, the DVB/CAR-PDMS fibre afforded high-resolution chromatograms when compared to the other fibres' chromatograms. We therefore decided to use this dual-coated fibre to characterize the cocoa and chocolate products and establish their aroma and volatile profiles.

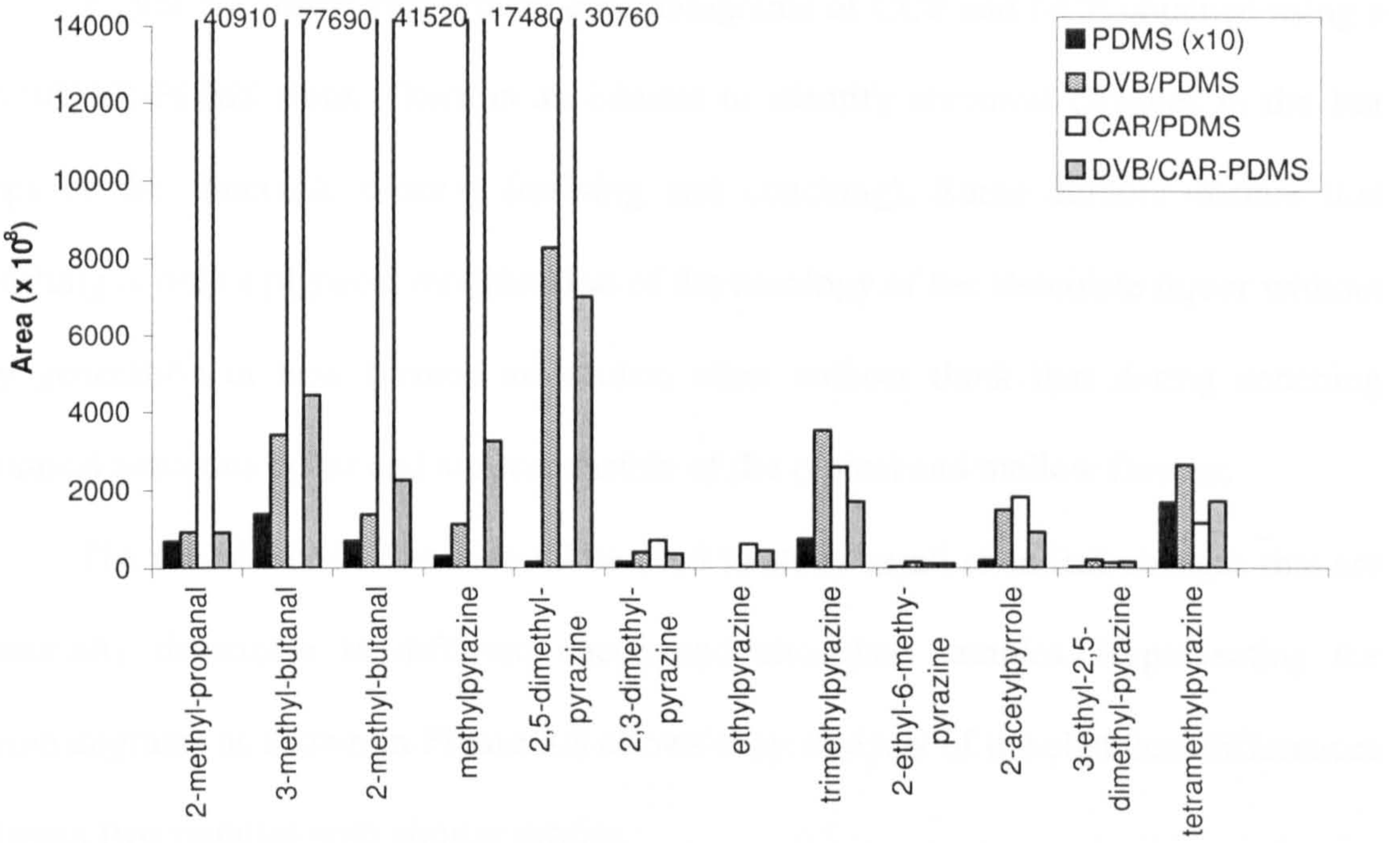


Figure 5.6 Uptake of the key odorant compounds from NCP tested by HS-SPME using the four fibre coatings (sampling at 60°C for 15 min.) Data are expressed as peak areas obtained by GC-MS analysis.

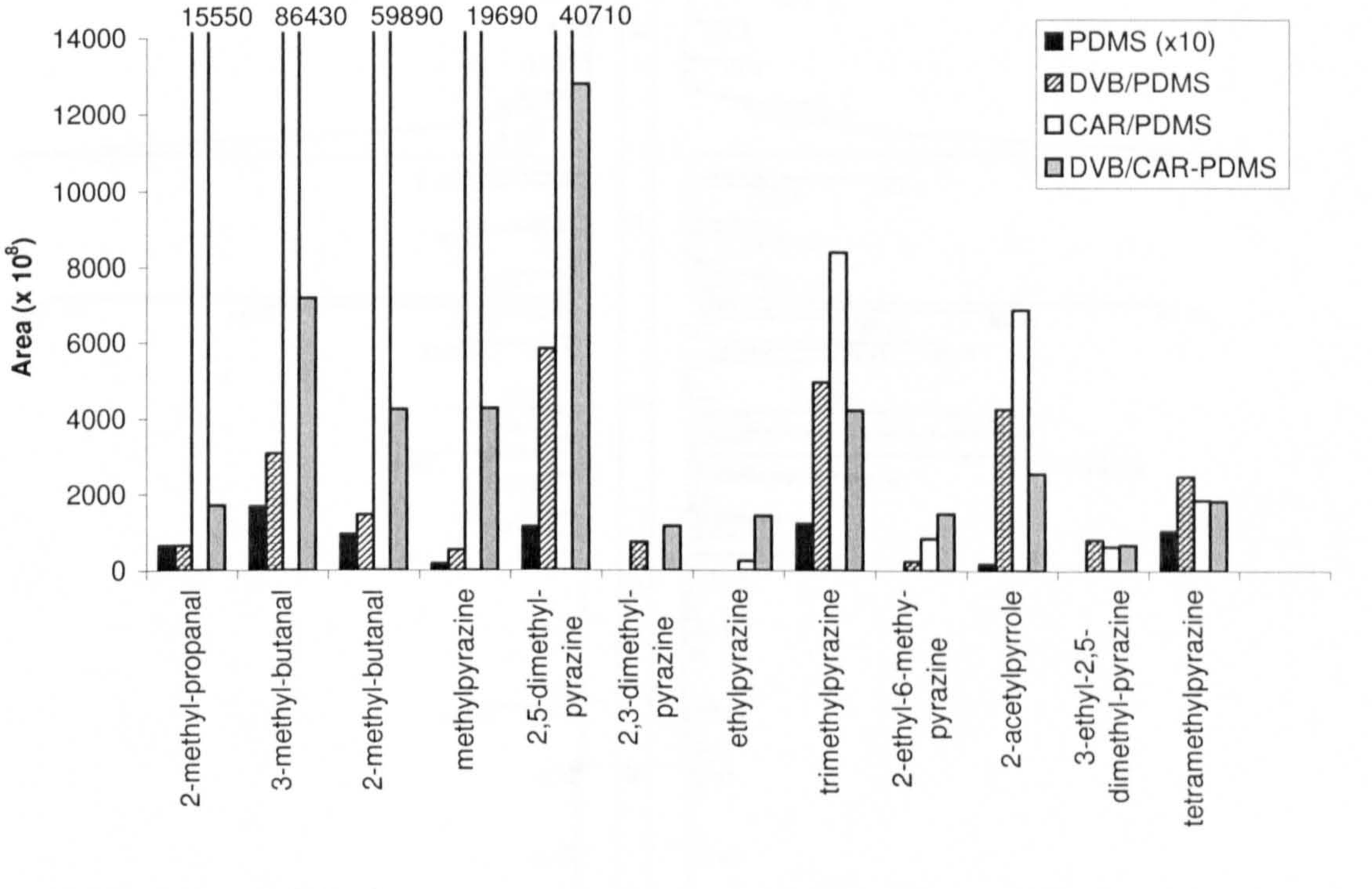


Figure 5.7 Uptake of the key odorant compounds from CCP tested by HS-SPME using the four fibre coatings (sampling at 60°C for 15 min.) Data are expressed as peak areas obtained by GC-MS analysis.

Figure 5.8 shows two typical chromatograms of CCP and NCP obtained using a DVB/CAR-PDMS fibre. There is an interest to identify chemical changes in the last steps of the chocolate process (refining and conching). Some authors declare that conching is only a physical modification of the rheology of the chocolate liquor without any generation of new flavour molecules, other authors think that during conching chemical reactions occur and are responsible of the typical and mellow flavour.

The use of SPME fibres could be used to study small chemical changes that are sensorially detectable in different cocoa and chocolate samples. Representing the chromatograms as shown in Figure 5.8 allows easy analysis of the chemical differences between two samples with similar profile.

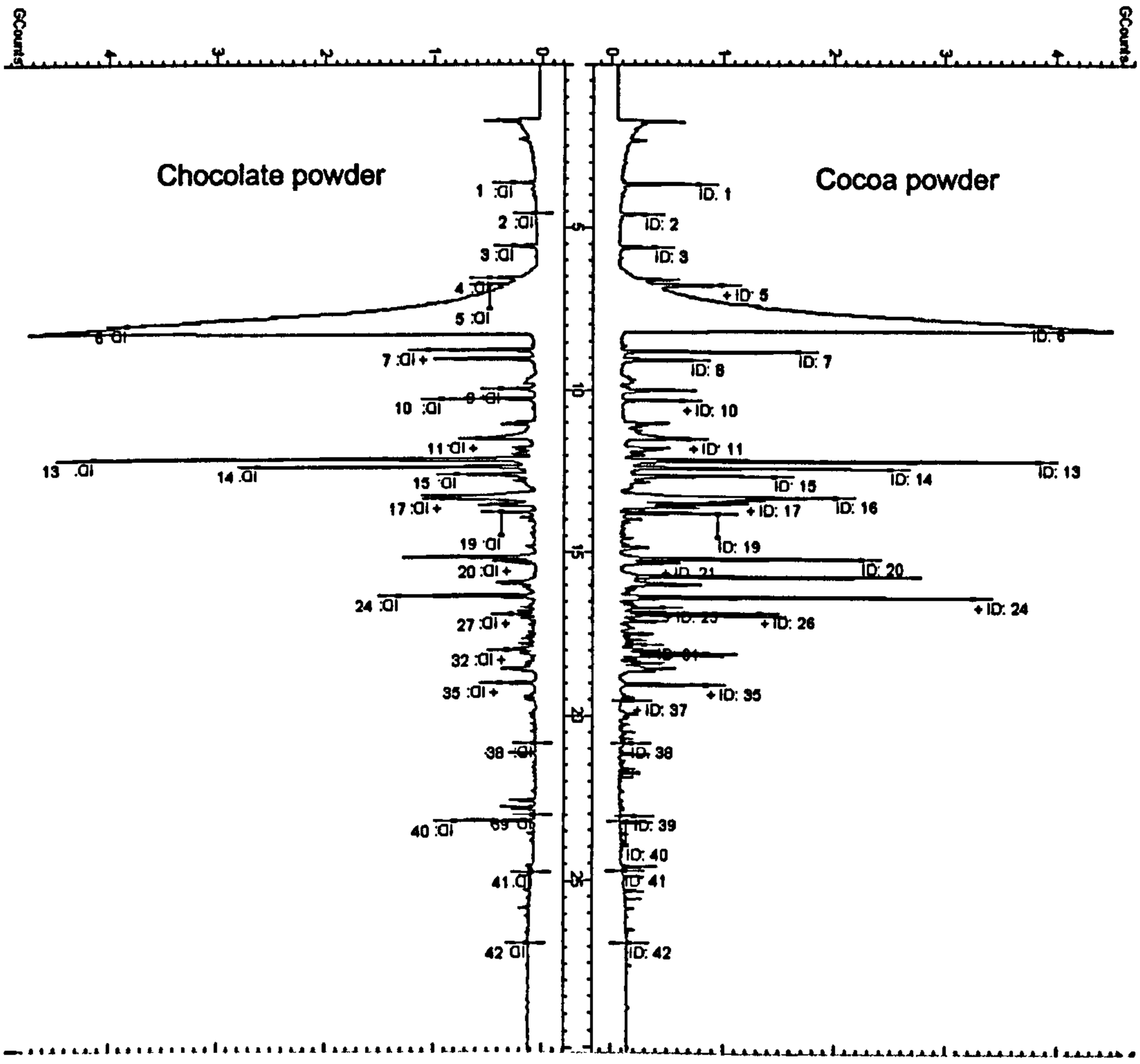


Figure 5.8 Typical HS-SPME chromatograms obtained using DVB/CAR-PDMS fibre of CCP and NCP. Peak assignments as described in Table 5.5.

5.1.2.2 Selection of extraction time and temperature

Temperature and time of extraction are two important factors that control sample recovery by the fibres [79]. Time affects the mass transfer of the analytes onto the fibre; optimum time is required for the fibre to reach its equilibrium. Temperature directly affects how fast this equilibrium is reached; favouring the diffusion and it also has an influence on the composition of the volatile phase, increasing the ratio of compounds with low vapour pressure (semi-volatiles). Consequently, these parameters were studied in order to establish the optimum extraction conditions.

To examine the effect of the time and temperature on extraction efficiency, samples were prepared as previously described and incubated at a given time and temperature (prior to sampling). Figure 5.9 shows a three dimensional representation of the total peak area versus extraction times and temperatures. The graph clearly shows that the equilibrium is reached within 15 min (total peak area remains constant after 10-12 minutes in all cases). The temperature also has an exponential effect on the total peak area, allowing optimum extraction at 80°C. However this temperature had to be discarded since chemical changes may occur in the sample at this temperature.

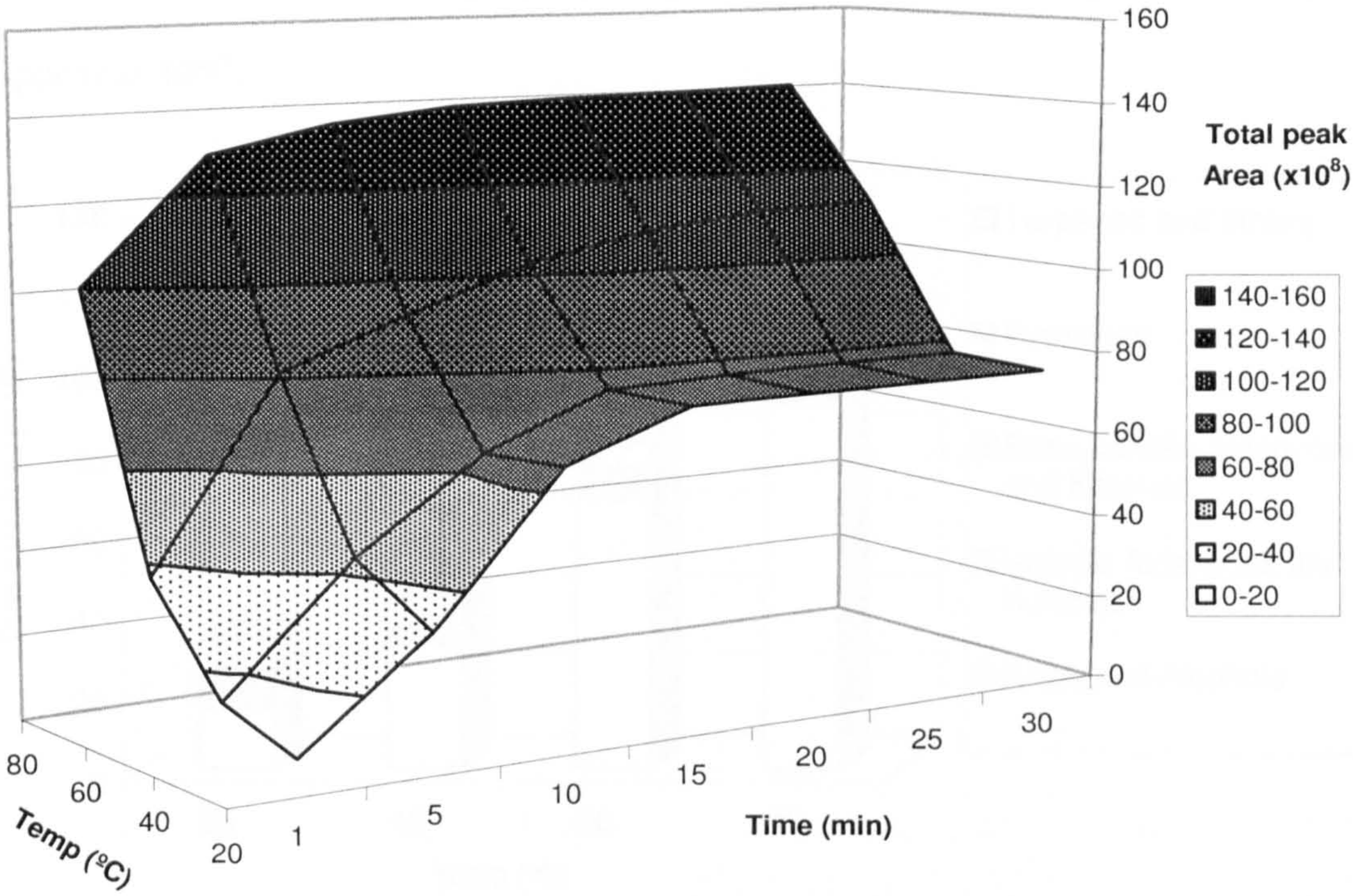


Figure 5.9 Effect of extraction time and temperature on the total peak area (x-axis: time in min; y-axis: total peak area expressed in total ion counts; z-axis: temperature in °C). CAR/DVB-PDMS fibre was used.

The extraction was investigated at four different temperatures (25, 40, 60 and 80°C) and the compounds extracted were divided into five main compound family: pyrazines, volatile aldehydes/ketones, semi-volatile aldehydes/ketones, acids/alcohols, and terpenes and others (Figure 5.10). In general, an increase of the total peak area can be observed when the temperature of extraction is increased. The "acid and alcohol" fraction are better extracted at higher temperatures. The "pyrazine" and the "semi-volatile aldehydes and ketones" fractions behave similarly at low temperatures (weak peaks) and from 60ºC, their extraction efficiency is improved. Volatile compounds have a higher vapour pressure at room temperature; at 25ºC their concentration in the gas phase is higher than at higher temperatures when the presence of semi-volatiles starts to became important. On the other hand, there is a dramatic decrease in the "volatile aldehyde and ketone" fraction (30% at 25ºC) with increase of the temperature (only 5%

at 60ºC). Peaks for 2-methyl propanal, 2-butanone or 3-methyl butanal almost disappear at 40ºC.

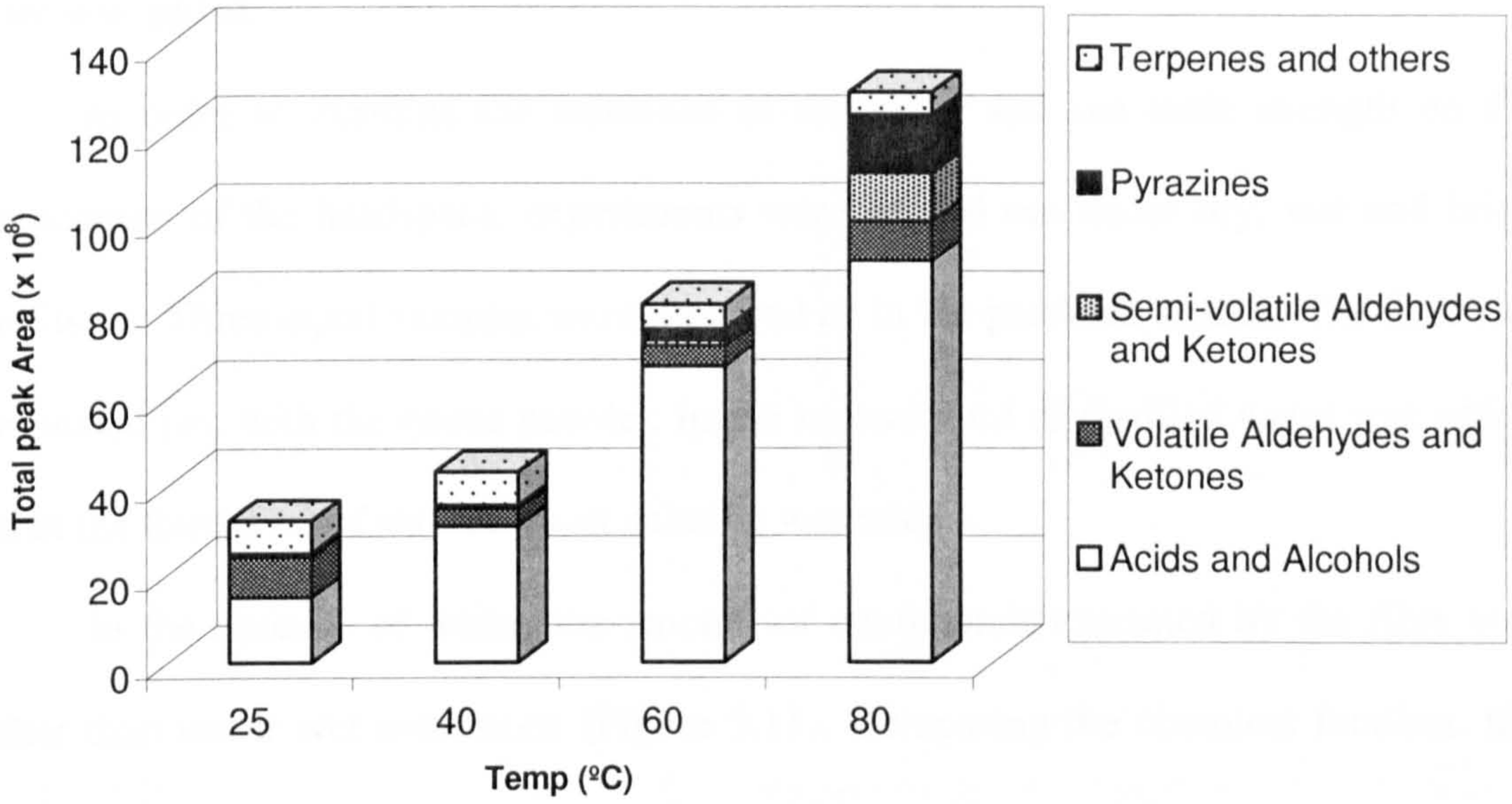


Figure 5.10 Effect of extraction temperature on the peak area of the main compound families (x-axis: temperature in ºC; y-axis: total peak area expressed in total ion counts). CAR/DVB-PDMS fibre was used and 5 min for extraction time.

Taking into account these results, the optimum conditions were chosen to be 60ºC for the extraction temperature and 15 minutes for the extraction time.

5.1.2.3 Study in wet, dry and brine conditions

Most of the samples matrices of works published on food volatiles analysis using the HS-SPME technique are aqueous.[68, 77, 88, 89, 97] Many foods are aqueous solutions like wines, juices or foods with high water content that can be milled to produce a liquid sample. Other times with solid foods, like cheese, chocolate, seeds, etc. the tendency is to pulverize the solids and perform the sampling over a water suspension of the ground food. [103, 104] The increase of the ionic strength by saturation of aqueous solutions with salt, enhance the volatile recovery by between 2 to 20-fold. [95, 105]

In our case, we used a direct analysis of the volatiles coming from solid particles, the cocoa and chocolate powders were confined in a headspace vial where no water was added.

In order to examine the influence of the water and the ionic strength on the composition of the headspace, experiments were carried out under dry, wet and brine conditions. Three equal samples were prepared as in the previous studies. The first vial was sealed just with the cocoa powder, in the second 8 ml of distilled water was added and in the third 8 ml of saturated salt solution was added.

In the absence of water, the amount of compounds extracted by the fibre was higher than under wet conditions (Figure 5.11). Comparing the chemical families, the largest difference was found in the "acid and alcohol" group, being the least well extracted. These results could be explained by the fact that acids and alcohols are very soluble in water, decreasing their presence in the volatile phase. The "pyrazine" group presented only weak signals in plain water which by increasing the ionic strength of the media increased significantly. The polarity of their nitrogen gives pyrazines an acceptable solubility in water, which in presence of NaCl makes them less soluble in water thus increasing their presence in the volatile fraction.

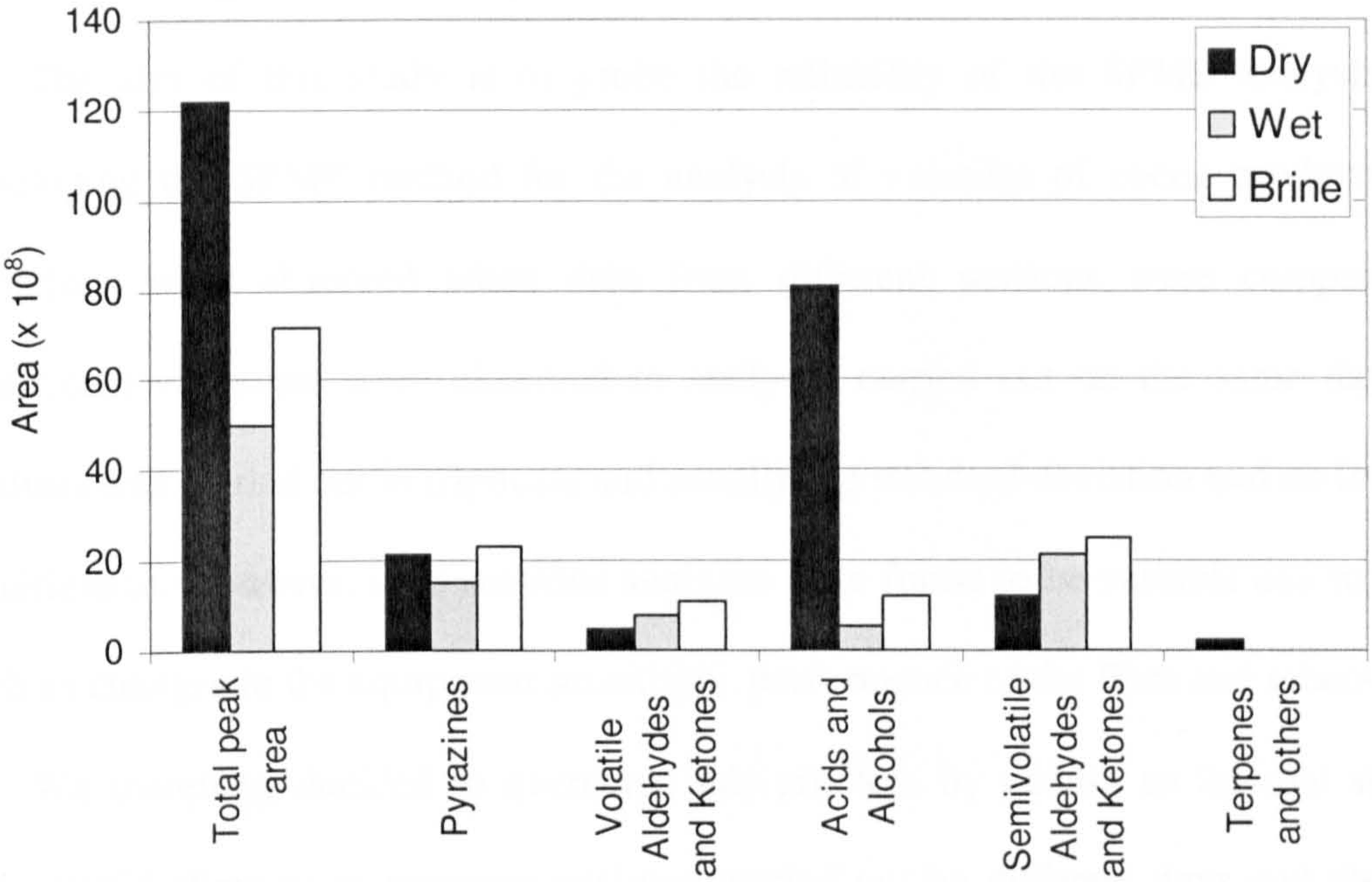


Figure 5.11 Effect of dry, wet and brine conditions on the headspace compounds in NCP by SPME. CAR/DVB-PDMS fibre was used (sampling at 60ºC for 15 min).

Three new pyrazines were tentatively identified under wet and brine conditions, 3,5-Dimethyl-2-isobutylpyrazine (RI 1197), 2-Isoamyl-6-methylpyrazine (RI 1258) and 2,6-Dimethyl-5-isopentylpyrazine (RI 1325). These new pyrazines have longer carbon chains, increasing their hydrophobicity. As a consequence, their presence in the volatile phase is higher and can be detected. On the other hand, a few compounds could not be detected under wet conditions such as dimethylpropanedioic acid, 2,3-butanodiol and α-pinene.

The convenience of using water, brine or dry conditions depends on the targeted compounds. The extraction of these targeted compounds and the lack of extraction of interferences will point to the best conditions. In our case, the results show that water is not desirable for analysis; its performance is enhanced when the ionic strength is increased (brine). Under dry conditions, alcohols and acids exhibit better extraction than the other groups when brine is used.

5.1.2.4 Quantification by SPME

The aim of this study is to probe the reliability of the SPME analysis. After developing the SPME method for the analysis of volatiles of cocoa products, some variations were observed when data from different sessions were compared. No significant variations were observed in analyses carried out on the same day. Each analysis was carried out in triplicate and usually the standard deviation had no important significance. However, inter sessions analyses were found to be variable due to factors, such as changes in the equipment sensitivity, performance of the fibre and others.

We therefore, decided to overcome this problem by adding an internal standard. This would allow us to compare analyses carried out on different days and also make the analysis semi-quantitative. The internal standard is subjected to the same equipment conditions (GC-MS sensitivity and SPME fibre performance) than the analytes but its amount is known and constant for all the samples. If the analytes amounts (peak area) are reported normalized versus the internal standard peak area *A/A(IS)* the variability due to the performance of the system can be corrected.

Isopropyl alcohol and toluene were preliminary chosen as internal standards because they are not naturally occurring in cocoa products, they have retention times that do not mask analyte peaks and have structural similarities with some of the compounds groups extracted from cocoa. Their recovery results revealed the 2-propanol was not suitable due to its very poor extraction. On the other hand, toluene presented excellent recovery (Figure 5.12).

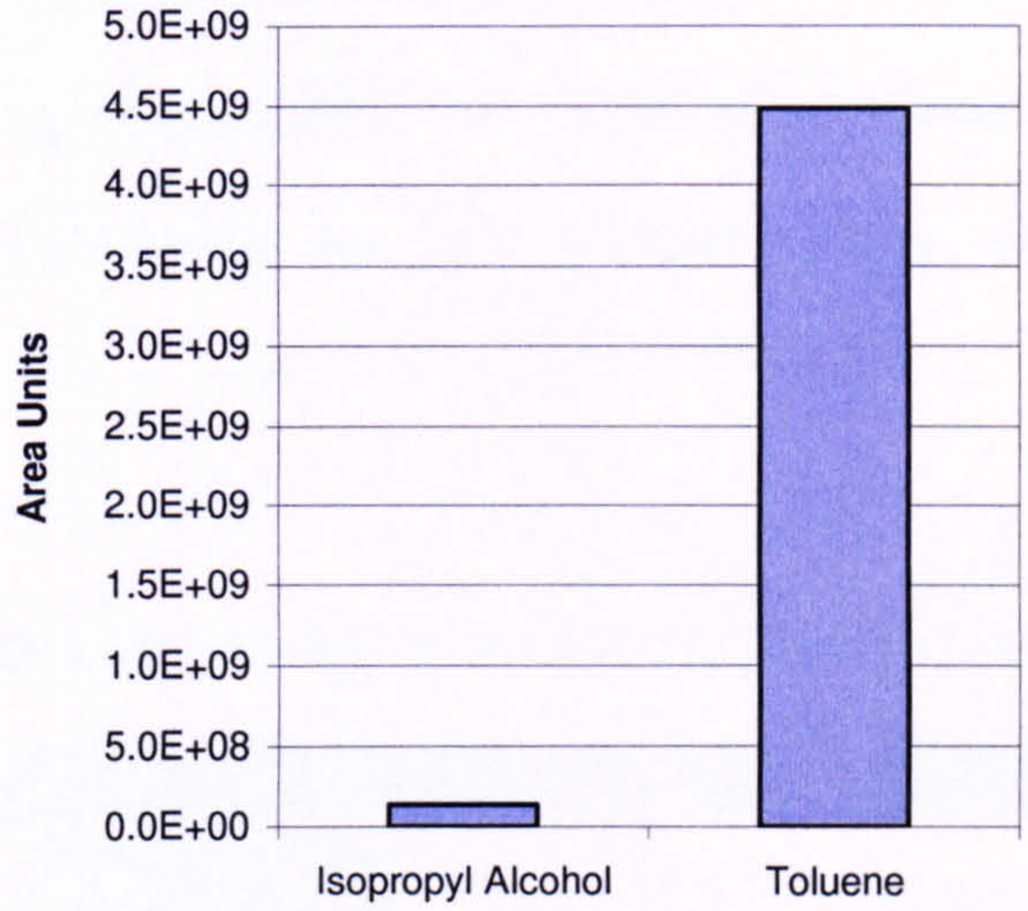


Figure 5.12 Area units obtained for 1 mg of standard extracted using a DVB/CAR-PDMS fibre in optimized conditions

The amount of toluene in the samples was adjusted to a smaller quantity to avoid saturation of the detector and also to prevent it from masking peaks of interest.

In order to assess whether a quantitative analysis could be carried out using SPME fibres, five water solutions of trimethylpyrazine from 0.2 to 4 mg were prepared. Fifty µg of internal standard (IS) was added to each solution. Figure 5.13 shows the representation of the experimental data, before and after normalisation with the internal standard.

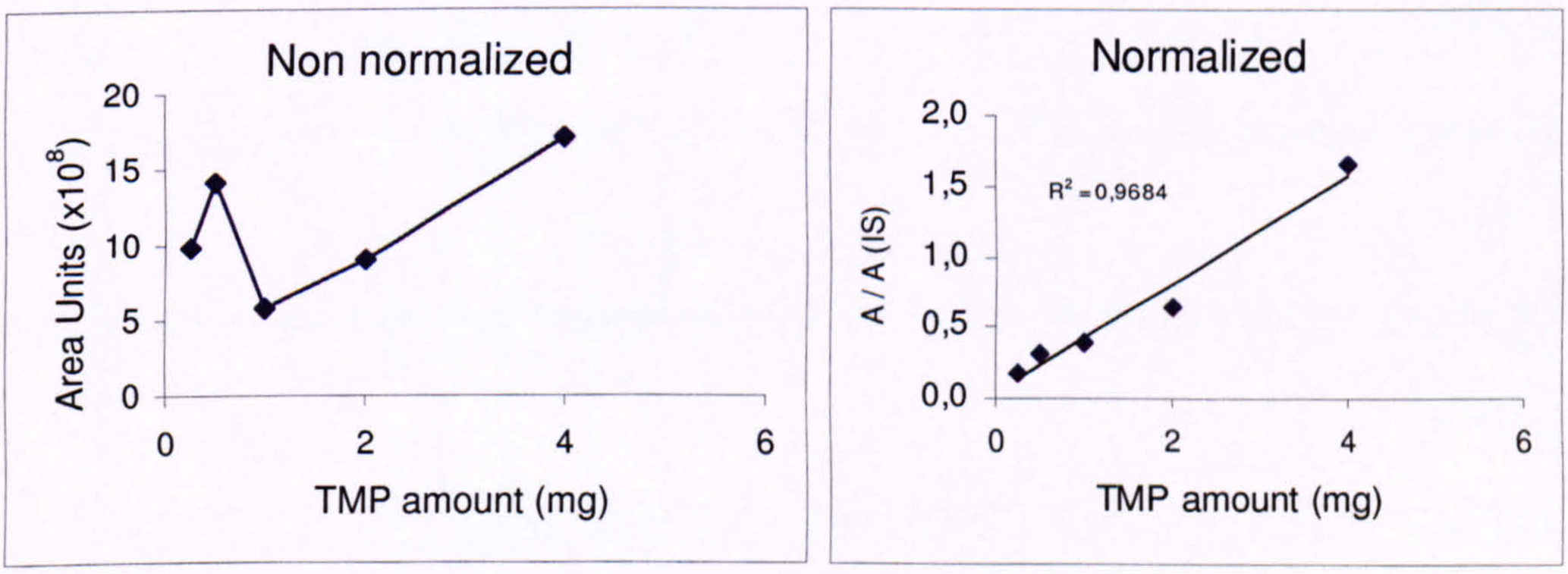


Figure 5.13 Effect of the Internal Standard on the calibration curve of the Trimethylpyrazine.

Next, a water (5 mL) solution containing seven standard compounds, regularly found in cocoa, was prepared (Table 5.6). Four 1/3 dilutions of this solution were prepared to obtain a calibration curve. In each dilution, 50 µg of toluene was added as internal standard (IS).

Table 5.6 List of compounds and their amounts in the initial solution (H_2O 5 mL)

N°	Compound	Amount (mg)
1	Isobutylaldehyde	1
2	Isovaleraldehyde	1
3	2-methylbutylaldehyde	0.5
4	2,3-Dimethylpyrazine	1
5	Trimethylpyrazine	0.5
6	Tetramethylpyrazine	0.5
7	1-furfurylpyrrole	0.5
IS	Toluene	0.05

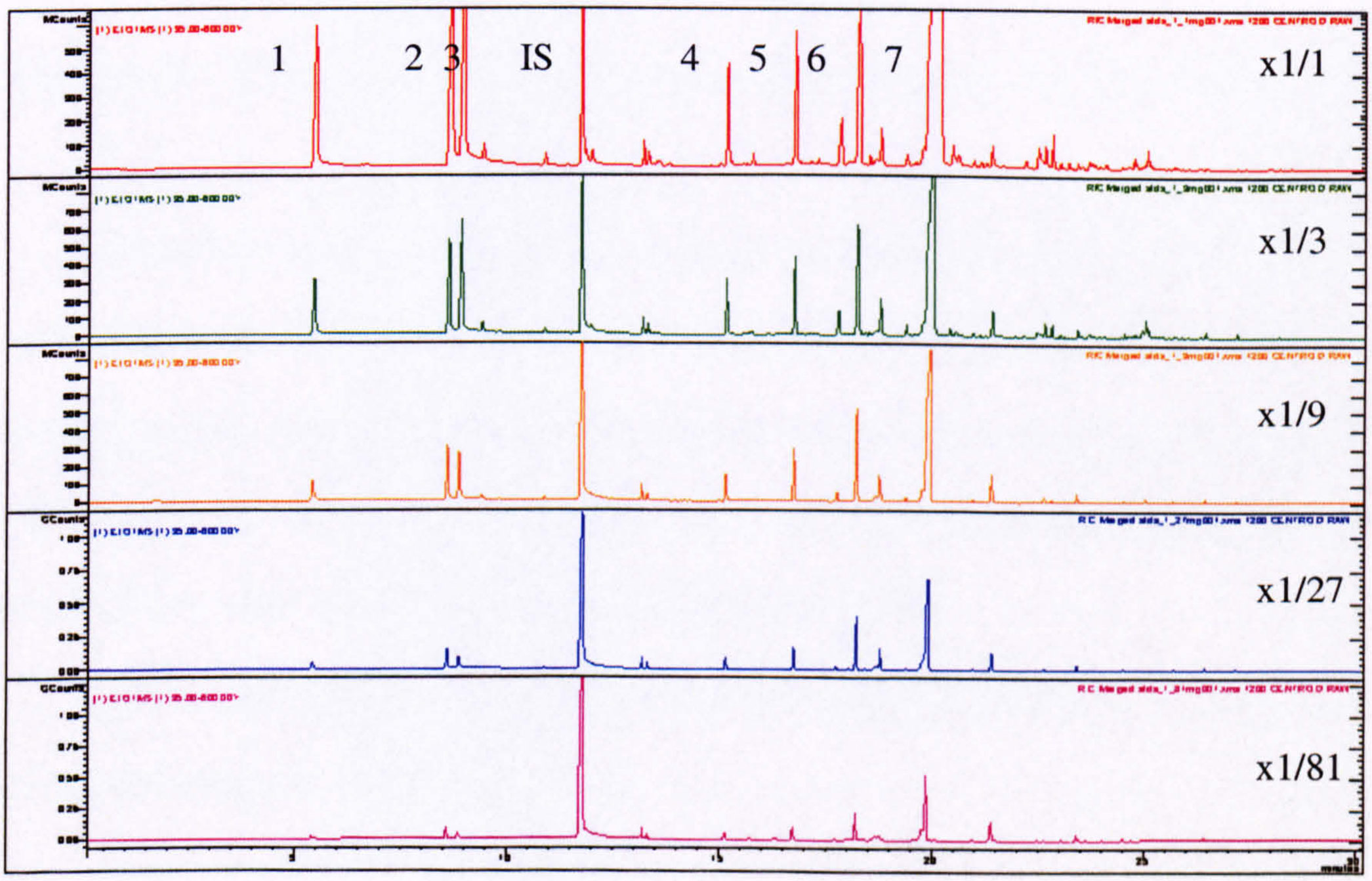


Figure 5.14 Chromatograms recorded after dilution of the standards.

Figure 5.14 shows the chromatograms of the dilutions of the standards solution. The areas under the peaks clearly show that this decrease is proportional to their amount in the solution. The representation of the normalized peak areas with the internal standard peak area versus the concentration are represented in Figure 5.15. All the graphs present excellent correlation with regression coefficients $R^2 > 0.98$.

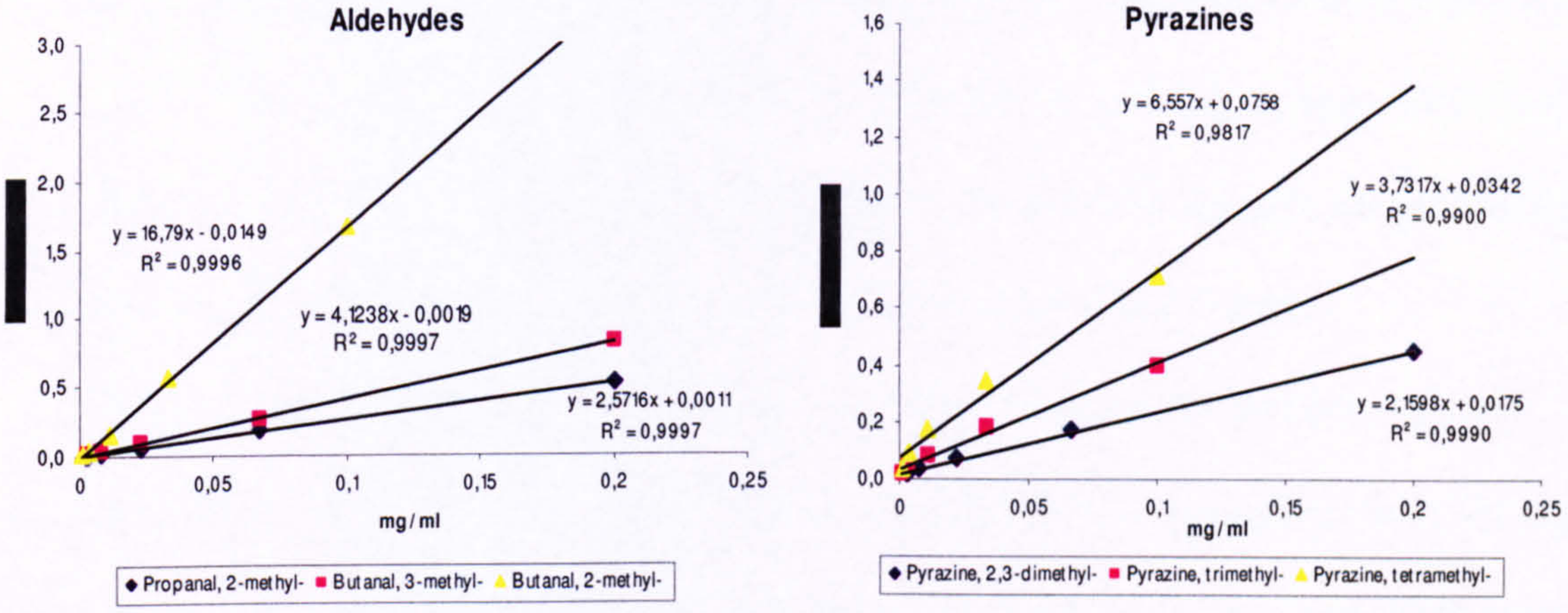


Figure 5.15 Calibration curves of some representative standards obtained using SPME.

Quantitative determination can be carried out by the use of an internal standard which requires a calibration curve of each compound. Quantification in equivalents can also be used by using the method of the internal standard, in which ratios between the responses factors of the individual compounds versus the internal standard must be known. In our case, toluene was used as an internal standard.

Subsequent semi-quantitative determination was accomplished with reference to the internal standard.

5.1.3 Conclusion

The use of SPME fibres to analyse volatiles from cocoa products was optimized. Parameters that influence the performance of these fibres were studied.

From the four fibres assessed, the **DVB/CAR-PDMS** fibre was chosen as the most appropriate fibre. Good recoveries and a good distribution and proportion along the chromatogram of the extracted volatiles and semi-volatiles are its best qualities.

Even though the greater the incubation temperature the higher extraction, **60°C** was chosen to demonstrate a good equilibrium between a good recovery and be a temperature that insured no damage or generation of artefacts in the sample during sampling. At this temperature, the equilibrium was reached after 15 min.

Direct solid SPME was found to have the greatest recovery although the use of saturated aqueous salt solution reported the best extraction of semi-volatile even though with a considerable decrease of the alcohols and acids. Thus, both sampling methods are valid and the choice will depend on interest of the analysis.

The addition of the internal standard to the samples was successful. The data presented shows that after normalization the areas under each peak are proportional to its amount and comparable. The internal standard resolves problems encountered due to variations of factors such as fibre performance, desorption in the injector, equipment sensitivity, etc. These factors still affect the internal standard area and the other compounds; however, it is now possible to normalise the results against the internal standard that is constant.

The linearity of the calibration curves demonstrates that it is possible to perform quantitative analysis with SPME.

On the other hand, it is necessary to mention that the matrix is a key factor in the SPME analysis. The matrix controls the equilibrium between gas phase and liquid/solid

phase. Changes in the matrix have a direct repercussion on the composition of the gas phase, thus in the fibre extraction.

5.2 Evolution of the volatile profile of chocolate during its industrial process

Chocolate is the main product manufactured from cocoa. During its industrial process, the cocoa is mixed with the rest of the ingredients (mainly sugar) and then refined and conched (Section 1.2). Various reports have studied how these steps affect the chemical composition of the final product. [24, 56, 59, 106] The evolution of the volatile profile of chocolate during its industrial process was investigated. The SPME methodology developed in section 5.1.2 was employed, using toluene as internal standard. This study was also used to test the reliability of the method.

The samples were taken from the facilities of Natra Cacao S.L. (Valencia, Spain) by an authorized operator who filled three buckets of 20 kg, after each process stage (mixing, refining and conching) from a 55% cocoa dark chocolate batch. The buckets were hermetically sealed and sent to our laboratory where they were stored at room temperature until their analysis.

5.2.1 Results

The CAR/DVB-PDMS SPME fibre was used to extract the volatiles from the headspace of a sealed vial containing 4 g of sample and 50 µg of internal standard (toluene). Samples were analysed in triplicate to assess the reproducibility of the method.

Although chocolate is a cocoa product, the matrices of the cocoa powder and chocolate are very different. Chocolate at the sampling temperature (60°C) is totally melted, resulting in a fat suspension of solid particles (mainly cocoa and sugar), while

the cocoa powder which is reduced in fat and ground, is essentially cocoa solid particles. These matrix differences have an influence on the composition and proportions of the volatiles found in the headspace, thus the extracts were different to the ones obtained for the cocoa powder in the previous section.

The components were classified taking into account the non-browning reactions that are responsible for the major part of the flavour production (Table 5.7). Thus, aldehydes, mainly produced in the well known Strecker degradation of the Maillard reaction, are the first group. Nitrogen-containing compounds were grouped into pyrazines and pyrroles, which require amino acids or another nitro source for their production. Furans, furanones and pyranones have no nitrogen in their structure, and are non-enzymatic browning products generally produced in the dehydration processes of sugars. Acids and alcohols were grouped together as they are an important class of oxidized products that come from the fermentation step or are generated as by-products of other reactions or simple oxidation of aldehydes. Ketones and esters are a minor group with only four compounds including caffeine.

Some new compounds were tentatively identified such as furans, for example furfural or 1-(2-furanyl)-propan-1-one, a couple of pyrazines like 3,5-diethyl-2-methyl-pyrazine or 2.6-dimethyl-5-isopentyl-pyrazine, two acids (hexanoic acid and 2-ethyl-hexanoic acid) previously not detected and significant in this samples.

Table 5.7 Compounds extracted from samples of different steps of the chocolate process. (Area/IS Area)

No	Compounds	RI	Mixing	Refining	Conching
3	2-methyl-Propanal	550	0.114 ±0.001	0.191 ±0.022	0.186 ±0.012
7	3-methyl-Butanal	653	0.235 ±0.015	0.372 ±0.025	0.373 ±0.030
8	2-methyl-Butanal	662	0.425 ±0.015	0.491 ±0.064	0.439 ±0.066
28	Benzaldehyde	981	1.982 ±0.228	2.229 ±0.138	1.528 ±0.117
34	Benzeneacetaldehyde	1062	0.809 ±0.051	0.843 ±0.049	0.526 ±0.028
53	5-Methyl-2-phenyl-2-hexenal	1480	0.013 ±0.002	0.017 ±0.002	N.D
Aldehydes			3.578 ±0.265	4.144 ±0.164	3.051 ±0.254
17	Methylpyrazine	832	0.991 ±0.065	1.154 ±0.082	0.735 ±0.017
23	2.5-Dimethyl-pyrazine	921	1.225 ±0.038	1.590 ±0.110	0.581 ±0.008
24	Ethyl-pyrazine	926	0.130 ±0.022	0.151 ±0.002	0.073 ±0.004
25	2.3-Dimethyl-pyrazine	928	0.309 ±0.087	0.438 ±0.050	0.203 ±0.002
29	2-Ethyl-6-methyl-pyrazine	1013	0.089 ±0.004	0.117 ±0.012	0.040 ±0.001
30	Trimethyl-pyrazine.	1011	0.969 ±0.024	1.302 ±0.101	0.350 ±0.023
32	1H-Pyrrole-2-carboxaldehyde	1018	0.296 ±0.017	0.395 ±0.045	0.219 ±0.003
35	2-Acetylpyrrole	1072	0.637 ±0.033	0.890 ±0.090	0.437 ±0.009
36	3-Ethyl-2.5-dimethyl-pyrazine	1086	0.152 ±0.021	0.212 ±0.036	0.057 ±0.002
37	Tetramethyl-pyrazine	1094	1.811 ±0.098	2.178 ±0.227	0.679 ±0.075
43	3.5-Diethyl-2-methyl-pyrazine	1167	0.051 ±0.003	0.068 ±0.006	0.059 ±0.010
47	2-Butyl-3.5-methyl-pyrazine	1212	N.D.	0.020 ±0.012	N.D.
50	2.6-Dimethyl-5-isopentyl-pyrazine	1325	0.039 ±0.002	0.048 ±0.008	0.016 ±0.001
Pyrazines & Pyrroles			6.936 ±0.356	8.837 ±0.681	3.604 ±0.151
13	3.5-Dimethyl-dihydro-furan-2-one	766	0.061 ±0.007	0.039 ±0.023	0.068 ±0.005
19	Furfural	842	0.450 ±0.016	0.486 ±0.034	0.342 ±0.017
21	2-Furanmethanol	854	0.248 ±0.016	0.258 ±0.013	0.213 ±0.009
22	1-(2-Furanyl)-ethanone	878	0.176 ±0.012	0.206 ±0.012	0.101 ±0.010
31	1-(2-furanyl)-propan-1-one	1035	0.296 ±0.017	0.395 ±0.046	0.219 ±0.003
44	2.3-Dihydro-3.5-dihydroxy-6-methyl-pyran-4-one	1182	0.239 ±0.041	0.274 ±0.033	0.154 ±0.021
54	3.4-Dihydro-8-hydroxy-3-methyl-2-Benzopyran-1-one	1565	0.015 ±0.001	0.019 ±0.008	N.D.
Furans. furanones & pyranones			1.484 ±0.100	1.679 ±0.108	1.097 ±0.049
6	Acetic acid	630	58.604 ±4.608	72.363 ±6.386	59.343 ±6.418
10	3-Hydroxy-2-butanone	707	0.442 ±0.035	0.524 ±0.021	0.351 ±0.053
12	Dimethylpropanedioic acid	755	0.847 ±0.227	0.925 ±0.234	0.555 ±0.028
15	2.3-Butanediol*	782	20.036 ±1.364	23.724 ±1.355	19.986 ±0.625
16	2.3-Butanediol*	792	8.567 ±0.482	9.212 ±0.686	7.504 ±0.466
18	3-Methyl-butanoic acid	837	4.734 ±0.335	5.459 ±0.367	2.851 ±0.165
20	2-Methyl-butanoic acid	847	1.463 ±0.051	1.641 ±0.118	0.832 ±0.059
27	Hexanoic acid	946	0.772 ±0.015	1.255 ±0.100	0.595 ±0.072
33	Benzyl Alcohol	1048	0.161 ±0.016	0.217 ±0.009	0.123 ±0.009
39	2-Ethyl-hexanoic acid	1128	10.245 ±1.076	18.437 ±1.595	7.327 ±1.552
41	Phenylethyl Alcohol	1133	1.282 ±0.066	1.803 ±0.180	0.739 ±0.061
42	Benzoic Acid	1157	0.218 ±0.020	0.256 ±0.050	0.126 ±0.015
51	1-Butanol. 3-methyl-. benzoate	1404	0.139 ±0.014	0.170 ±0.013	0.070 ±0.001
Acids & Alcohols			107.51 ±7.08	135.99 ±10.12	100.40 ±9.47
1	Acetone	495	0.106 ±0.016	0.223 ±0.039	0.320 ±0.062
2	Methyl ester acetic acid	521	0.177 ±0.031	0.218 ±0.038	0.223 ±0.008
4	2.3-Butanedione	581	0.127 ±0.029	0.209 ±0.010	0.104 ±0.013
55	Caffeine	1783	0.034 ±0.024	0.056 ±0.015	0.029 ±0.006
Ketones & Esters			0.444 ±0.100	0.706 ±0.071	0.677 ±0.076

All runs preformed with extraction at 60°C for 15 min in dry conditions. Column 30 m x 0.25m m ID 1 µm IF Factor 4. **N.D.** Compound not detected.

5.2.2 Discussion

In general, the behaviours were similar for all the groups (Figure 5.16). The refined sample had the largest recovery of volatiles and the conched had the minor. Our hypothesis about this finding relates to the size reduction of the particles and the fat interaction. The mixed ingredients (55% cocoa liquor, 44% cane sugar) were introduced into a six-roller refiner, where they were subjected to high pressures. The temperature in the roller can be elevated for short periods of time due to friction and particle reduction can favour the generation and release of volatiles.

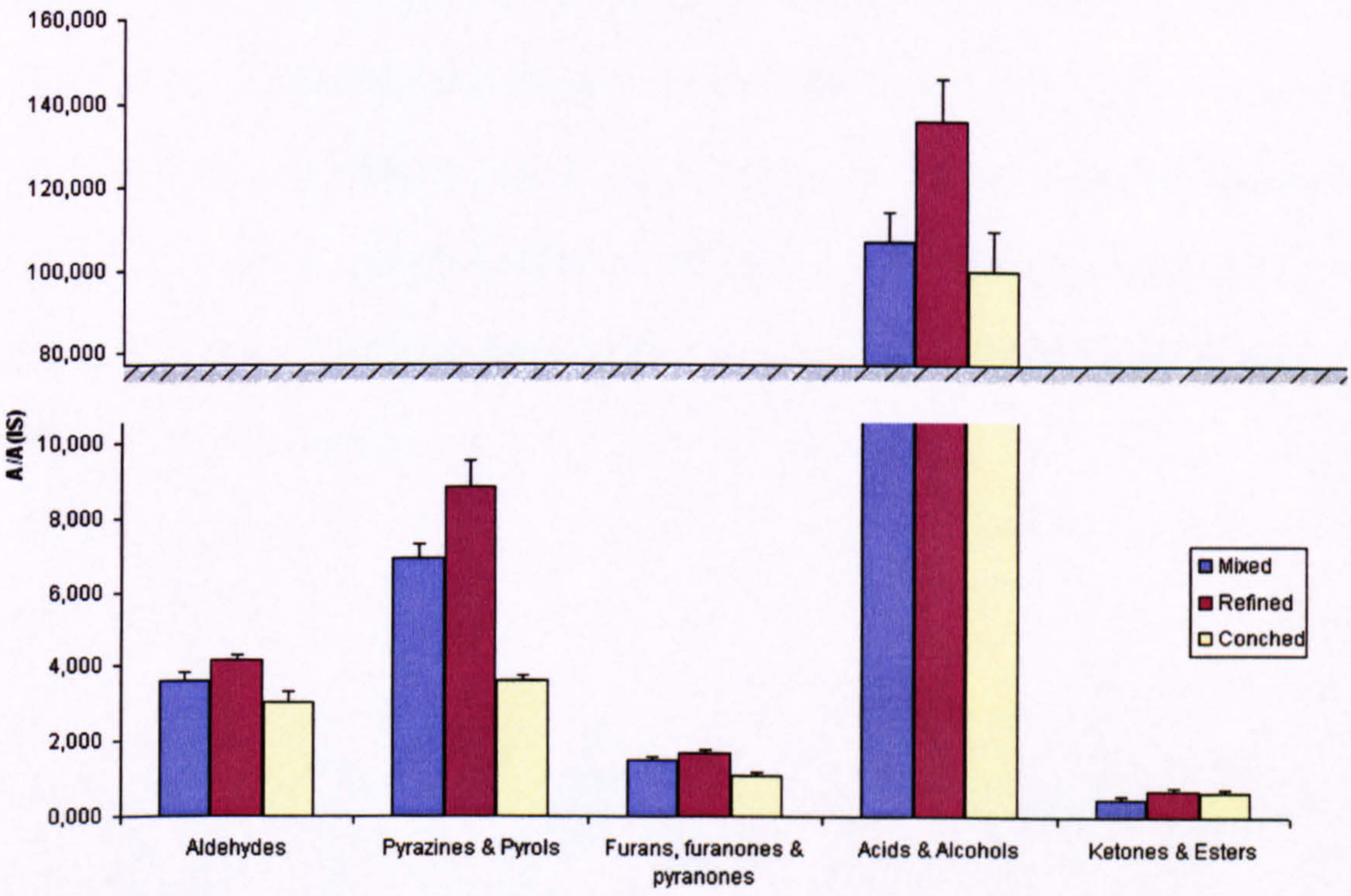


Figure 5.16 Representation of the values for each component group for the three-sampled process stages of the chocolate manufacture. Due to the big difference between the acids and alcohols group and the others, the scale has been cut to accommodate the higher part of the bars.

A general decrease was observed after the conching. This can be explained by the loss of volatiles due to heating, intense kneading and long processing time (around 8 hours). Conching takes place in specially designed kneaders that are open to promote

the elimination of some off-flavours like acetic acid. Another important aspect of the conching is the coating with fat of those new small particles generated during the refining. This coating may affect the capacity of the compounds that are located in the solid particles to be released. Volatiles might be trapped in the solid particles that are surrounded by the fat (Figure 5.17), explaining the general decrease in volatiles after the conching. This reduction is especially important in the pyrazines and pyrroles group with more than 50% decrease. The low polarity of these compounds and their high solubilisation in fat may offer an explanation. Some authors reported increases of alkyl pyrazines and short aldehydes after the conching. [24, 59, 106] In these reports, analyses were carried out using traditional extraction methods like steam distillation and organic solvent extraction of the distillate, essentially defating the samples. The coating of the particles was removed and had no effect on the extraction. In our extraction method, we sampled the volatiles and semi-volatiles that the chocolate releases into the headspace, in a manner that is closer to what the human nose would smell avoiding the generation of any artefact.

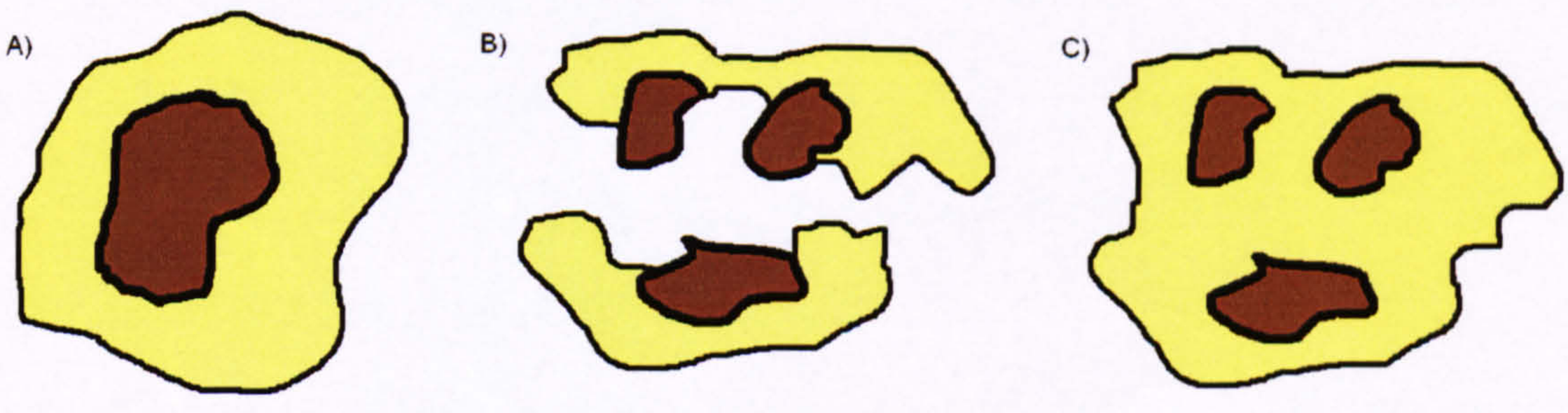


Figure 5.17 Scheme of the state of the solid particles versus the fat in the states of the chocolate process A) Mixing B) Refining C) Conching

The acid and alcohol group was the largest recovery with an important difference compared to the others groups. Looking at particular components in this group, acetic acid, usually accounted for more than 50% of this group. This large presence of the acids and alcohols can be attributed to the influence of the fat in the matrix. Chocolate is composed of around 30% cocoa fat, which is essentially apolar and in this environment of long chain fatty acids, the compounds with high polarity such as acids and alcohols leave the matrix increasing their concentration in the headspace. This correlates with the results obtained in our study. This concentration is considerably higher than that extracted from cocoa powder.

Looking at the performance of the analytical methodology, the results were satisfactory. In average, the standard deviation for the samples (done in triplicate) was reduced from 22 % (samples without internal standard) to 7 % (samples with internal standard). The use of toluene as internal standard allowed us to increase both the reproducibility and robustness of the method.

5.2.3 Conclusion

The sample matrix had a strong influence on the extraction of volatiles from the headspace. In the case of chocolate, the fatty matrix enhances the release of polar and hydrophilic compounds. Acid and alcohol group presented the highest recovery with the acetic acid being the most extracted compound.

The composition of the volatile fraction depends on the physical processes that occurred in these industrial stages. The volatiles extracted in the refined samples were higher than in the mixed samples. The particle reduction is one explanation for this increase. Big particles are broken into smaller ones favouring the release of volatile

compounds. The other explanation is the production of more volatiles due to the physical process involved in refining, i.e., rollers cause sudden high temperature changes due to friction leading to volatile formation. Conched samples presented the smallest recovery. During this stage, the new surfaces created in the refining step were coated with fat; thus, the heating (70-90°C) and long conching times (8-12 hours) are probably responsible for this poor extraction as volatiles can be lost in the open conche.

Finally, the results allowed us to confirm the sensitivity and robustness of the methodology. The use of internal standard allows us from now on to work with only one analysis per sample.

5.3 Characterization of the cocoa powders

Several cocoa powders were acquired at the beginning of the project from Natra Cacao. S.L (Valencia. Spain). Table 5.8 shows the list of cocoa powders received with their description.

Table 5.8 List of cocoa powders with their fat content and sensorial description. All these cocoa powders are commercially available from Natra Cacao S.L.

Name	Fat content	Alkali	Sensorial description
G-10 G-20	10-12 % 20-22 %	No	Very bright brownish colour. Natural taste. slightly acidic and fruity.
T-1 T-10 T-20	0-1 % 10-12 % 20-22 %	Yes	Dark red colour. Intense bitter chocolate taste and strong aroma.
A-10	10-12 %	Yes	Light brown colour. Mild chocolate taste and cocoa aroma.
M-10	10-12 %	Yes	Light reddish-brown tones. Mild in aroma and taste. cocoa just slightly astringent
C-10	10-12 %	Yes	Dark reddish colour. Rounded chocolate taste and aroma.
V-10	10-12 %	Yes	Dark brown chocolate colour. Sweet spicy aroma and full cocoa taste.

There were two natural (G-10 and G-20) and seven alkalized (T-10, T-20, A-10, M-10, C-10, V-10) cocoa powders with two different fat contents (10-12% and 20-22%), obtained by pressing processes. There was also T-1 (0-1% of fat) a non-fat cocoa obtained by supercritical fluid (CO_2) extraction from T-10. The cocoa powders were stored in hermetically sealed boxes protected from the direct sun light at room temperature.

The cocoa powders were characterized by analysing some parameters like the volatiles profile, caffeine and theobromine content, pH, total polyphenol content (TPC), amount of free amino groups (FAG).

These cocoa powders have been produced following confidential recipes, which were not disclosed, but the manufacturer did provide some information such as pH and other indications about used processes (Table 5.9). The pH was measured as a 10% aqueous suspension of cocoa powder.

Table 5.9 Guidelines of the processes used to obtain the diverse cocoas and their pH.

Name	Fat content	Alkali	Red factor	Indicated pH	Measured pH*
G-10 G-20	10-12 % 20-22 %	No	-	5.6	5.80
T-1 T-10 T-20	0-1 % 10-12 % 20-22 %	Yes	-	8	7.96
A-10	10-12 %	Yes	-	7	6.80
M-10	10-12 %	Yes	+	7	6.75
C-10	10-12 %	Yes	+	8	7.80
V-10	10-12 %	Yes	-	7	7.10

* measured with pH meter.

Regular nib roasting process (Section 1.2.3.4) was used to roast the cocoas. The broken cocoa nibs were introduced into a big drum heated at 130ºC; this temperature

was maintained for 5 min. The alkali solution (50% aqueous K_2CO_3) was added in a step prior to roasting.

G-10 and G-20 are natural cocoa powders; these cocoas were not alkalised during the process. All the other cocoa powders in the Table 5.9 were alkalised. The indicated pH directly relates to the amount of alkali added during their processes. The higher the indicated pH, the higher the amount of alkali used.

The red factor refers to the air and water introduced to produce reddish colours in the cocoa powder. These favour oxidation with the melanoids and polyphenols producing red colour notes. [12]

5.3.1 Volatile profiles

Four grams of each cocoa powders were sealed in a head space vials with IS (toluene). The sample was incubated for 15 min at 60°C. The CAR/BVD-PDMS SPME fibre was used to sample the volatiles (Table 5.10 & Figure 5.18).

Overall, T-1 is the cocoa powder with the highest volatiles recovery, followed by A-10 and G-10. The *fat content* obviously plays an important role in the ability of these compounds to be extracted efficiently. A similar effect was observed when the refined and the conched samples were compared in the chocolate manufacture study (Section 5.2). The coating of the solid cocoa particles with fat led to a general decrease in volatiles extracted (Figure 5.17). In this case, the absence of fat allows release of the volatiles, increasing their concentration in the headspace of the vial. This effect can also be observed in samples from the same family that differ in the fat content. For example, **G-10** (10 % fat) presents a larger recovery than **G-20** (20 % fat), the same occurs in the **T** family of cocoa powders.

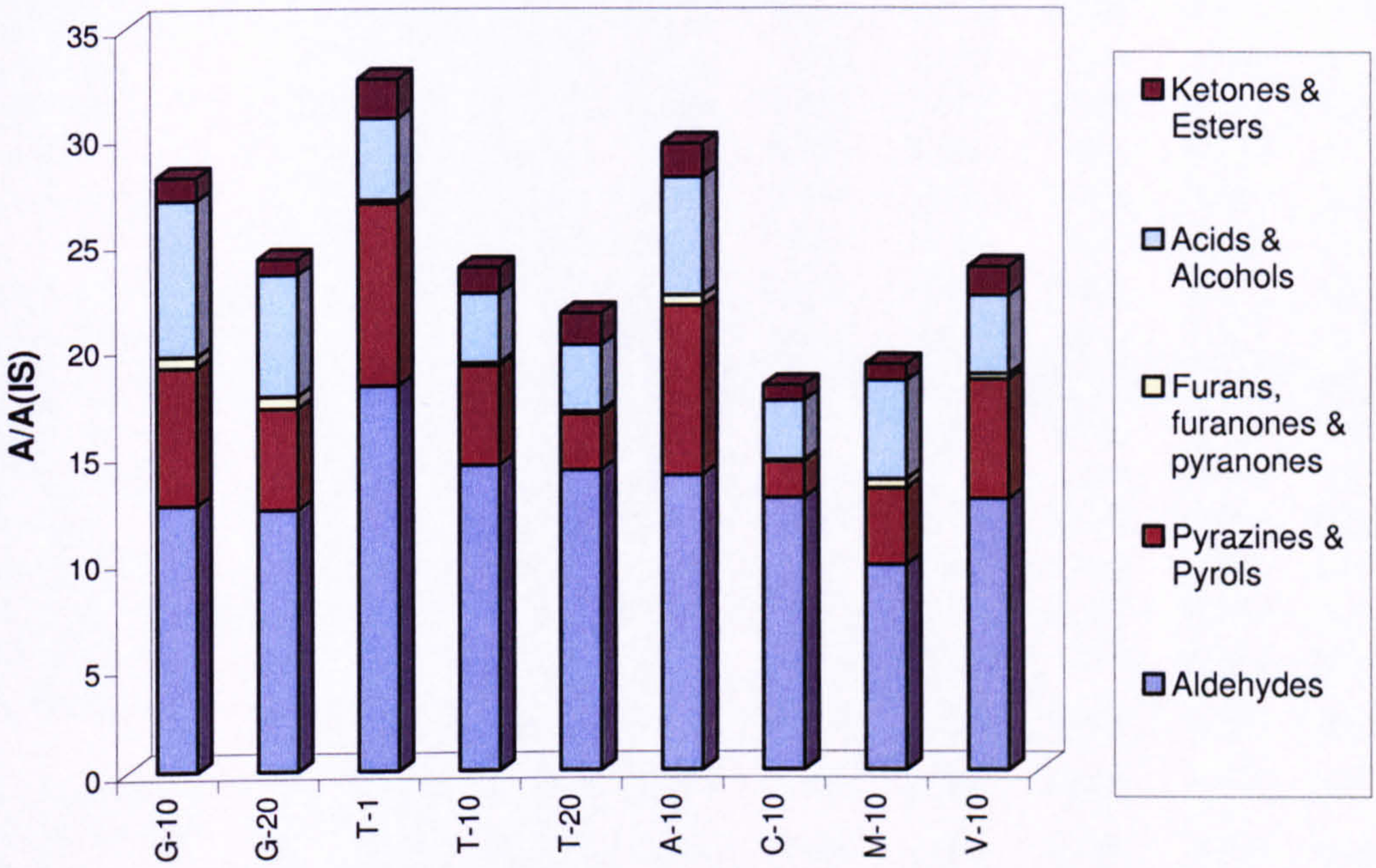


Figure 5.18 Bar representation of the volatiles extracted by SPME for each cocoa powder classified by groups.

Looking at different cocoa powders with the same fat content to compare other factors, we notice that natural cocoa powders had richer volatile profiles than the alkalized cocoas (with the exception of **A-10**). The *pH/alkalisation* has a significant decreasing effect. This effect is, logically, more acute in the acid and alcohol group where the grade of alkalisation is clearly reflected and cocoa powders with highest pH, **T** and **C** families, presented the poorest profiles. Furans, furanones and pyranones were also poorly found in these strong alkalized cocoas having their smallest concentration. It is clear that the alkalisation affects this family. The furans, furanones and pyranones group is mainly responsible for the caramel notes.

Cocoas with positive *red factor*, **M-10** and **C-10**, (also alkalised) had the poorest overall volatiles profile of the cocoa powders studied. The use of extra amount of water and injection of air during their roasting process is probably the cause of this loss of

Table 5.10 Volatile profile of the cocoa powders available in the study [A/A(IS)].

Compounds	G-10	G-20	T-1	T-10	T-20	A-10	C-10	M-10	V-10
2-Methyl-propanal	0.460	0.459	0.913	0.678	0.712	0.572	0.498	0.288	0.420
3-Methyl-butanal	1.090	1.403	2.362	1.895	1.747	1.644	1.360	0.724	1.192
2-Methyl-butanal	0.961	0.977	2.479	1.752	1.493	1.258	1.513	0.750	1.161
Benzaldehyde	5.813	5.581	7.979	6.391	6.351	7.018	6.287	5.125	5.545
Benzene acetaldehyde	4.106	3.840	3.860	3.544	3.701	3.254	3.067	2.717	4.350
Nonanal	0.013	N.D.	N.D.	N.D.	0.014	N.D.	N.D.	N.D.	N.D.
3.5-Dimethyl benzaldehyde	0.065	0.036	N.D.	0.033	0.055	0.052	0.012	0.035	0.035
5-Methyl-2-phenyl-2-hexenal	0.037	0.021	0.494	0.062	0.043	0.044	0.027	0.013	0.025
Aldehydes	12.545	12.318	18.087	14.355	14.116	13.843	12.765	9.651	12.728
Methyl-pyrazine	0.538	0.611	0.445	0.425	0.311	0.742	0.203	0.419	0.460
2.5-Dimethyl-pyrazine	0.863	0.941	1.549	1.271	0.726	1.234	0.440	0.936	1.544
Ethyl-pyrazine	0.236	0.257	0.132	0.115	0.067	0.329	0.063	0.203	0.207
2.3-Dimethyl-pyrazine	0.341	0.266	0.111	0.105	0.073	0.146	0.007	0.057	0.071
2-Ethyl-6-methy pyrazine	0.401	0.344	0.544	0.318	0.129	0.737	0.099	0.312	0.415
Trimethyl-pyrazine	1.488	0.753	1.408	0.947	0.504	1.583	0.319	0.642	1.086
3-Ethyl-2.5-dimethyl-pyrazine	0.472	0.334	1.354	0.664	0.259	1.087	0.204	0.294	0.746
Tetramethyl-pyrazine	0.955	0.535	0.490	0.389	0.279	0.685	0.099	0.129	0.235
3.5-Diethyl-2-methyl-pyrazine	0.343	0.119	0.650	0.168	0.054	0.486	0.055	0.122	0.249
2-Butyl-3.5-methyl-pyrazine	0.101	0.056	0.493	0.118	0.034	0.243	0.042	0.085	0.147
2.6-Dimethyl-5-isopentylpyrazine	0.168	0.068	1.223	0.001	0.045	0.356	0.051	0.106	0.247
2-Carboxaldehyde-pyrrole	0.113	0.134	0.087	0.028	0.054	0.162	0.050	0.097	0.091
2-Acetylpyrrole	0.405	0.319	0.137	0.100	0.076	0.220	0.063	0.154	0.108
Pyrazines & Pyrroles	6.427	4.740	8.624	4.652	2.611	8.013	1.694	3.558	5.607
Dihydro-2-methyl-3-furanone	0.010	0.009	N.D.	N.D.	N.D.	0.008	N.D.	N.D.	N.D.
Furfural	0.178	0.216	0.031	0.029	0.030	0.125	0.023	0.080	0.052
2-Furanmethanol	0.065	0.081	0.011	0.019	0.012	0.101	0.016	0.140	0.084
2-Acetilfuran	0.099	0.095	0.046	0.038	0.031	0.055	0.029	0.040	0.031
1-Propanone-2-furanyl	0.113	0.133	0.083	0.028	0.052	0.163	N.D.	0.097	0.090
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0.044	0.013	N.D.	N.D.	N.D.	N.D.	N.D.	0.042	N.D.
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0.006	0.011	0.009	0.012	0.011	0.018	0.005	0.010	0.005
Furans. furanones & pyranones	0.515	0.557	0.181	0.126	0.135	0.469	0.073	0.407	0.262
Acetic acid	2.684	1.532	0.648	0.438	0.366	1.201	0.374	1.647	0.974
3-Methyl-butanoic acid	2.482	2.273	1.744	1.537	1.647	2.733	1.639	1.904	1.471
2-Methyl-Butanoic acid	1.030	0.961	0.228	0.186	0.198	0.493	0.141	0.308	0.296
Benzyl Alcohol	0.171	0.149	0.237	0.192	0.179	0.187	0.158	0.120	0.153
Phenylethyl Alcohol	1.044	0.811	0.973	0.952	0.746	0.981	0.481	0.657	0.825
Benzoic Acid	0.017	0.033	N.D.	0.014	N.D.	0.028	N.D.	0.013	0.014
Acids & Alcohols	7.428	5.758	3.831	3.319	3.136	5.624	2.793	4.649	3.732
Acetone	0.390	0.245	0.421	0.253	0.379	0.360	0.169	0.190	0.278
Acetic acid. methyl ester	0.143	0.100	0.476	0.478	0.373	0.609	0.209	0.293	0.552
2.3-Butanedione	0.287	0.104	0.075	N.D.	0.077	0.072	0.040	0.037	0.060
2-Butanone	0.246	0.251	0.811	0.501	0.653	0.468	0.300	0.262	0.449
Isoamyl benzoate	0.024	0.048	0.027	0.028	0.023	0.044	N.D.	0.010	0.019
Ketones & Esters	1.090	0.748	1.809	1.260	1.506	1.553	0.717	0.793	1.357

All runs preformed with extraction at 60ºC for 15 min in dry conditions. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4 N.D. Compound not detected.

volatiles. The water eliminated via evaporation will behave like in the steam distillation (section 5.1.1.1) dragging out volatiles and the injection of air favours this effect as well as oxidising reactions. The pyrazine and pyrroles group presents their poorest values on these cocoa powders with positive red factor. The alkalisation and the red factor had a negative effect on the production of pyrazines and pyrroles, therefore **C-10**, with the highest pH (8) and positive red factor, had the lowest concentration. These results are in agreement with those previously presented by Bonvehí and Ventura. [41] They argued that at high pH, the partial oxidation (deamination) of the free amino is increased and this leads to an underestimation of amino acids actually consumed by the Maillard reaction.

5.3.2 Other analyses

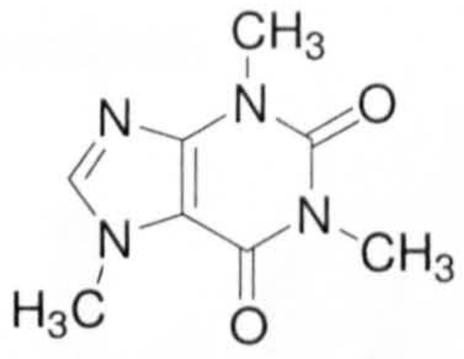
Other parameters studied were the content in caffeine and theobromine, the amount of free amino groups and the total polyphenol content (Table 5.11).

The methylxantines (caffeine and theobromine, Figure 5.19) are the main compounds responsible for the bitterness of the cocoa. It is well known that cocoa is the main natural source of theobromine and its concentration is much higher than that of caffeine. Their concentration was determined by reverse phase HLPC analysis using a UV detector of the methanolic extracts. In general, it was found that the ratio between theobromine and caffeine is around 18, this result is in disagreement with the ones reported in the USDA National Nutrient Database for Standard Reference (Release 18, 2005) for unsweetened cocoa powder. The value of theobromine is similar but the value of caffeine is around 50% higher, giving a ratio theobromine/caffeine of nine.

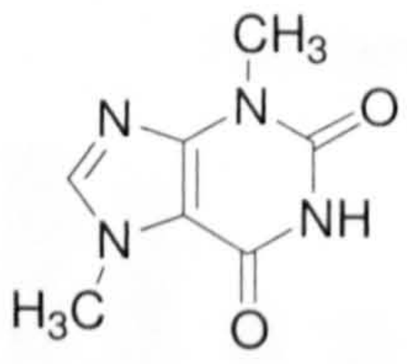
Table 5.11 Content of caffeine (mg/g), theobromine (mg/g), total polyphenol (mg GAE/g) and free amino group (mmols –NH2/g) for each cocoa powder.

	Caffeine (mg/g)	**Theobromine (mg/g)**	**Total polyphenol (mg GAE/g cocoa)**	**Free $-NH_2$ (mmols $-NH_2$ per g cocoa)**
G-10	1.73	23.96	125.0	0.150
G-20	1.53	19.49	115.0	0.116
T-1	---	---	104.8	0.080
T-10	1.62	23.50	72.5	0.074
T-20	1.19	21.48	69.7	0.073
A-10	1.83	21.59	65.9	0.114
C-10	0.65	28.31	63.3	0.070
M-10	0.83	26.52	47.9	0.100
V-10	1.20	26.56	88.4	0.111
R^2	0.9992	0.9994	0.9994	0.9989
ST2R	5.3%	2.4%	3.1%	4.6%

Determinations were carried out using an external standard calibration curve. Regression coefficient square and the deviation of the standard reanalyzed after the sequence of samples in its second level of concentration.



Caffeine



Theobromine

Figure 5.19 Structures of Caffeine and Theobromine

The total phenolic content (TPC) is directly related to the antioxidant properties. Polyphenols have been submitted to intense studies in recent years to determine their health properties. The antioxidant capacity of the polyphenols gives them "quasi magical" virtues. They have been reported to exhibit anti-carcinogenic, anti-atherogenic, anti-ulcer, anti-thrombotic, anti-inflammatory, and many other healthy properties. [107-109] Polyphenols were quantified using the Folin-Ciocalteu method

adapted from Georgé. [110] The Folin-Ciocalteu method is a colourimetric analysis based in the reduction of phosphotungstic and phosphomolybdic acids during the phenol oxidation. Blue coloration of the complex is followed at 760 nm and reflects the quantity of polyphenols, expressed as gallic acid equivalent (GAE) (Figure 5.20).

The quantification of primary amines from amino acids, peptides and proteins is interesting since these are involved in the Maillard reaction (Section 2.2) and may be used as an indicator of the flavour development capacity of the cocoa powders. The free amino group (FAG) was determined using a spectrophotometric assay developed by Church. [111] This method is based in a specific reaction between *o*-phthaldialdehyde (OPA) and 2-mecaptoethanol with the primary amines of amino acids, peptides and proteins. The absorption of OPA adducts of proteins, amino acids and peptides are very similar, at λ_{max} 334 nm (Figure 5.20).

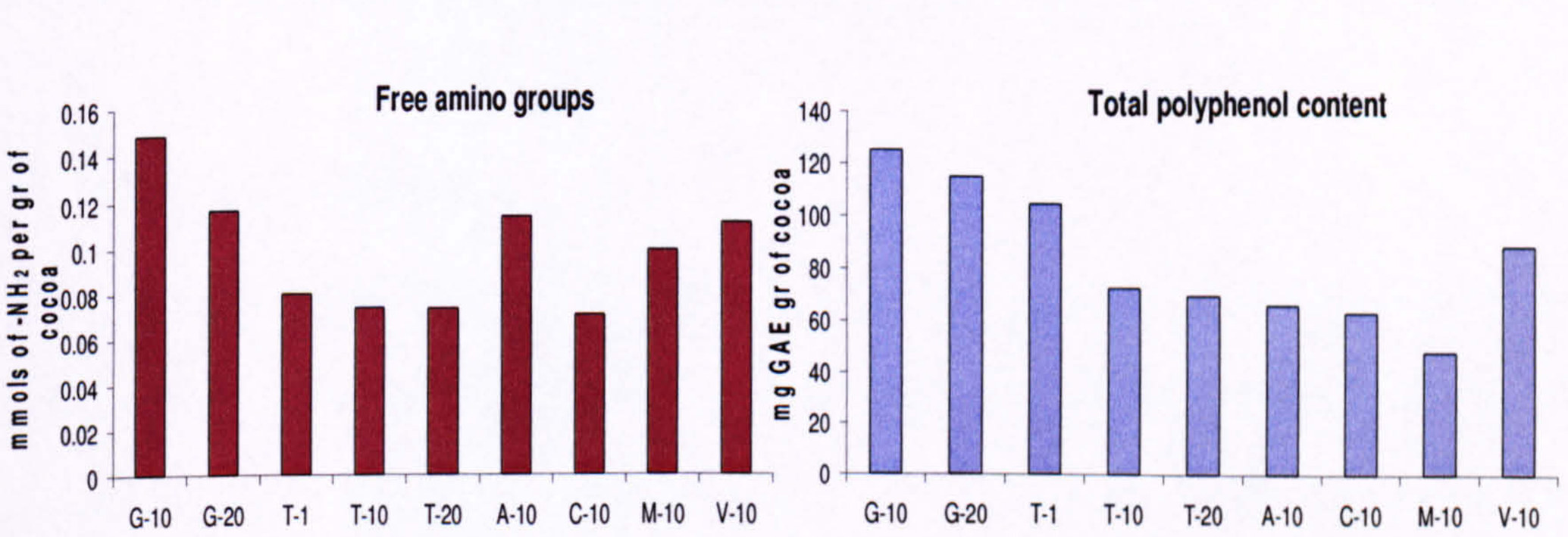


Figure 5.20 Determination of FAG (in mmol –NH2 per g of cocoa, right) and TPC (mg GAE per g cocoa, left) in cocoa powders.

The alkalisation step produces a change in the colour and in the flavour of the cocoa. In general, non-alkalized cocoas (**G** family) were found to contain higher amounts of polyphenols and free amines than the alkalised cocoas (Figure 5.20). The alkalisation step decreases the nutritional value of these cocoas [13]. Our results

indicate that the polyphenols are directly affected by the alkalisation and by the red factor, with the two cocoa powders with red notes (**M-10** and **C-10**) being the lowest. This also shows that the introduction of extra air and water during the process has a detrimental effect on the amount of polyphenols. The combination of the extra air and water to obtain the red effect has also oxidising effects which probably decrease the polyphenol content.

On the other hand, the free amines are clearly driven by the pH of the cocoa. Cocoa powders with higher pH (**T** and **C** families) contain the lowest amount of free amines. Bonvehí and Ventura [41] reported that samples roasted under alkaline conditions (pH 7.20 – 7.92) exhibited darker brown colour formation. These effects were explained by the alkalization of the nibs favouring the oxidative deamination of free amino acids by some polyphenols to produce brown products (polymerization). This explanation is in agreement with the results of FAG we obtained.

5.4 Sensorial evaluation

The development of this project relies heavily on the characterisation of the generated products by chemical analysis, which we have widely been discussed in this chapter, but also on their sensorial evaluation. The sensorial evaluation will determine the acceptance of the products on the market. This parameter is the most important because it will be the key factor for its commercial success.

The sensorial evaluation of the flavour of cocoa and chocolate was carried out by a sensorial panel of Natraceutical S.L. located in their facilities (Valencia, Spain). At the beginning of the project, there was no internal sensory panel in the company and all the sensory evaluation were carried out externally. The decision to carry out this project led the company to implant a permanent trained sensory panel within the company. The

researcher was actively involved in the set-up and training of the sensory panel. The first task was to identify sensorial descriptors suitable for cocoa and chocolate. Several were found in the literature. [112, 113]

- The *aroma* descriptors correspond to the sensations that are perceived from the volatiles of the sample by the olfactory system (Table 5.12).
- The *flavour* descriptors communicate the whole feelings that are perceived in the mouth when food is eaten (Table 5.13).
- The *off-flavour* descriptors group the unpleasant sensations that can be found from a food sample (Table 5.14).

Table 5.12 *Aroma* descriptors for cocoa and chocolate. [38, 112-114]

DESCRIPTORS	DEFINITIONS
Chocolate	Typical aroma of the dark chocolate, with milk and high content in cocoa.
Cocoa	Characteristic of the cocoa liquor, like the aroma when the African bean is roasted.
Sweet spicy	Intense aroma characteristic of vanilla.
Caramel	Weakly, burnt sugar flavours, sweet and pleasant, similar to "toffee".
Diary	Typical milky aroma.
Nutty	This aroma is reminiscent of the odour and flavour of fresh nuts (distinct from rancid nuts) and not of bitter almonds
Roasted	Aroma linked with toasted seeds, where the intensity goes from green to burnt, according to the temperature and roasting time.
Spicy	This aroma descriptor is typical of the odour of sweet spices such as cloves, cinnamon and allspice. Tasters are cautioned not to use this term to describe the aroma of savoury spices such as pepper, oregano and Indian spices
Fruity	This aroma is reminiscent of the odour and taste of fruit. The natural aroma of berries is highly associated with this attribute.

Table 5.13 *Flavour* descriptors for cocoa and chocolate. [38, 112-114]

DESCRIPTORS	DEFINITIONS
Chocolate	Typical flavour of the dark chocolate, with milk and high content in cocoa.
Cocoa	Typical flavour of cocoa paste, e.g. aroma produced when cocoa seeds from West Africa (Ivory Coast….) are toasted.
Caramel	Weakly, burnt sugar flavours, sweet and pleasant, similar to "toffee".
Sweet	This is a basic taste descriptor characterized by solutions of sucrose or fructose.
Milky	Flavour of the fresh milk and its non-fermented derivates
Nut	This Flavour is reminiscent of the odour and flavour of fresh nuts (distinct from rancid nuts) and not of bitter almonds.
Sweet Spicy	Aromatic and sweet flavour characteristic of the vanilla.
Toasted	Flavour linked with toasted seeds, where the intensity goes from green to burnt, according to the temperature and roasting time.
Bitter	A primary taste characterized by the solution of caffeine, quinine and certain alkaloids. This taste is considered desirable up to a certain level and is affected by the degree of roast brewing procedures.
Astringent	This attribute is characteristic of an after-taste sensation consistent with a dry feeling in the mouth
Fruity	This flavour is reminiscent of the odour and taste of fruit. The natural aroma of berries is highly associated with this attribute
Acid	A basic taste characterized by the solution of an organic acid

Table 5.14 *Off-flavour* descriptors for cocoa and chocolate. [38, 112-114]

DESCRIPTORS	DEFINITION
Mouldy	Wet room, mould and fungal growing.
Fermented	Milk out of date.
Chemical	This odour descriptor is reminiscent of chemicals, medicines and the smell of hospitals
Smoky	The aroma is associated with smoke produced when burning wood.
Rancid	This aroma descriptor includes two terms that are associated with odours reminiscent of deterioration and oxidation of several products. Rancid as the main indicator of fat oxidation mainly refers to rancid nuts and rotten is used as an indicator of deteriorated vegetables or non-oily products
Alcoholic	Flavour present in alcoholic drinks due, in general, to ethyl alcohol.

The panellists were chosen amongst the staff of the company. Fourteen people were selected to be part of the sensorial panel, based on their natural ability to recognise and discriminate between the four basic flavours: acid, salty, bitter and sweet. They were subjected to a ranking aptitude test with standards of these four flavours in different concentrations (Table 5.15).

Table 5.15 Basic flavour standards and concentrations used during the aptitude test.

Flavour	Standard	Concentration limits
Acid	Citric acid	1.3 to 14 g/L
Salty	Sodium Chloride	0.34 to 2 g/L
Bitterness	Caffeine	0.16 to 0.5 g/L
Sweetness	Sucrose	0.94 to 12 g/L

The fourteen people were then trained to be expert panellists in the chosen sensorial descriptors (Table 5.16). A two-month training programme was organised which included 16 sessions of 90 minutes. The sensorial panel was educated to recognise the flavours and the intensities of the chosen descriptors.

Table 5.16 Descriptors standards used to train the sensorial panel.

Flavour	Standard	Concentration limits
Cocoa	Natural cocoa powder	0.1 % to 4 %
Chocolate	Dark chocolate (70%)	0.1 % to 4 %
Bitterness	Caffeine	0.16 to 0.5 g/L
Toasted	Over roasted cocoa powder	0.05 % to 1 %
Caramel	Caramelized sucrose	0.2 % to 4 %
Vanilla	Vanillin	10 to 200 mg/L

The standards were obtained from the commercially available products of Natra Cacao S.L. Natural cocoa powder and dark chocolate (70%) were used without any modification. Natural caffeine was obtained from Natraceutical S.L.. To express the toasted descriptor an over-roasted cocoa powder was used as standard. To obtain this

standard, natural cocoa powder was heated in an oven for 30 min at 150ºC. The standard for the caramel descriptor was obtained by caramelising a 50 % sucrose water solution. The vanilla standard was vanillin, the main compound responsible of the natural vanilla pod's flavour. All these standards were prepared in aqueous solution and in semi-skimmed milkshakes at different concentrations. At the beginning, the standards were evaluated individually but after some sessions, the natural cocoa was fortified with some of the other standards to obtain slightly different natural cocoa powders.

The first two sessions were open. The panellists had knowledge of the descriptors and concentrations of each sample. The objective of these sessions was to teach them to memorise the correct flavour for each descriptor. These open sessions were followed by "remembering" sessions. Panellists should demonstrate a minimum level of discrimination ability of about 70% correct matches.

Ranking, paired-comparison and triangular tests were used to evaluate the evolution of the sensorial panel. [115] All these processes were carried out at the facilities of Natraceutical S.L. (Valencia. Spain).

At the end of the training period, the panel was ready to evaluate and score the cocoa and chocolate flavours produced during this project.

Chapter 6

6 Study of the volatiles development of cocoa products by thermal processing technology

Thermal processing is a technology for the preparation of cocoa products that involves the use of a reactor in which the cocoa products and chocolate making ingredients are mixed and heated under controlled conditions including the temperature, pressure and stirring power. The reactor (IKA LR2000P, Appendix 10.3) is a closed system that promotes the chemical reactions between the ingredients due to increased temperature and pressure retaining the volatiles. Under such conditions Maillard reactions and caramelisation, the most important non-enzymatic browning reactions, responsible for the generation of aroma and brown coloured molecules (hence browning), can be enhanced.

Maillard reactions involve the reaction of amino compounds and reducing sugars while caramelisation involves only sugars. Both reactions need elevated temperatures to take place. [116, 117] The factors affecting the Maillard reaction and caramelisation include temperature, time, moisture content, concentration, pH and nature of the ingredients. Therefore, the effect of these factors on the volatile profile was the subject of this study. [118-121]

The experiments were designed to characterise the chemical and sensorial evolution of the volatiles:

- The volatiles evolution was evaluated using the SPME methodology optimised in our laboratories (Section 5.1.2).
- The sensorial evaluation would be carried out using the sensorial panel at the facilities of the industrial partner. Unfortunately, an important part of the

sensorial study could not be achieved because the company's strategy changed one and half years into the project leading to the company pulling out of the project. The sensorial evaluation was therefore continued by the researchers who were actively involved in the set-up of the panel of tasters. These personal evaluations lack statistical relevance but give us a general evaluation of the sensorial properties of these products.

6.1 Influence of the reaction time and temperature

The chocolate-making ingredients were reacted in the IKA reactor and the influence of temperature and time on the evolution of the volatile profile was studied. Non-enzymatic browning and caramelisation are endothermic reactions therefore the temperature will be an important factor for the development of these processes.

6.1.1 Chemical evolution of the volatile profile

Since Maillard reactions usually occur at temperatures over 80°C and caramelisation at temperatures over 100°C, we decided to carry out experiments at 70, 90, 110 and 130°C. The other parameters were kept constant: 20% fat natural cocoa powder (500 g), sucrose (500 g) and water (550 g) were added to the reactor before starting the experiment. Due to the heating process, the pressure inside the reactor rose: 0.1 bar at 70°C, 0.5 bars at 90°C, 1.2 bar at 110°C and close to 3 bar at 130°C.

Reaction times were chosen in function of the reaction temperature, 4 hours for the higher reaction temperature (130°C) experiment, 8 hours for 110°C experiment, since high temperatures promote reactions faster and for the two lower reaction temperatures the reaction time was extended to 10 hours to ensure the volatile profile

had evolved since lower temperatures have slower kinetics. Sample were taken at time = 0, 1, 2, 4 hours and at the low temperatures 10 hours.

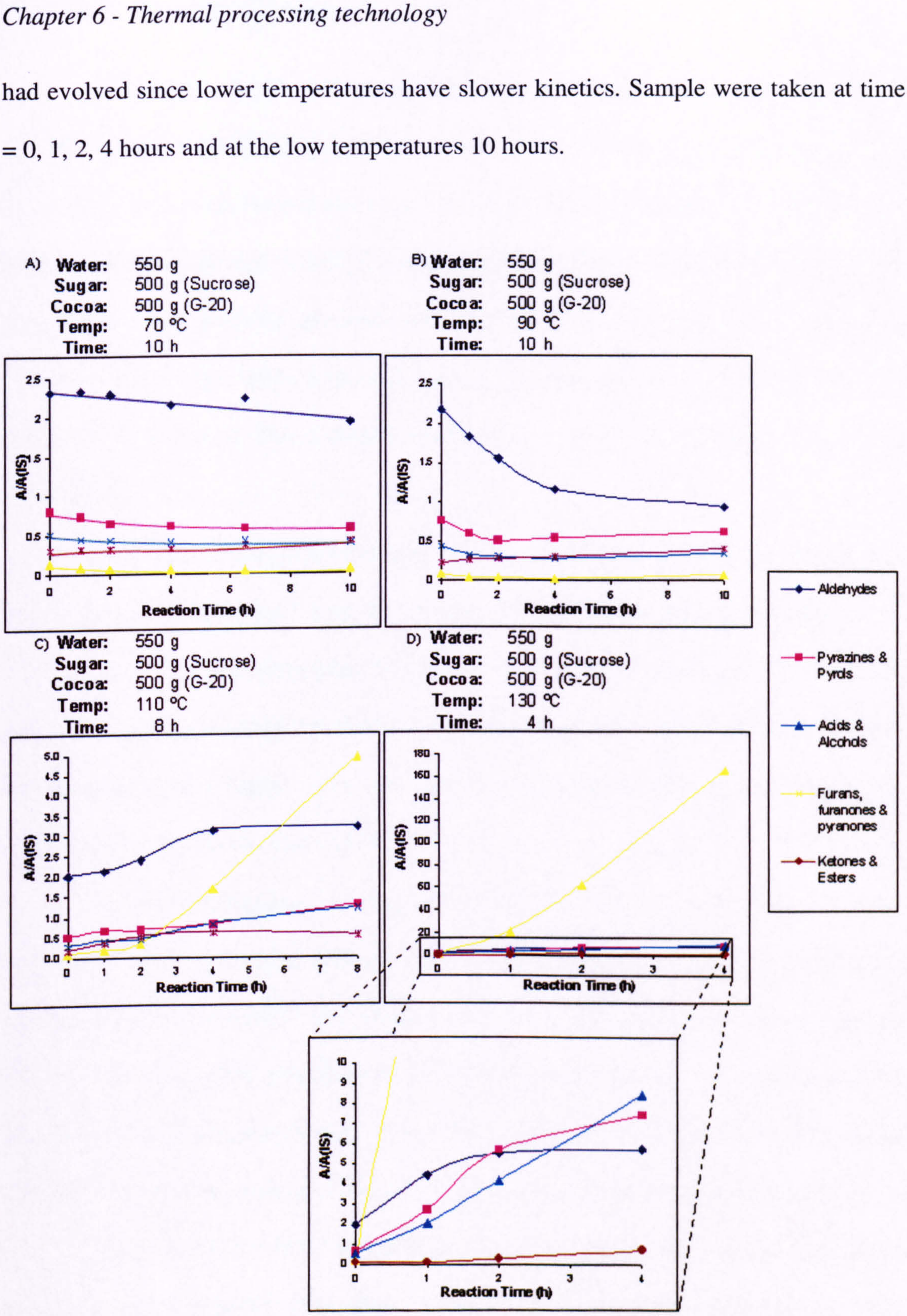


Figure 6.1 Effect of the temperature on the volatile profile. A) 70°C, B) 90°C, C) 110°C, D) 130°C, D') 130°C (zoom of D)). Normalized area, A/A(IS), versus reaction time. Experiment conditions are summarized above individual graphs.

The volatile analysis of the samples was performed using our optimised HS-SPME-GC-MS method developed in section 5.1.2. Almost fifty compounds were identified by comparison with pure standards or tentatively identified by comparing the mass spectra or/and by retention index data with the NIST98 database.[98] The compounds were grouped into different categories for easier discussion; aldehydes, pyrazines & pyrroles, acids & alcohols, furans & furanones & pyranones and ketones and esters (Figure 6.1). Tables with the individual compound evolution can be found in appendix 10.4.

On perusal of the graphs (Figure 6.1) one immediately notices that the scale of the Y-axes is not the same for all the representations. The graphs corresponding to the 70°C and 90°C reaction temperatures have a maximum Y-axis reading of 2.5, the 110°C has a max reading of 5 and the 130°C a max reading of 180. This clearly indicates that the production of volatiles increases with the temperature, with a more significant increase for temperatures above 110°C.

The X-axis represents the reaction time and here we can observe that the lower reaction temperatures indeed require longer reaction times to reach a plateau. In fact, the volatile analyses for these longer reaction times (70°C and 90°C experiments were run for 10 hours) show that only secondary reactions which decrease the concentration of volatiles in the headspace took place and these temperatures were too low to produce volatiles. The total amount of volatiles and semi-volatiles recovered decreased by 17 % for 70°C and 36 % for 90°C experiment. This degradation of volatiles practically doubles from 70 to 90°C. This effect can be clearly seen in the aldehyde evolution. Aldehydes are reactive functionalities, and under these conditions, they can react to produce by-products such as alcohols or carboxylic acids, decreasing their

concentrations. These results suggest that to have detectable browning reactions, the temperature must be above 90ºC otherwise only degradation can be observed.

Looking at the volatile profile after 4 hours for each temperature (Figure 6.2), the graph clearly shows the direct relationship between production of volatiles and temperature. There is a sudden increase in volatile production at 110ºC; before this temperature there was no sign of non-enzymatic browning, since most of the categories remain constant with the only exception of the degradation of the aldehydes commented above and attributed to parallel secondary reactions that occur faster at higher temperatures. The rest of the volatiles remain practically constant until 90ºC. At 110ºC or higher reaction temperatures, there was clear evidence of caramelisation and Maillard reactions. Practically all groups had an increase at these temperatures and all the compounds within these groups are well known non-enzymatic browning products. This increase is very significant for furans, furanones and pyranones group (yellow curve), 37 times higher at 110ºC and >3000 times higher at 130ºC when compared to 90ºC after 4 hours of reaction.

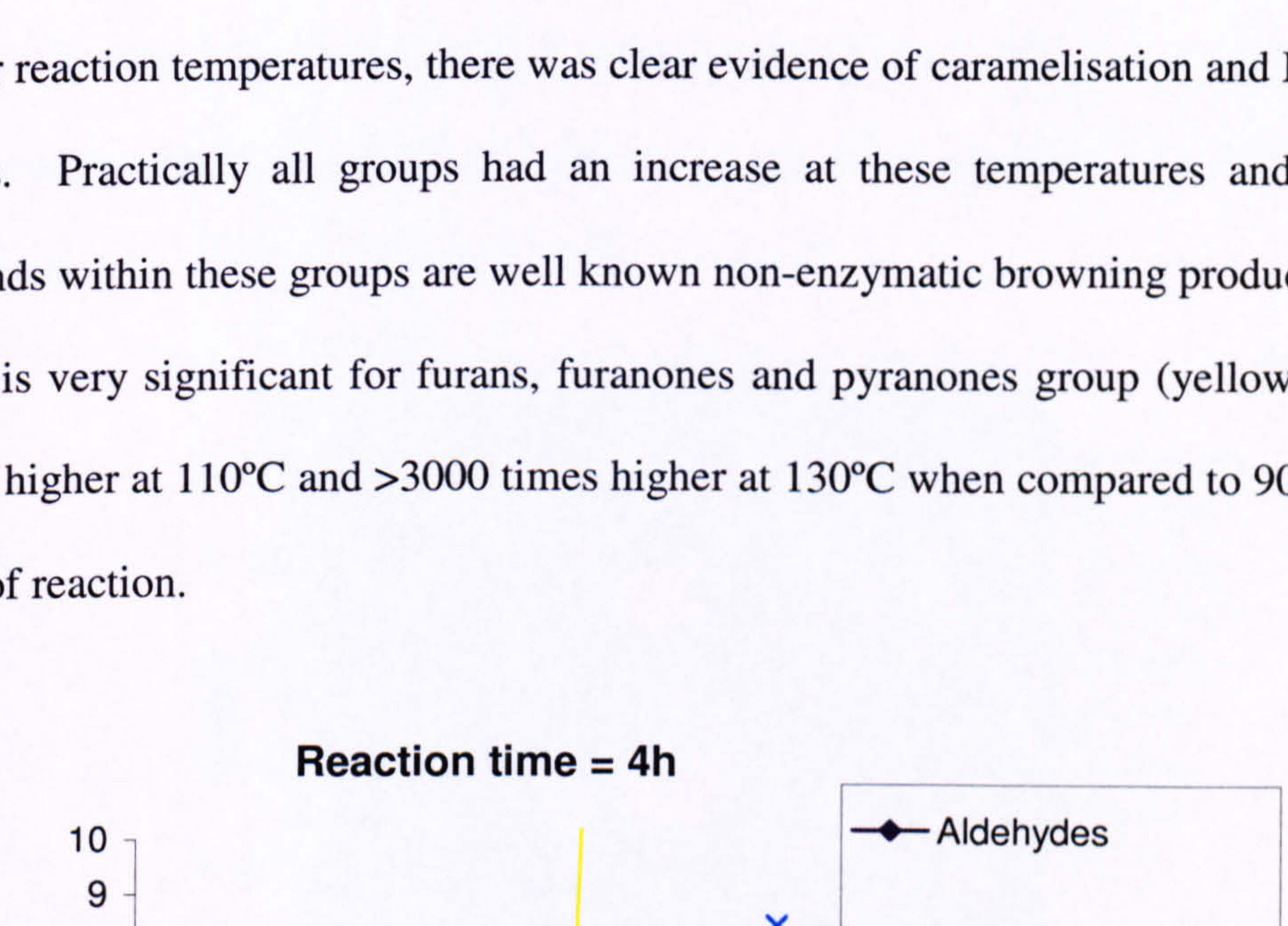


Figure 6.2 Evolution of the volatiles after 4 hours of reaction versus reaction temperature (°C).

6.1.1.1 Evolution of the furans, furanones and pyranones group

The **furans, furanones and pyranones group** (Figure 6.3) presented an increase of more than three orders of magnitude, becoming the largest group of compounds present in the headspace. This group remained constant until 110ºC followed by a steep increase with time at 130ºC resulting in an exponential evolution with the temperature. The massive increase observed for this group was mainly caused by one product; furfural was responsible for 75% of the total amount of this group (at 130ºC and 4 hours) and was the largest recovered over all the categories. Other furans (Figure 6.4) were found in minor quantities but having similar behaviour as for example 2-acetylfuran, 5-hydroxymethyl-2-furfural (HMF), 2-furanmethanol or the dimer 2-(2-furanylmethyl)-5-methylfuran.

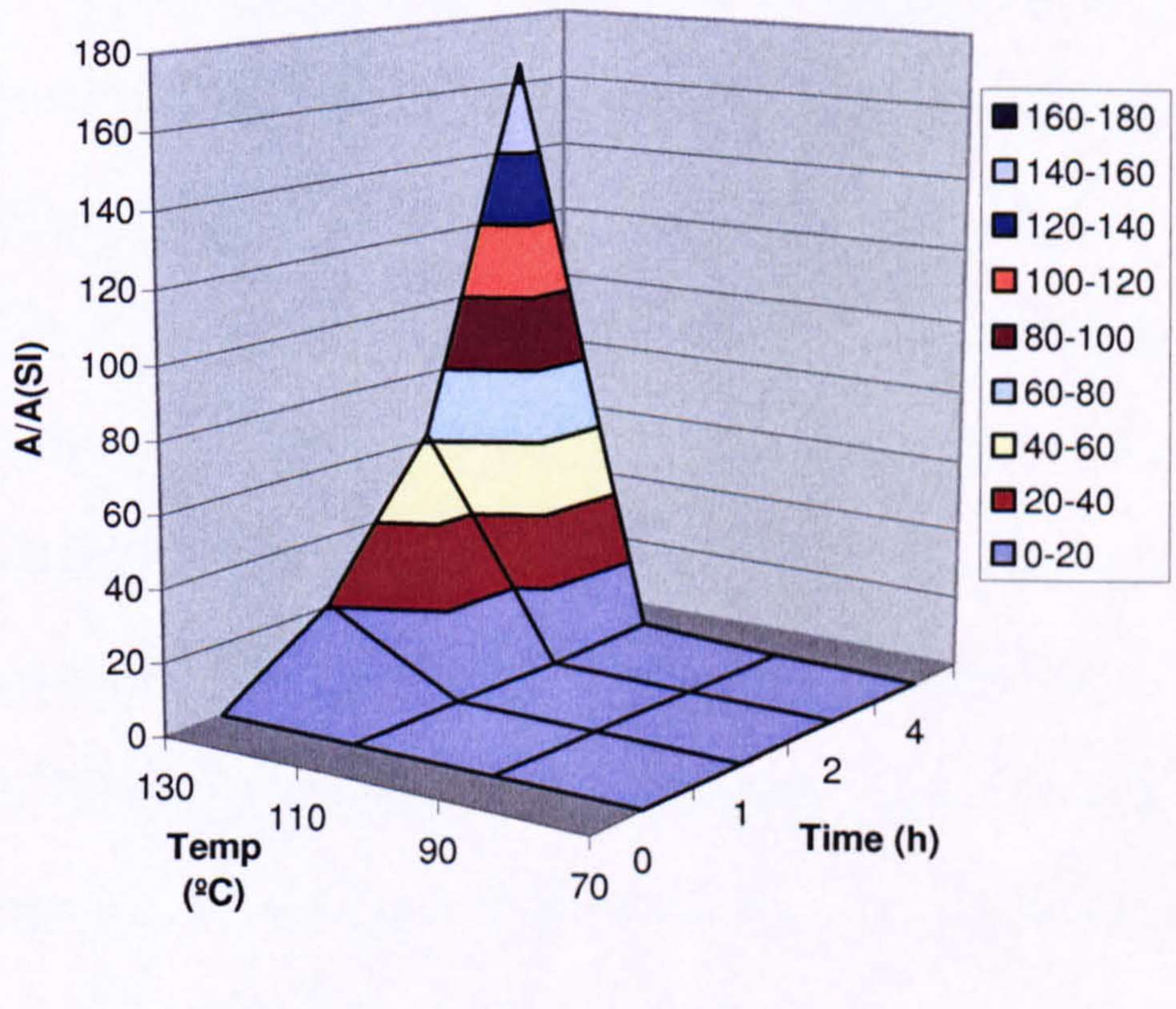
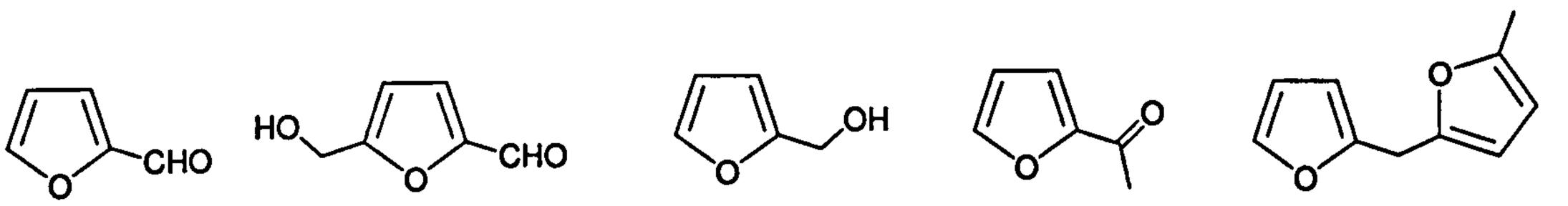


Figure 6.3 Three dimensional representation of the furans, furanones and pyranones group. X-axis: time in hours; Y-axis: temperature in °C; Z-axis: normalised areas.



Furfural | 5-Hydroxymethyl -2-Furfural (**HMF**) | 2- Furanmethanol | 2-Acetylfuran | 2-(2-Furanylmethyl)-5-Methylfuran

Figure 6.4 Structures the furans found in the reaction volatile fraction.

Caramelisation of the sugars is the main responsible for the formation of high quantities of the furans, furanones and pyranones group. Furfural and furans are well-known caramelisation products however; they can be formed as well through Maillard reaction pathways involving nitro compounds (Figure 2.2).

The formation of furfural is typical of the caramelisation of C_5 sugars (Figure 6.5), while hydroxymethyl furfural (HMF) results from the C_6 sugars. In our case, we used sucrose, which is a dimer of two six-carbon monosaccharides, glucose and fructose. The effect of the temperature and the acid media due to the cocoa (pH 5.7) breaks down the sucrose to form a mixture of the two monosaccharides (inverted sugar). The main caramelisation product expected was the HMF but instead furfural was the major product identified by HS-SPME analysis. We could not find a plausible explanation for the formation furfural instead of the HMF. The formation of furfural from HMF was reported under Curie-Point-Pyrolysis conditions at 300°C, [122] because furfural is a thermodynamically stable degradation product of HMF, but these conditions are dramatically different and more severe than the conditions used in our experiments. Others have reported that using high pressures and hydrothermal conditions both species are present with the HMF being the largest. [123] No report describing the formation of furfural from C_6 sugars as main product has been found. [122-124]

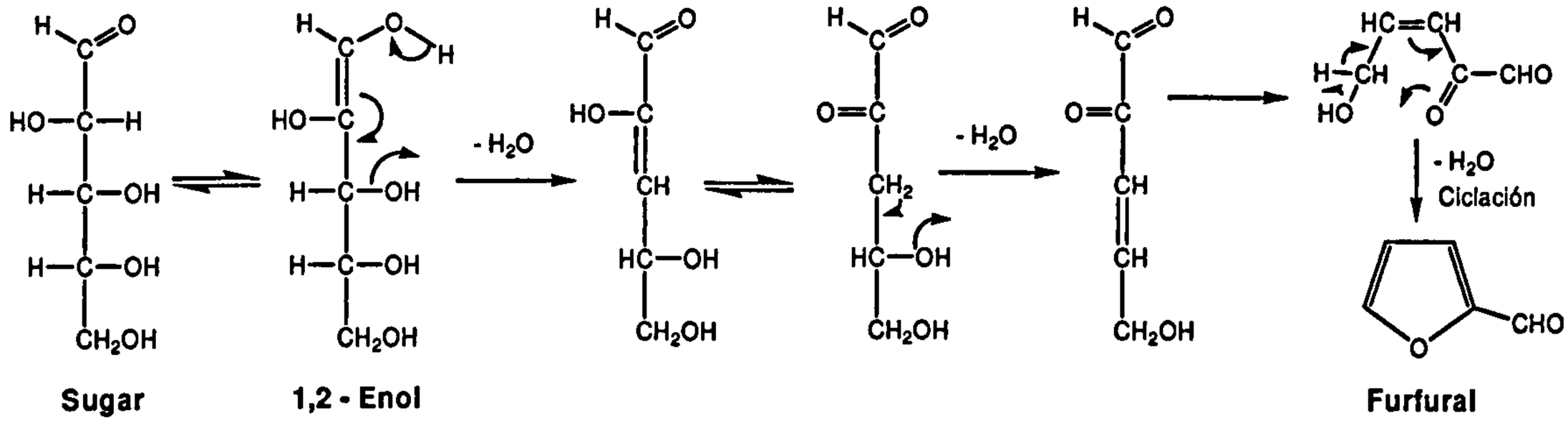


Figure 6.5 Formation of furfural via dehydration of C_5 sugars (caramelisation)

Almost certainly, other furans, furanones and pyranones were formed through other caramelisation pathways or from sub-reactions of furfural with other components in the mixture.

The introduction of sucrose into the reactor in large excess, the increase in production of the furans, furanones and pyrroles group after 110°C [122] and the burnt off-flavour developed in the sample (typical flavour of over-caramelized products) confirmed caramelisation was occurring. The confirmation of this hypothesis led us to plan experiments where the ingredients and their proportions would be modified to control caramelisation.

The other chemical groups presented some similarities with the furans group albeit on a lower scale. There was no significant variation until 110°C and at 130°C some significant changes of the profile occurred.

6.1.1.2 Evolution of the aldehydes group

The **aldehydes group** (Figure 6.6) displayed a 3-fold increase in concentration at 130ºC compared to 90ºC after 4 hours of reaction (Figure 6.2, dark blue curve). The evolution is progressive with time and eventually reaches a plateau. This can be explained by the fact that the precursors have been mainly consumed during the reaction slowing down their formation plus the chemical instability of the aldehydes formed, (the aldehyde functional group is very reactive). Their formation would have therefore been affected by their subsequent transformation into other molecules such as alcohols or acids, slowing down or decreasing their formation.

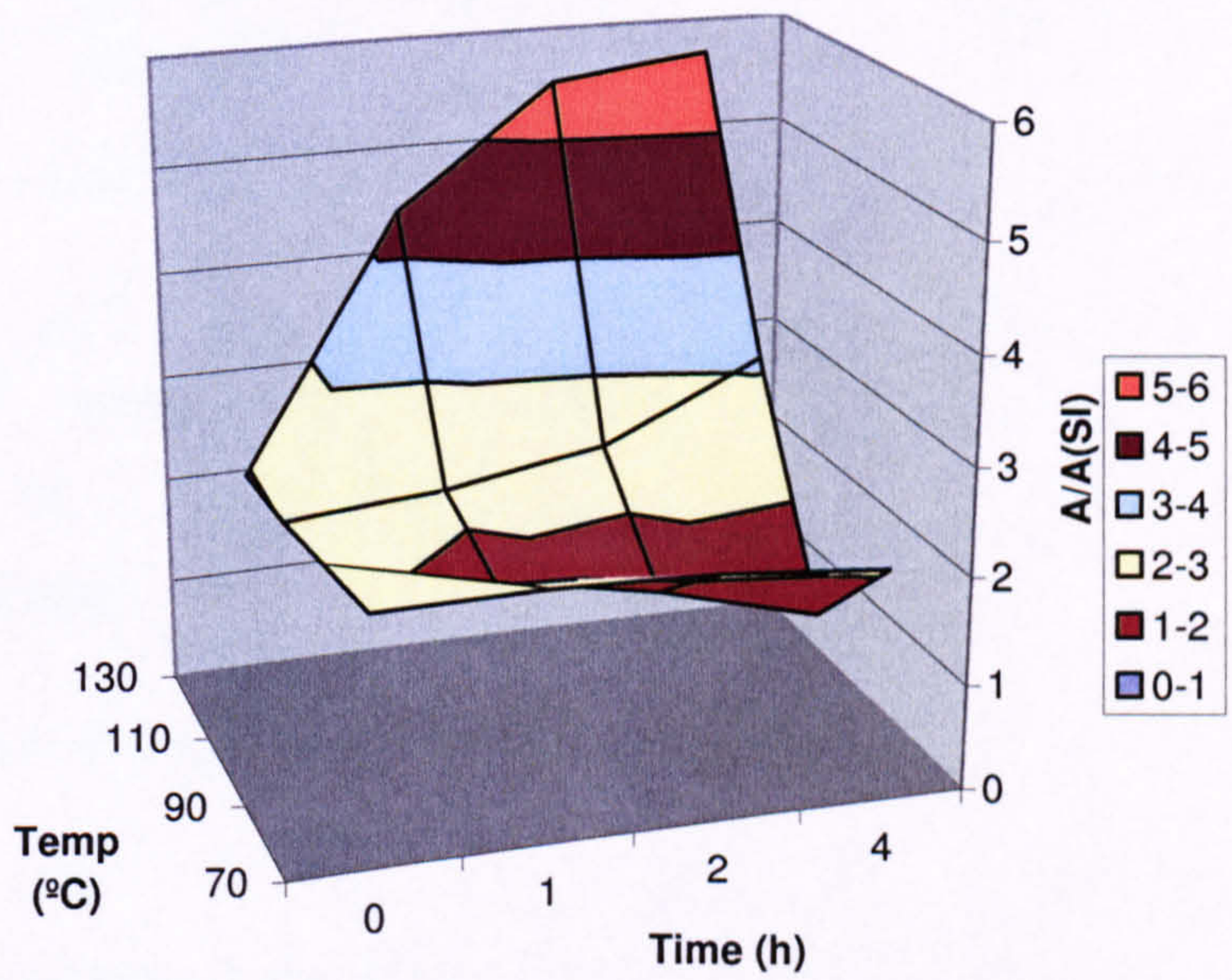


Figure 6.6 Three dimensional representation of the aldehydes group. X-axis : time in hours; Y-axis : temperature in °C; Z-axis : normalised areas.

The 3D representation of the aldehydes group (Figure 6.6) shows the evolution of the aldehydes against time and temperature. At low reaction temperatures (70 and 90ºC), the slope is slightly negative and after 4 hours of reaction, the amount of aldehydes is lower than at the beginning, especially for 90ºC experiment. On the other

hand, at higher reaction temperature (110°C - 130°C), the amount of aldehydes increases significantly. With time, the sharp slope levels to reach a plateau after 3 hours of reaction at 130°C and after 5 hours at 110°C.

These aldehydes come from the Strecker degradation of the amino acids (Figure 6.7). The Strecker degradation is one of the most important reactions leading to the final aroma compounds in the Maillard reaction. The aldehydes formed are determined by the starting amino acid, the R- group of the aldehyde being the R- group of the amino acid (Table 6.1).

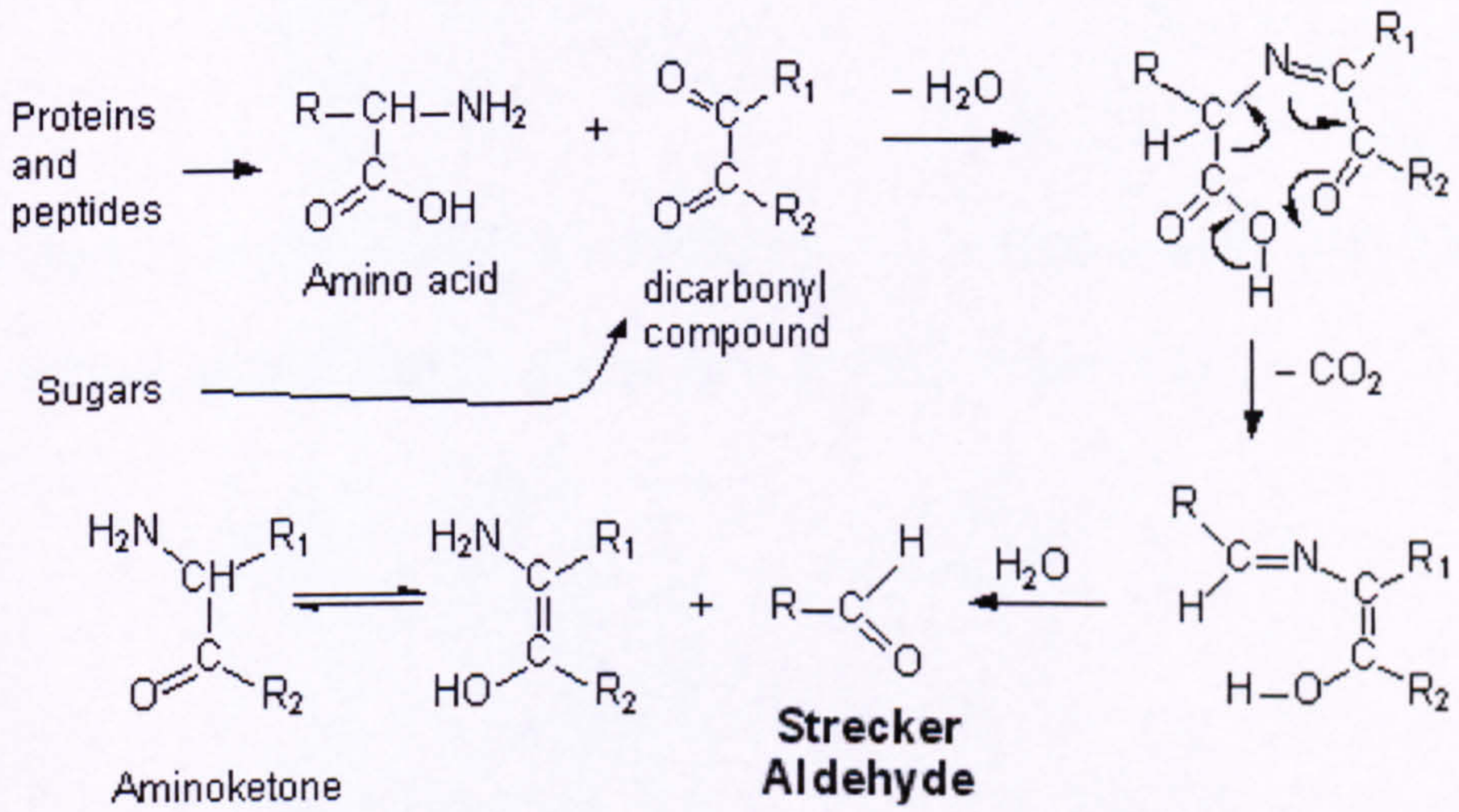


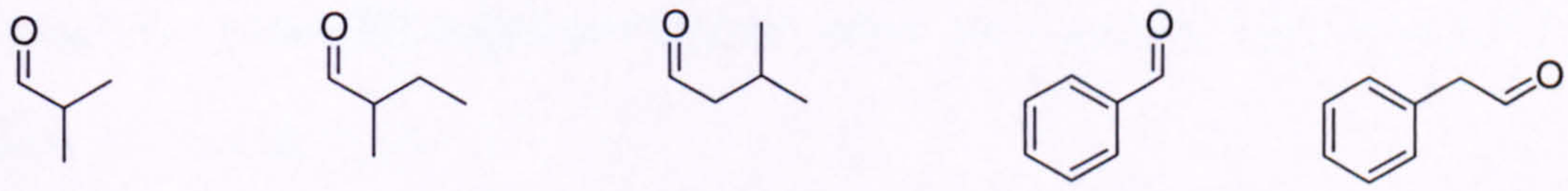
Figure 6.7 Mechanism of the Strecker degradation of amino acids

Table 6.1 Relationship between the amino acid and Strecker aldehyde

Amino Acids	Strecker Aldehydes
Alanine	Acetaldehyde
Glycine	2-Methylpropanal
Valine	3-Methylbutanal
Leucine	2-Methylbutanal
Phenylalanine	2-Phenylacetaldehyde
Methionine	Methional

Looking at individual amino acids that constitute the aldehydes group, not all the molecules behave in the same way (Figure 6.9). In fact, two subgroups can be clearly identified, the aliphatic aldehydes and the aromatic aldehydes (Figure 6.8).

- The aliphatic aldehydes comprise compounds like 2-methyl propanal, 2- and 3-methyl butanal.
- The aromatic aldehydes include products such as benzaldehyde and benzacetaldehyde.



2-methyl propanal 2-methyl butanal 3-methyl butanal benzaldehyde benzacetaldehyde

Figure 6.8 Structures of aliphatic and aromatic aldehydes recovered.

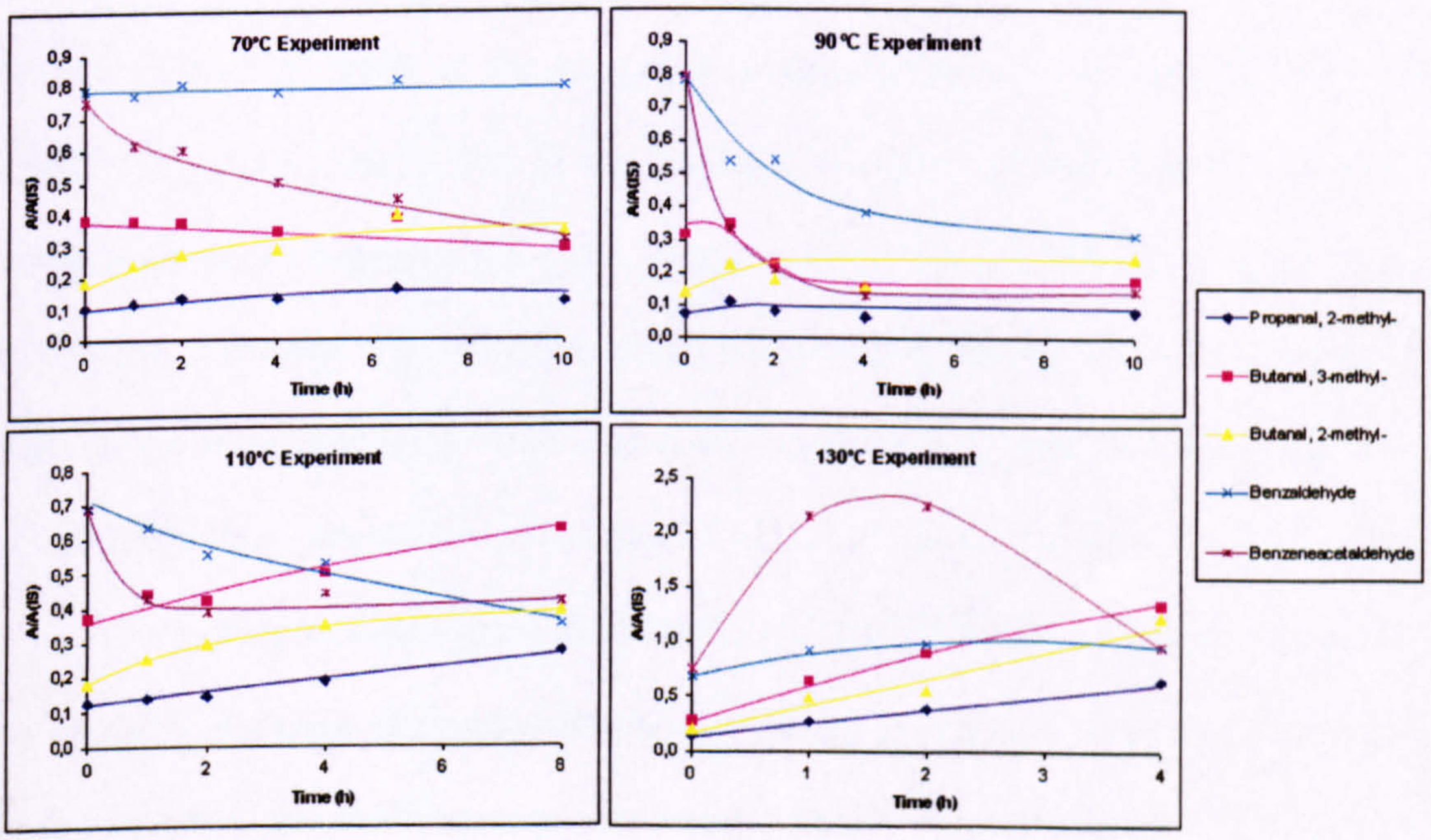


Figure 6.9 Evolution of the main aldehydes versus the reaction temperature used in the study.

The aromatic aldehydes, benzaldehyde (Figure 6.9, cyan curve) and benzacetaldehyde (Figure 6.9, purple curve), were the mainly responsible of the decrease of the aldehydes group at low temperature (70 and 90°C) experiments. For the 70°C experiment, benzacetaldehyde, which represented 35% of the whole aldehydes group, suffers from strong degradation, decreasing by 55%. On the other hand, 2-methylbutanal (Figure 6.9, yellow curve) had a significant increase doubling in amount. In the 90°C experiment benzaldehyde and benzacetaldehyde, representing 65% of the aldehyde group, again suffer from strong degradation decreasing by 55%. On the other hand and like in the 70°C experiment, 2-methylbutanal displays a significant increase doubling its amount.

The experiments carried out at higher temperature (110°C and 130°C) display linear increasing evolutions for the three aliphatic aldehydes. The aromatic aldehydes at 110°C show significantly softer decreasing evolution compared to the 90°C experiment. Their formation as result of the heating is compensating for their degradation. In the 130°C experiment, this effect, is even clearer and the benzacetaldehyde presents a strong increase during the early stages of the reaction passing through a maximum and decreasing at the end. The quick formation consumes all the precursors produced by the high temperature thus in the last stages the degradation remains the main effect. For benzaldehyde this was the only experiment with an increasing profile.

This group of compounds contributes actively to the flavour of the final product. For example the three aliphatic aldehydes (2-methyl propanal, 2- and 3-methyl butanal) were reported to have strong chocolate flavour by Counet et al. [24] The benzacetaldehyde has been attributed to have flowery and honey [24, 125] notes while benzaldehyde has been described as almond and burnt sugar.[125]

6.1.1.3 Evolution of the pyrazines and pyrroles group

The **pyrazines and pyrroles** group was highly dependant on temperature, with their presence increasing with the temperature. Their amount increased 14 times at 130°C in comparison with 70°C after 4 hours of reaction (Figure 6.2, pink curve). At 110°C, their concentration is increased by 50% compared to 70°C. This group obviously requires elevated temperatures to evolve.

The three-dimensional graph (Figure 6.10) shows the evolution of the pyrazines and pyrroles group versus the time and temperature. The formation of pyrazines and pyrroles is temperature-dependant and there was a significant increase between the experiments carried out at 110°C and the ones at 130°C. Even at 110°C, the production of pyrazines is minimal and a temperature of 130°C is needed to push their formation. With time, the slope increase is fairly constant for all the reaction times and maybe only after four hours reaction time, the slope seems to decrease slightly.

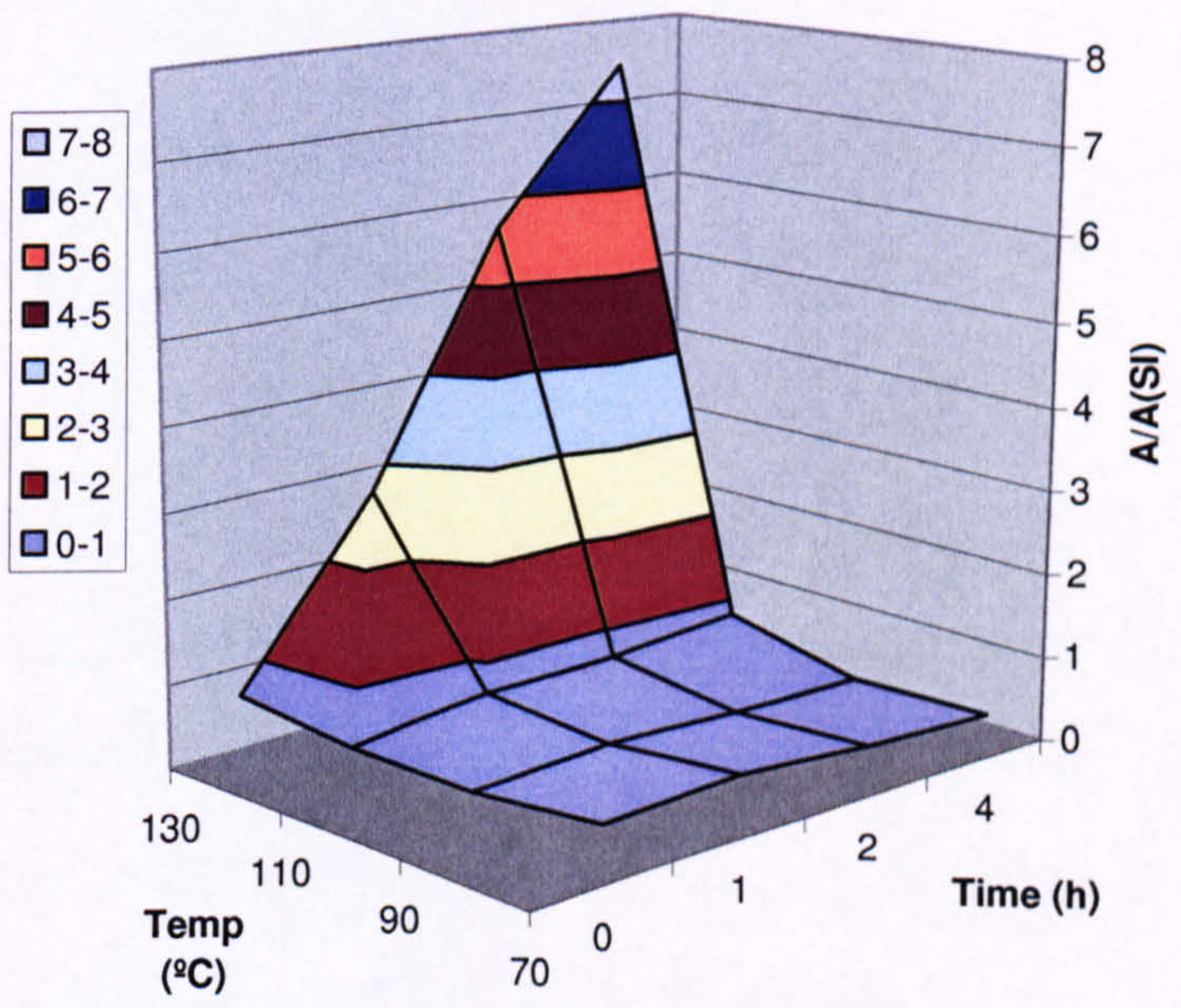
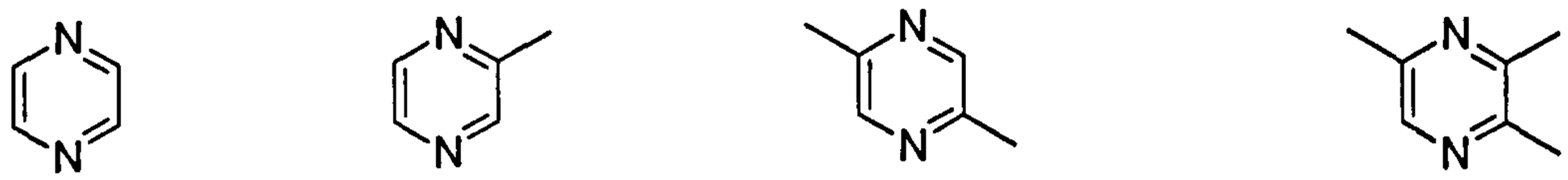


Figure 6.10 Three dimensional representation of the pyrazines and pyrroles group. X-axis : time in hours; Y-axis : temperature in °C; Z-axis : normalised areas.

Looking at individual molecules that make up the pyrazines and pyrroles group, all the pyrazines have a similar behaviour and 2,5-dimethylpyrazine, trimethylpyrazine and the methylpyrazine were the most recovered compounds. The pyrazines at 70°C and 90°C showed a flat evolution and only a slight decreasing tendency after long reaction times could be observed. This point the fact that pyrazines are chemically stable at these temperatures. From the 110°C experiment, the pyrazines had a positive evolution and this progression was confirmed in the 130°C experiment where pyrazines were produced in significantly higher quantities. The extracted pyrazines at the end of the 130°C reaction (4 hours of reaction time) were >10 times higher. The pyrazines that were mostly produced were 2,6-dimethyl-5-isopentylpyrazine, 2-methylpyrazine, 2-ethyl-6-methylpyrazine and the 2,5-dimethylpyrazine (Figure 6.11).



Pyrazine methyl pyrazine 2,5-dimethyl pyrazine trimethyl pyrazine

Figure 6.11 Structures of pyrazines recovered

Pyrazines are well documented as very important flavour components with very low thresholds. [126, 127] They have been found in cooked, roasted and toasted foods. Sensorially, they have been described as nutty and roasted aromas. Pyrazine formation during the Maillard reaction has been studied for many years, and was presented in chapter 2. The most direct route for pyrazine formation results from the cyclisation of two α-amino ketones, which could be formed during the Strecker degradation of the amino acid (Figure 6.12).

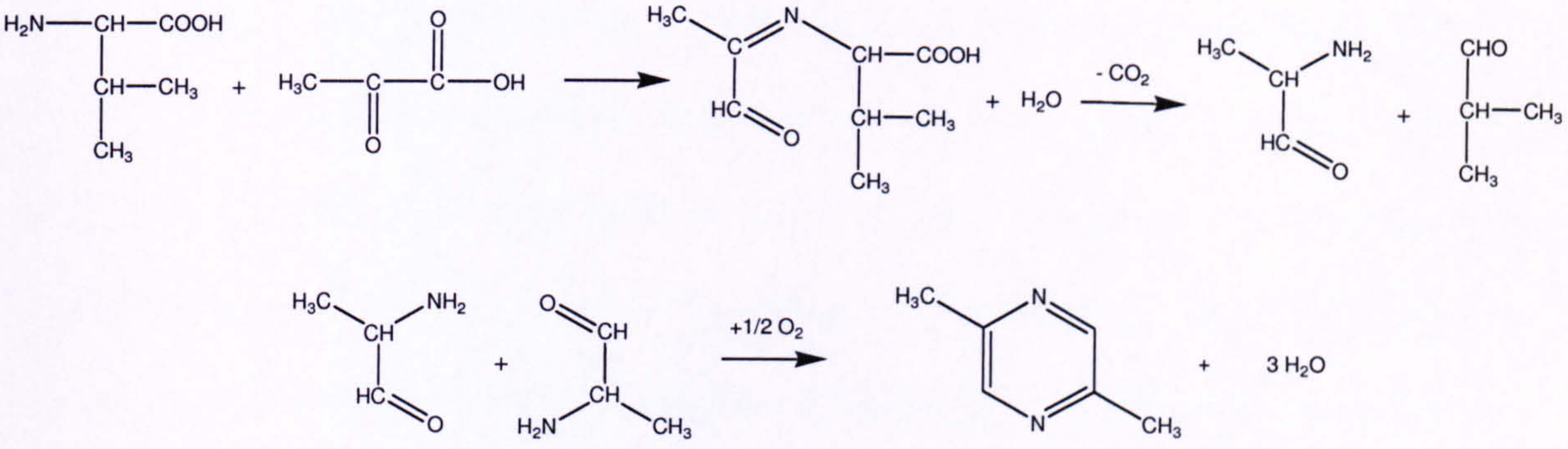


Figure 6.12 Formation of alkyl pyrazine via Strecker degradation

6.1.1.4 Evolution of the acids and alcohol group

The **acids and alcohols** group increased dramatically with the temperature. Their formation increased by 28-fold at 130°C in comparison at 90°C after 4 hours of reaction (Figure 6.2, cyan curve). In fact, it was the group with the second most important production after the furans, furanones and pyranones group. At 110°C, the increase was already evident when their amount rose by a factor of three. Overall this group had a very similar behaviour to the furans, furanones and pyranones group but less intense.

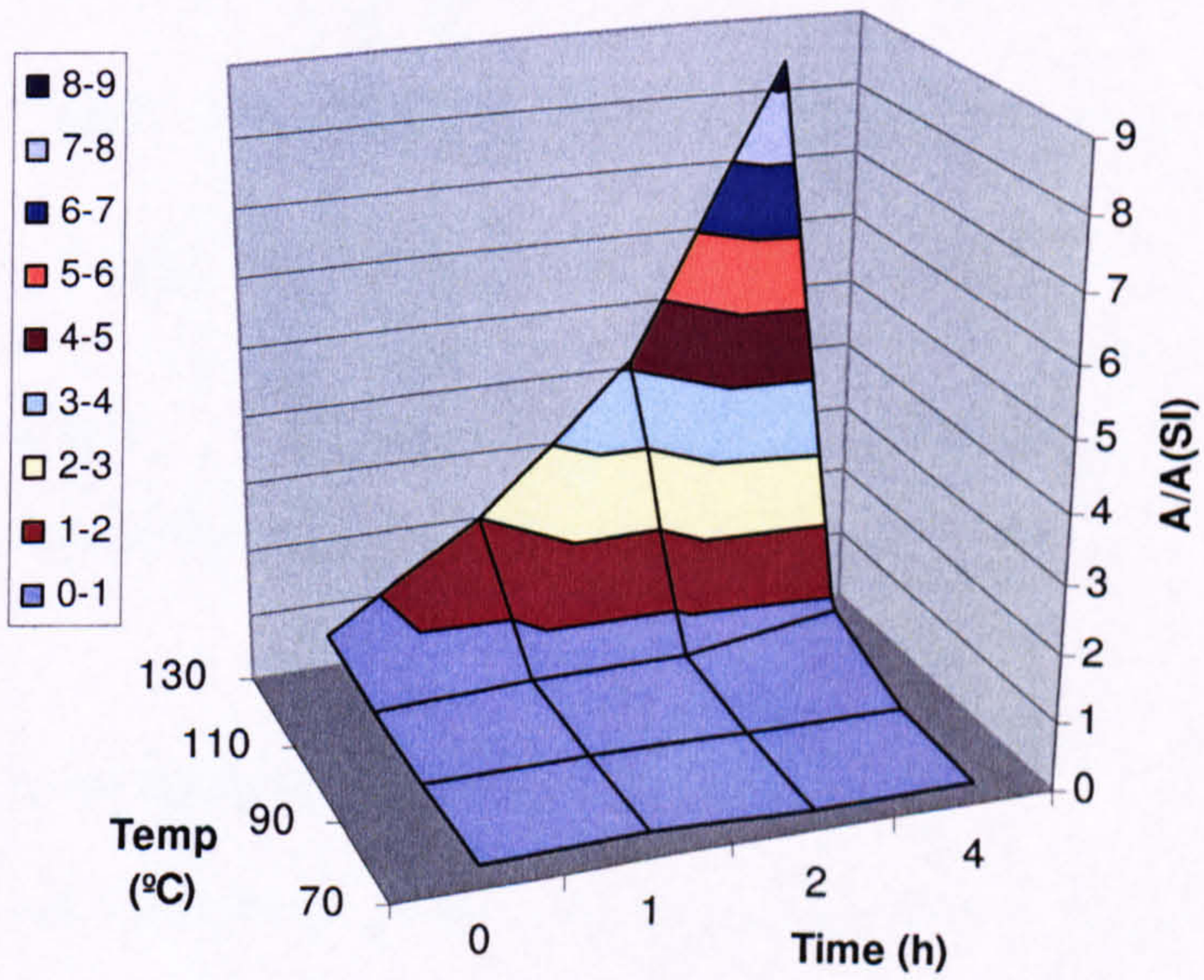


Figure 6.13 Three dimensional representation of the alcohols and acids group. X-axis : time in hours; Y-axis : temperature in °C; Z-axis : normalised areas.

The three dimensional representation for the alcohols and acids group (Figure 6.13) looks like the furans, furanones and pyranones group but with the Y-axis scale 20 times smaller. Such a coincidence and similitude between both groups can be explained because acetic acid is one of the most important by-products in the caramelisation reaction. Acetic acid belongs to the alcohols and acids group and accounts for 80% of this group. The production of acetic acid controls the evolution of the group.

The other components of the group include benzoic acid, 2- and 3-methyl butanoic acid, benzyl alcohol and phenylethyl alcohol which are in fact the alcohols or acids of some of the aldehydes reported previously (Figure 6.14). It is very tempting to assume that these acids and alcohols were made from the corresponding aldehydes. Their production could be the effect of the degradation of the aldehydes due to the thermal conditions when every signs pointed towards browning reactions were taking place.

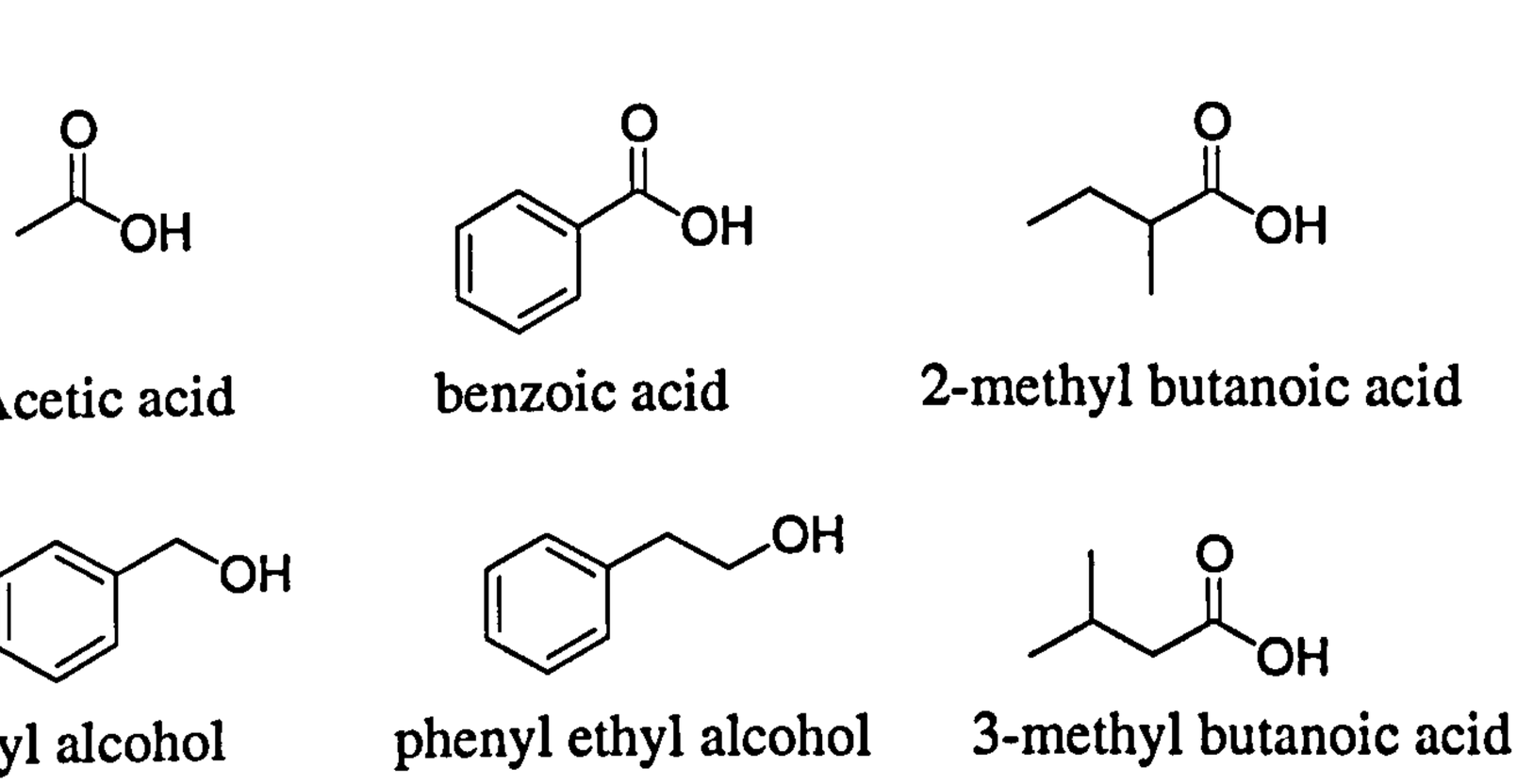


Figure 6.14 Structures of acids and alcohols recovered

6.1.1.5 Evolution of the ketones and esters group

The **ketones and esters** group presented only subtle development. It was the group that presented the lowest recovery and their variation was the least significant. After 4 hours of reaction, (Figure 6.2, purple curve) their recoveries remained low for all the temperature experiments and in comparison with the other groups that evolve

quite dramatically sometimes this group did not, even at high temperatures. Their extraction was the poorest of all the volatiles.

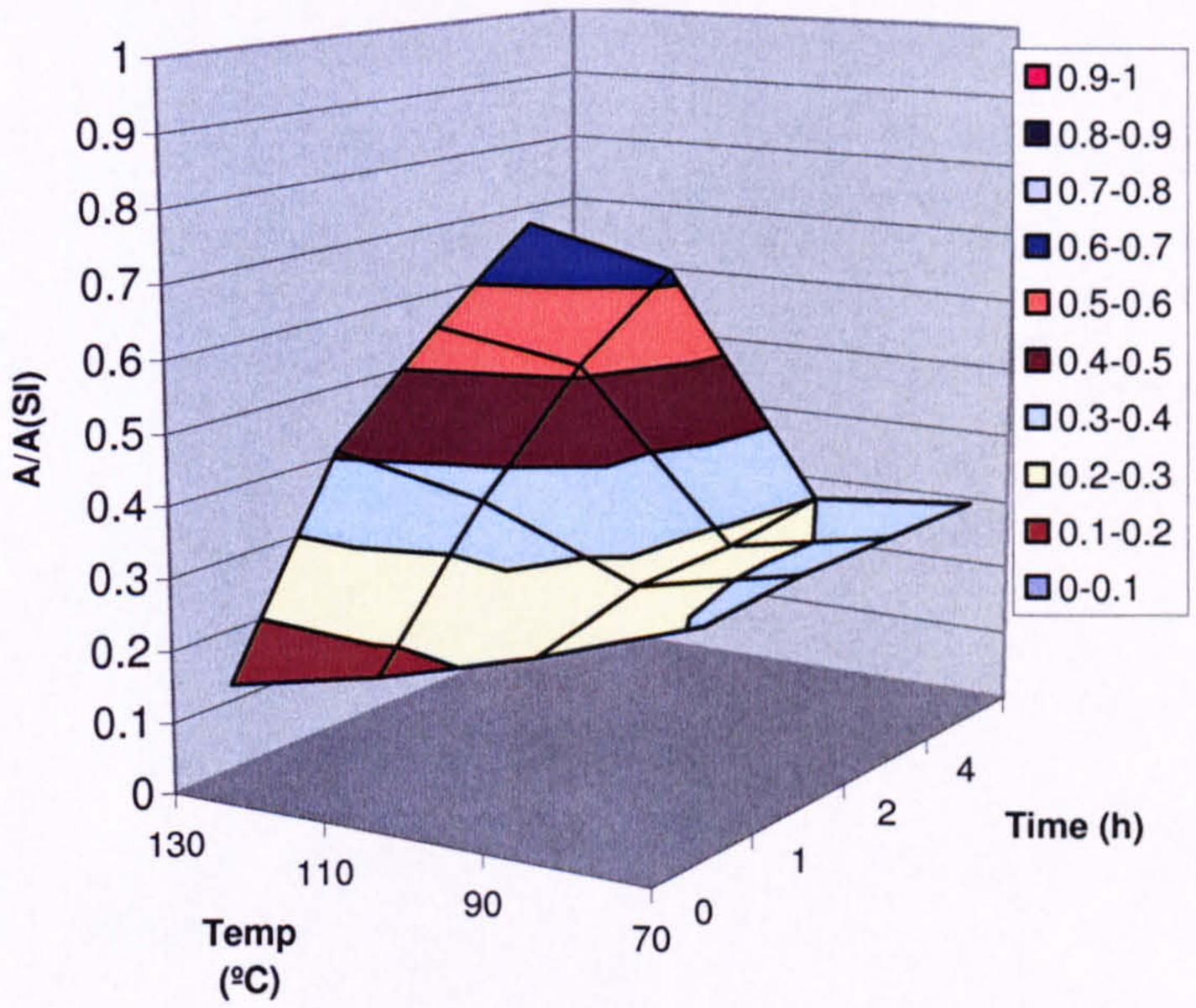


Figure 6.15 Three dimensional representation of the ketones and esters group. X-axis : time in hours; Y-axis : temperature in °C; Z-axis : normalised areas.

The evolution of the ketones and esters group with time and temperature is still positive. Overall, the increase is very small in comparison with the other groups analysed. The scale of the Y-axis only stretches out over one unit. Not many ketones and esters have been reported as typical non-enzymatic browning products and are not even specified in the general pathways. This is in agreement with the result found in our experiments.

6.1.1.6 Evolution of volatile profile with low sugar loading

An additional experiment was carried out to prove that the involvement of sucrose in the formation of the furans, with furfural as the main component. The experiment at 130ºC (Figure 6.1.D) was repeated using low loading of sucrose (20 g).

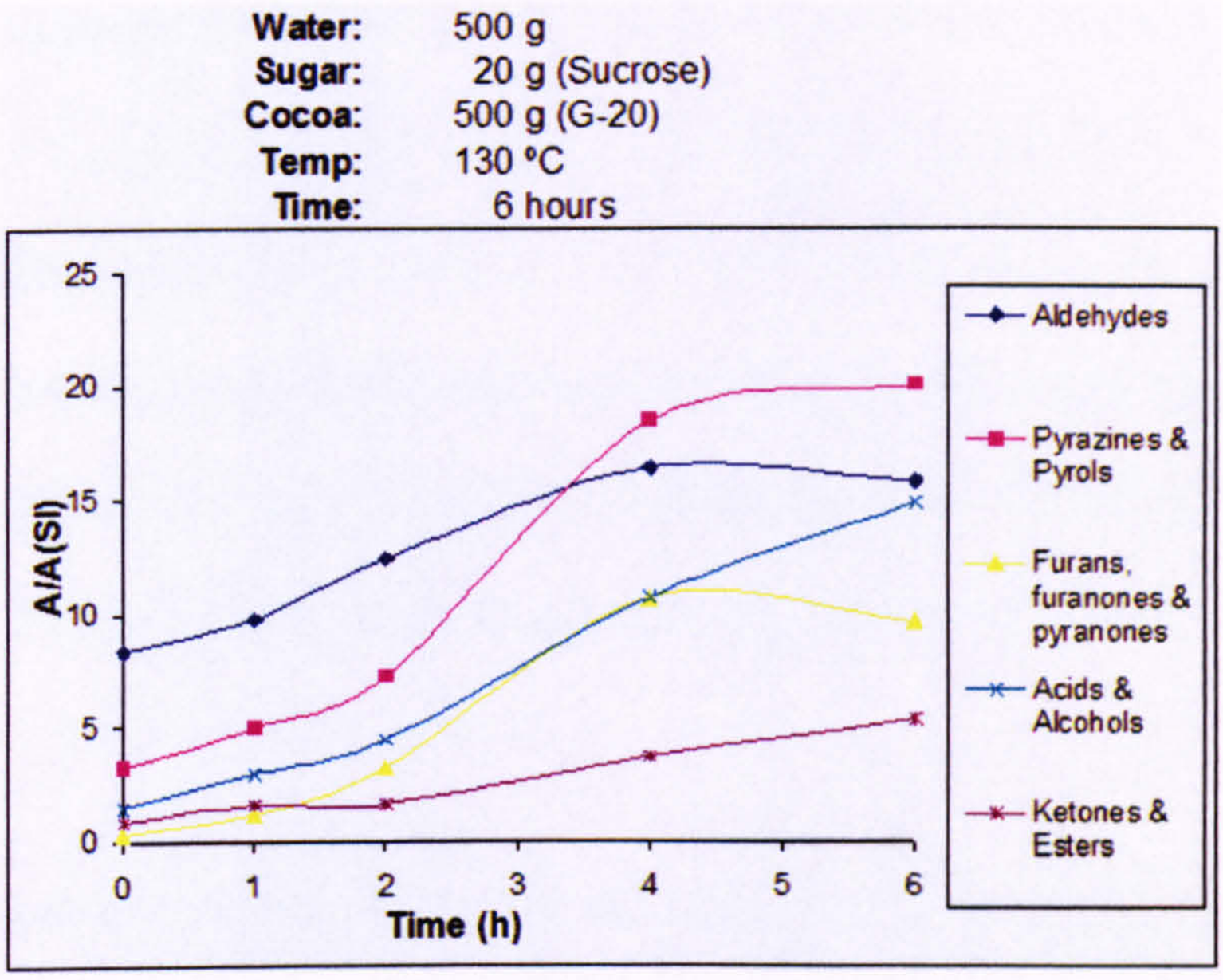


Figure 6.16 Evolution of the volatiles emulating the conditions of **Figure 6.1**.D but using a low loading of sugar.

The results (Figure 6.16) were as expected and the formation of furans (yellow curve) was kept in the same range as the others groups. Using less sucrose resulted in a decrease in the furans formation and even a plateau was reached after 4 hours of reaction. Their formation slowed down after 4 hours probably due to the consumption of the precursors and the degradation reaction of the furans become more important and their concentration decrease. Table with the individual compound evolution can be found in appendix chapter 10 in Table 10.21.

The results demonstrate that sucrose is responsible for the formation of the furans and therefore the furfural was indeed formed from sucrose. Surprisingly we could not find previous work relating to the formation of furfural as a main product from sucrose, therefore we think that this is the first report.

The other groups of volatiles had a similar behaviour as in the previous reactions. The acids and alcohols group (cyan curve) presented a considerably smaller

increase, but this can be explained since their formation is directly dependant on caramelisation of sugars.

6.1.2 Sensorial evaluation

All the samples were initially evaluated by the researcher at Salford, to gain a preliminary insight into the sensorial properties of the products. These evaluations were then confirmed by the trained panel of tasters set-up at the Natraceutical (Valencia, Spain).

The researcher at Salford indicated the acceptability of the generated samples with star (*) symbols, giving one star for unacceptable and five stars for excellent flavour (Table 6.2). The samples were also compared with each other.

Table 6.2 Personal sensorial evaluation of the acceptability of the products obtained varying the reaction time and temperature. (In all cases the recipe was 500g Water, 550 g sucrose, 500 g Natural Cocoa powder 20-22%, and 4 g vanillin).

Time (h) → ↓Temp (°C)	**1**	**2**	**4**	**10**
80		***	****	****
90	***	****	*****	****
100	****	***	**	**
110	***	**	*	
120	**	*		
130	*	*		

Acceptability * ** *** **** ***** → Bad … Excellent

Taking into account the aim of the project, the most acceptable flavour/aroma product was obtained when the reaction was carried out at 90 °C for 4 hours (*****). When the reaction was carried out a high temperatures (≥ 110 °C), the product was darker and usually was accompanied by a burnt off-flavour/aroma and after-taste. The effect of the temperature and its associated pressure, (130°C causes around 3 bars of over pressure) resulted in strong caramelisation of the sugar. The dehydration products, mainly furans, are well known to have burnt and bitter notes at elevated concentrations affording an appearance of unpleasant notes thus an unacceptable samples.

At short reaction times (1h), the reactions at low temperatures (70 and 90°C) had good acceptability scores; however, they did not get a very developed flavour, the flavour resembled that of plain sugared cocoa. At higher temperatures (100 – 110°C) the product presents a better development but with some shades of burnt or over roasted. At high temperatures (120°C – 130°C) the products presented over roasted note profiles but less intense than for longer reaction times, scoring poorly.

Batches carried out at long reaction times (10h), using mild temperatures (70-90°C), the flavour does not change very much in comparison with shorter times. Only some tiny shades of burnt could be felt.

After the preliminary sensorial evolution carried out at Salford, the sensory study was carried out at Natraceutical using their internal panel. As explained section 5.4, the formation of the sensory panel was planned at the beginning of the project and the training lasted 10 months. Members of the company staff were selected for their innate skills and used as tasters. The selected people were trained in the elemental basic aromas and flavours and in recognising the sensorial cocoa and chocolate descriptors interesting for our research.

In the first instance, only acceptability tests were carried out, in fact the acceptability test does not require to use of trained panellists because this test is based on how satisfactory the product is for the taster. Twenty people participated in this sensorial evaluation. Table 6.3 is the questionnaire used for the acceptability evaluation. It is based on a nine point scoring system (1: dislike very much to 9: like very much) which commonly used in such evaluation tests. [115]

Table 6.3 Questionnaire used for the acceptability evaluation

	Sample ID	____	____	____	____	____
9	I like very much					
8	I like much					
7	I like moderately					
6	I like a bit					
5	It is to me indifferent					
4	I dislike a little					
3	I dislike moderately					
2	I dislike much					
1	I dislike very much					

The products were tasted as milkshakes using sweetened (3% of plain sugar) skimmed milk. The first goal was to determine the optimum concentration of product to prepare the milkshake. Milkshakes with different percentages (%) of product were prepared by mixing the ingredients (chocolate, sugar, milk) and submitting them to a pasteurisation process (75°C for 15 min) for health and safety reasons. The product used to determine the optimum concentration in the milkshake was chosen by the researcher as the most acceptable (reaction for 4 hours at 90°C).

The panellists tasted the milkshakes and scored them for acceptability by filling in the questionnaire presented in Table 6.3.

The milkshakes appear acceptable until their concentrations reached saturation (Figure 6.17). From the quadratic adjustment, the maximum acceptability appears to be

between 4 and 5% of chocolate product, more accurately 4.4%, although statistically there is no significant difference between the three last points (3, 4 and 5% of product).

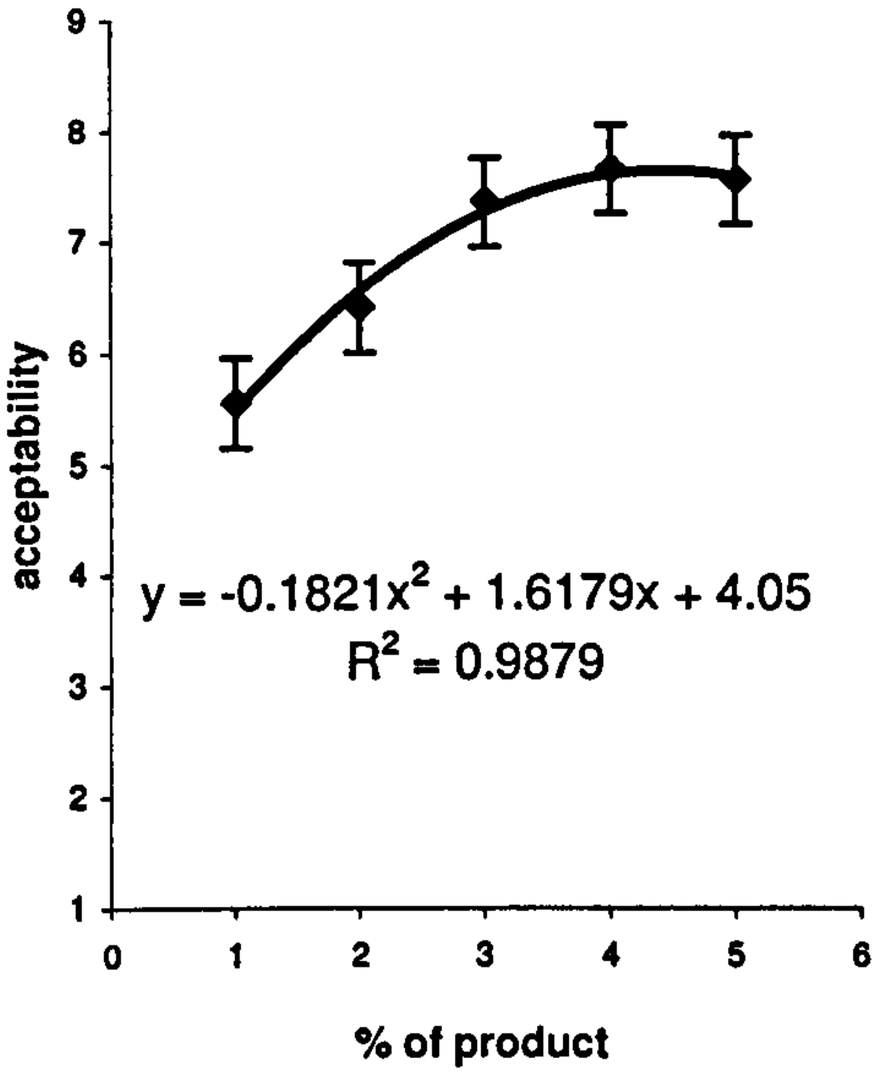


Figure 6.17 Acceptability versus the amount of product added to the milkshake. It represents the curve of adjustment of second order of the points

Once the proportion of product to use in the milkshake was established (4 % of product), the influence of the temperature used in the generation of the product was evaluated by the sensory panel. Six products obtained using the same reaction times (2 h) at different temperatures (80°C to 130°C) were assessed by the tasters for their acceptability using the questionnaire presented in Table 6.3.

Although there is no significant statistical difference between the first four points in the acceptability test on the reaction temperature (Figure 6.18), the point with the highest acceptability is the 90°C batch. Many panellists declared their preference for the products obtained using milder conditions and rejecting the samples generated using the higher temperatures. They described the samples as burnt, bitter, over-toasted and some cases of dizziness where observed. This may be attributed to the high

concentration of furfural in the highly caramelised samples, which is a known harmful chemical.

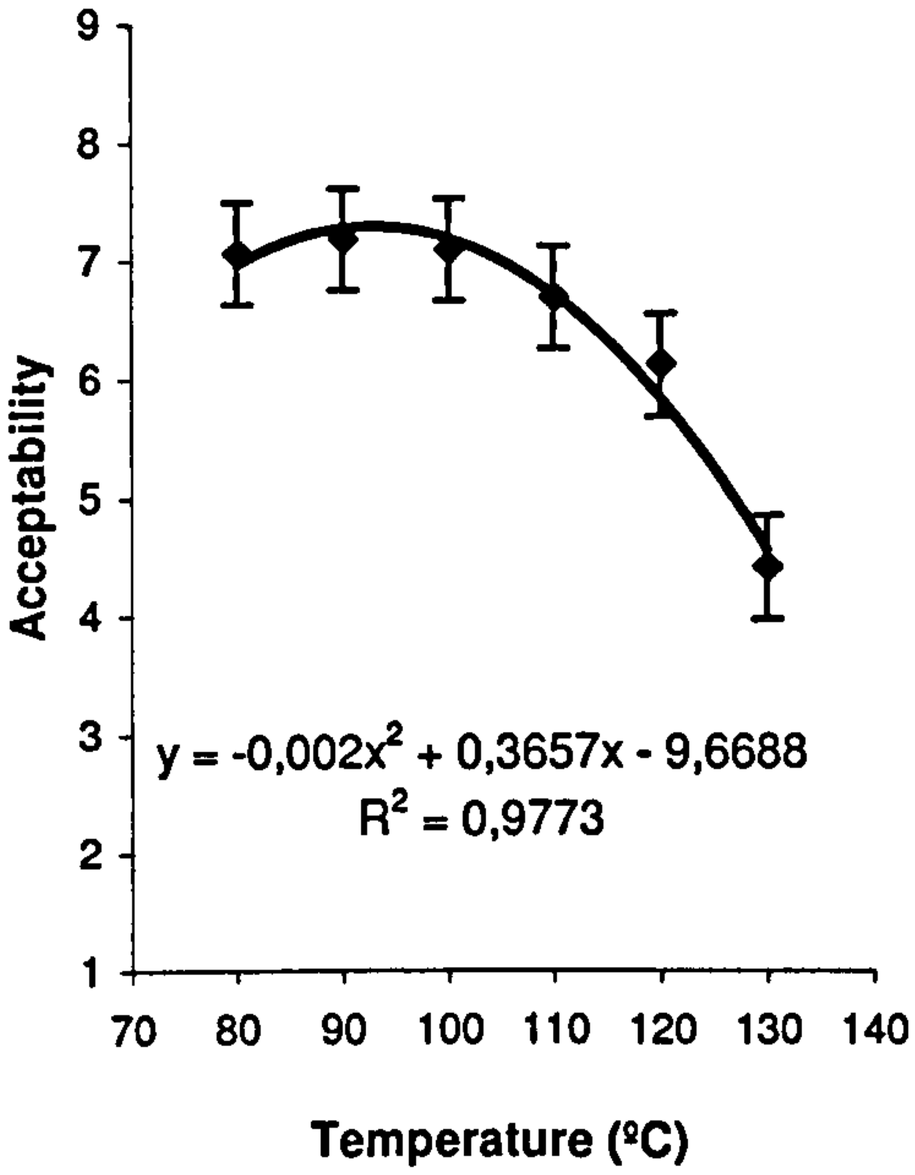


Figure 6.18 Representation of the acceptability versus the reaction temperature used in the generation of the final product from batches where the reaction time was kept constant (2 h).

Using the same methodology, the reaction time was assessed by the panel using the acceptability test (Table 6.3). This time, products from batches where the temperature used was the same (90°C) but the reaction times were different (from 1 to 10 h).

This time (Figure 6.19), the variation of the acceptability is smaller than in Figure 6.18, although some similarities can be observed. There was no significant difference between the last 3 points (2, 4 and 10 hours) but the highest acceptability was obtained for the batch that was prepared using 4 hours of reaction time.

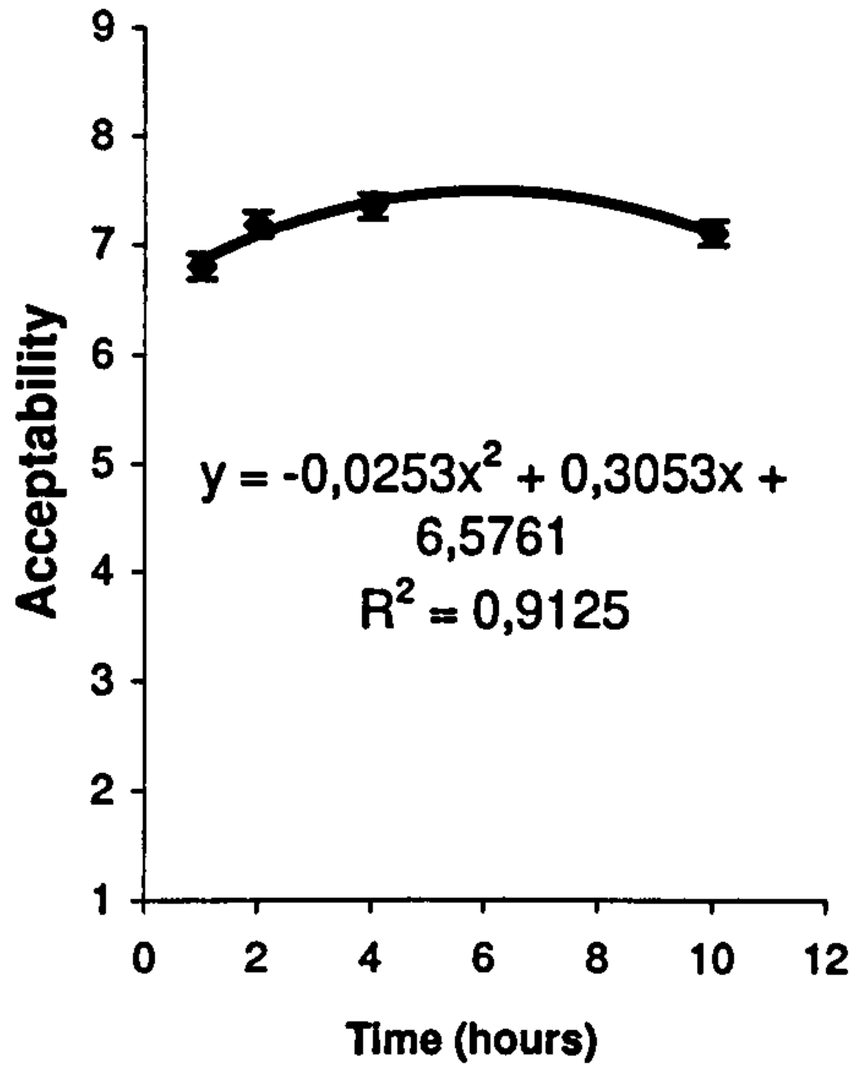


Figure 6.19 Representation of the acceptability versus the reaction time from batches where the reaction temperatures were kept constant (90°C).

6.1.3 Conclusion

Although we observed more chemical changes at high temperatures (≥100°C), the products generated at lower temperatures were more sensorially acceptable (≤90°C). Likewise longer reaction times (10 h) usually resulted in more chemical changes with no great influence on the sensorial acceptability of the final product.

Because the company sponsoring the project was more interested in developing a potentially commercial product, it was decided to continue the project with conditions that would afford sensorially acceptable products. Therefore the chocolate product produced by reacting cocoa powder, sugar and water in the reactor at 90°C for 4 hours was the most acceptable product.

6.2 Influence of the fat content

At this point, it was thought that the cocoa fat could be a factor that could inhibit the reactions between the molecules responsible of the flavour and volatiles development. It is well established that the molecules responsible for the typical cocoa

flavour and their precursors reside in the solid particles and not in the cocoa fat. It was thought that the fat in the cocoa powder could act as a shield surrounding the particles slowing down the aromatic reactions during the thermal processes (Figure 5.17).

To study this hypothesis, three experiments were planned using commercial alkalised cocoa powder with three different fat contents, 0-1%, 10% and 20%. The use of an alkalised cocoa was imposed by its availability of the non-fat form.

The 10% and 20% fat cocoa powders were produced in the regular cocoa factory using different parameters in a hydraulic press (10% of the maximum reduction reached). The non-fat cocoa powder is obtained from one of the reduced fat cocoa powders using extractive methods. Solvents such as hexane or other alkanes can be used for this purpose but are not very recommended due to their toxicity and their important sensorial influence. In our case, supercritical CO_2 was used to extract the fat and afford a quality final product.

6.2.1 Chemical evolution of the volatile profile

The reactions were carried out at 90°C for 4 hours since this was found to produce the most acceptable product. The ingredients and their ratio were modified; the 20% fat natural cocoa powder was substituted by three different fat content alkalised cocoa powders (T-1, T-10 and T-20) in this study (Table 6.4). The amount used was also adjusted to obtain an equivalent amount of cocoa solid in the reactor, thus 500 g of 20% fat cocoa was used, 444 g of 10% fat cocoa and 400 g of the non-fat cocoa. Sucrose was substituted by fructose because of its higher Maillard reactivity. Actually, sucrose is not a Maillard-reactive sugar unless it is hydrolyzed to form equimolar quantities of glucose and fructose which have significant Maillard reactivity. Sucrose hydrolyses at low pH (pH < 3), at low temperatures or at higher temperatures (> 80°C).

Hydrolysis may also occur at pH 7 under typical processing conditions.[128] Since the temperature used was 90°C, fructose was used instead of sucrose to facilitate the reactions. Sensorially, fructose has a similar behaviour to sucrose but it is 50% sweeter. The amount of fructose used was therefore reduced by half compared to the sucrose employed. The proportion of water was increased by 50 g to decrease the viscosity of the mixture because the use of non-fat cocoa resulted in a viscosity of the mixture, which became critical for the mechanical stirring.

Table 6.4 Amounts of ingredients used to study the influence of fat

Ingredients			
Cocoa	Alkalized cocoa (**T-1,T-10,T-20**)		
	400 g (0% fat)	**444 g** (10% fat)	**500 g** (20% fat)
Sugar	**250 g** (fructose)		
Water	**600 g** (mineral water)		

The evolution of the chemical groups during the thermal process at 90°C was evaluated using our SPME method. The results look very similar for all three different fat content cocoas (Figure 6.20). The aldehydes group (dark blue curve) presented significant and constant decrease in all three experiments, most probably caused by some degradation reactions that affect their global presence in the volatile phase. This behaviour was already observed and discussed in the previous study at the same reaction temperature. The group of the pyrazines and pyrroles (pink curve) decreased slowly with time maybe because the temperature and the time degrade the pyrazine skeleton producing small changes in their concentrations during the reaction. The other groups of compounds did not present significant changes in their concentrations during the reaction and remained constant with time. Tables with the individual compound evolution can be found in appendix chapter 10 from Table 10.22 to Table 10.24.

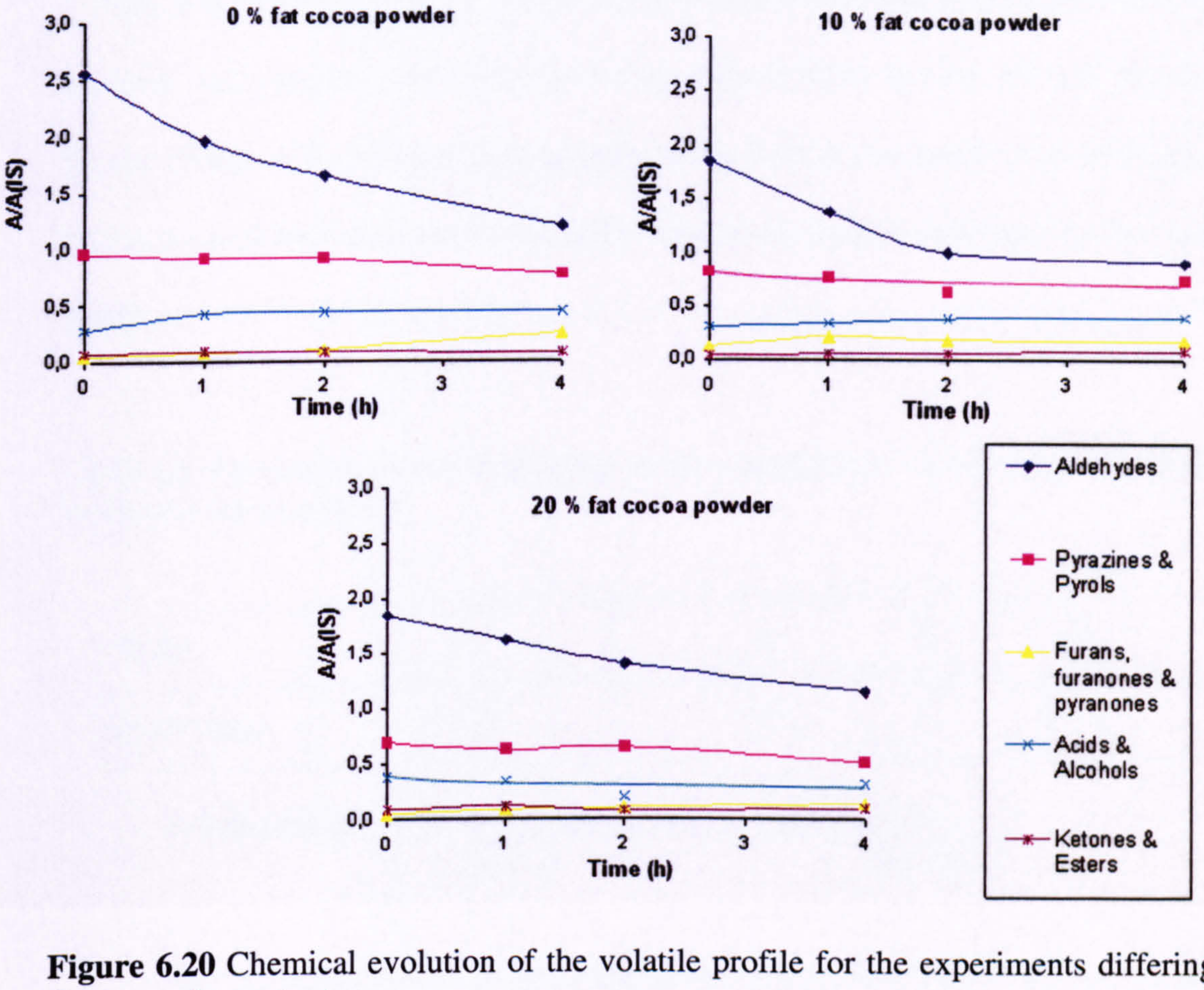


Figure 6.20 Chemical evolution of the volatile profile for the experiments differing in the cocoa fat content.

As was concluded in the previous study, a temperature of 90ºC is too low to allow significant Maillard reactions to occur or at least low enough not to produce a detectable amount of Maillard products. The significant changes in the chemical composition of the volatiles are degradation reactions mainly for the aldehydes that influence the overall volatile fraction concentration. The main difference in the three experiments was at the start (t = 0) where the cocoa fat content changes the proportions of the chemical groups. As was presented in section 5.3, the fat content affects the capacity of volatilisation of the compounds, allowing more volatile compounds to be liberated from the non-fat cocoa powder.

6.2.2 Sensorial evaluation

The Researcher at Salford carried out a personal sensorial evaluation of the three products. All samples looked similar in appearance (dark brown) and had acceptable flavour (Table 6.5). The product prepared with the non-fat cocoa powder seemed to display a *chocolatier* note compared to the others two products and therefore was scored highest.

Table 6.5 Personal sensorial evaluation of the experiments carried out using different fat content cocoa powder.

% fat	0	10	20
Evaluation	*****	****	****

Acceptability * ** *** **** ***** → Bad … Excellent

The sensorial analysis was completed using the trained sensorial panel at Natraceutical. This study was the first to be evaluated by the panel after their training period. The objective was to determine whether the fat content delays the aromatic development during the thermal reaction. Three samples obtained with different fat content cocoa powders (0%, 10%, and 20%) were evaluated by Profile test. [129]

The Profile test is a descriptive analysis where different sensory attributes (descriptors) are rated. Several descriptors were chosen for the evaluation session:

- *Aroma* : chocolate, cocoa, toasted, caramel, and vanilla,
- *Flavour* : chocolate, cocoa, toasted, caramel, bitterness, and vanilla.

These descriptors were proposed because they describe the major sensory attributes of the cocoa products. The panellists were trained with standards for each descriptor as was explained in section 5.4.

In order to avoid possible contributions of the skimmed milk to the sensory profile, the samples were prepared in water with 4% of the chocolate sample and 3% of sugar. In the evaluation session, the tasters evaluated two descriptors for each of the three samples plus a control sample per session (Figure 6.21). Three sessions were needed to evaluate the six flavour descriptors (chocolate, cocoa, toasted, caramel, bitterness, and vanilla), five in the case of the aroma because bitterness can only be evaluated as flavour.

Session 1: Chocolate and cocoa descriptors

Session 2: Toasted and caramel descriptors

Session 3: Vanilla and bitter descriptors

Figure 6.21 Organisation of the evaluation sessions

For the evaluation sessions, the questionnaire (Figure 6.22) displayed the descriptors in two groups, aroma and flavour with a 10 cm straight line drawn next to each of them. The taster had to evaluate the samples by marking the line with 0 being "descriptor not detectable" and 10 being "very strong and intense descriptor". The marks were then measured to give a score between 0 and 10.

Figure 6.22 Example of a questionnaire used during the evaluation using cocoa and chocolate descriptors.

Please, assess the FLAVOUR/AROMA of these three samples and mark the intensity for each descriptor.

Cocoa

0 — 10

Code	cm

Chocolate

0 — 10

Code	cm

Because the panel was newly trained, a "training/reminding" session was carried out before each evaluation session using standards for each descriptor. During this session, the standards were used as a reminder of the qualities of the descriptor examined in the evaluation session. This pre-evaluation session ended with a simple ranking test of three samples where the descriptor was added in different proportions.

In the evaluation session, three samples plus a control were given to the tasters with a Profile Test questionnaire. The marks on the questionnaire were transformed intro scores and compiled in a spreadsheet using Microsoft® Excel™:

- The Ranking test results were analysed using the Kramer's tables. [130, 131]
- The Profile test results were analysed using the Stat Graphics™ program by ANOVA and Chi-Squared distribution. [132]

All these statistical methods are indicated to determine if the differences in a parameter evaluated several times (in our case using the sensory panel) for diverse samples have significance depending on established confidence's interval. The distribution of results for one sample and one parameter, descriptor in our case, is

calculated by the statistical methodology. Two samples are considered statistically different (significance) when their distributions are not superimposable.

Applying the Kramer table to the results of the Ranking test (Appendix 10.2) indicates that the panel was able to distinguish between the aromas of cocoa, chocolate, caramel, toasted and vanilla and the flavours of cocoa, chocolate, caramel, and bitterness. Vanilla flavour presented some difficulties to be clearly distinguish, therefore, additional training was needed for this descriptor.

The results from the Profile test were analysed for their statistical significance are presented as averages in Table 6.6.

Table 6.6 Mean of the scores of the sensorial profile test for each descriptor.

Descriptors	**Samples**		
	0% Fat	**10% Fat**	**20% Fat**
Cocoa Aroma	2.05^{a}	3.39^{a}	3.98^{a}
Chocolate Aroma	6.86^{a}	5.82^{a}	4.93^{a}
Cocoa Flavour	2.6^{a}	4.11^{ab}	5.43^{b}
Chocolate Flavour	6.95^{a}	6.35^{a}	5.37^{a}
Toasted Aroma	4.40^{a}	4.14^{a}	3.6^{a}
Caramel Aroma	5.36^{a}	4.77^{a}	5.96^{a}
Toasted Flavour	4.96^{a}	4.58^{a}	5.51^{a}
Caramel Flavour	6.84^{a}	5.91^{a}	3.65^{b}
Vanilla Aroma	6.22^{a}	4.48^{b}	5.86^{ab}
Vanilla Flavour	6.24^{a}	4.9^{ab}	4.6^{b}
Bitterness	5.76^{a}	4.49^{a}	4.19^{a}

Means with different letters within a row are significantly different ($P < 0.05$)

The panel results had a wide distribution giving a low significance between the samples.

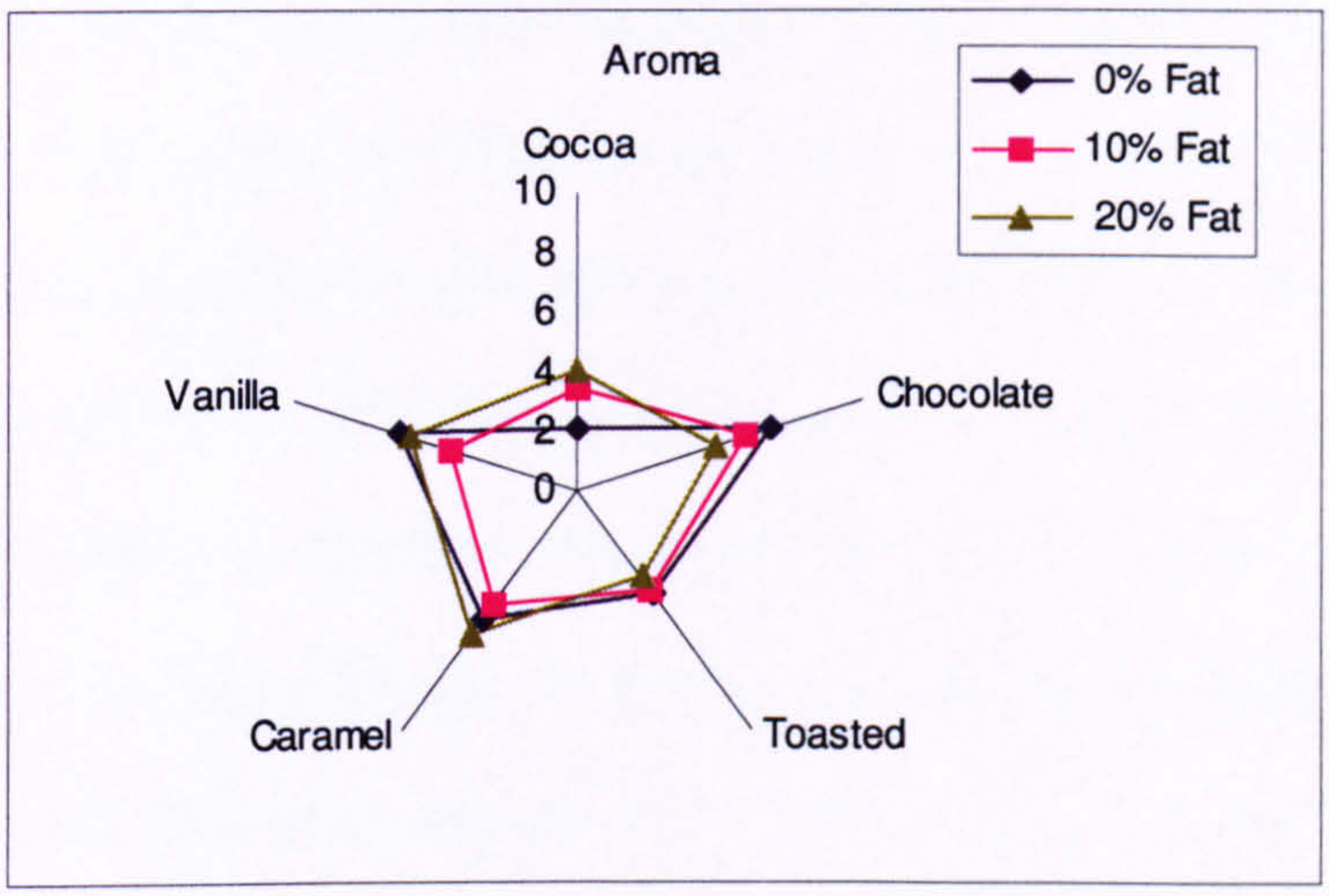


Figure 6.23 Profile representation of the aroma descriptors

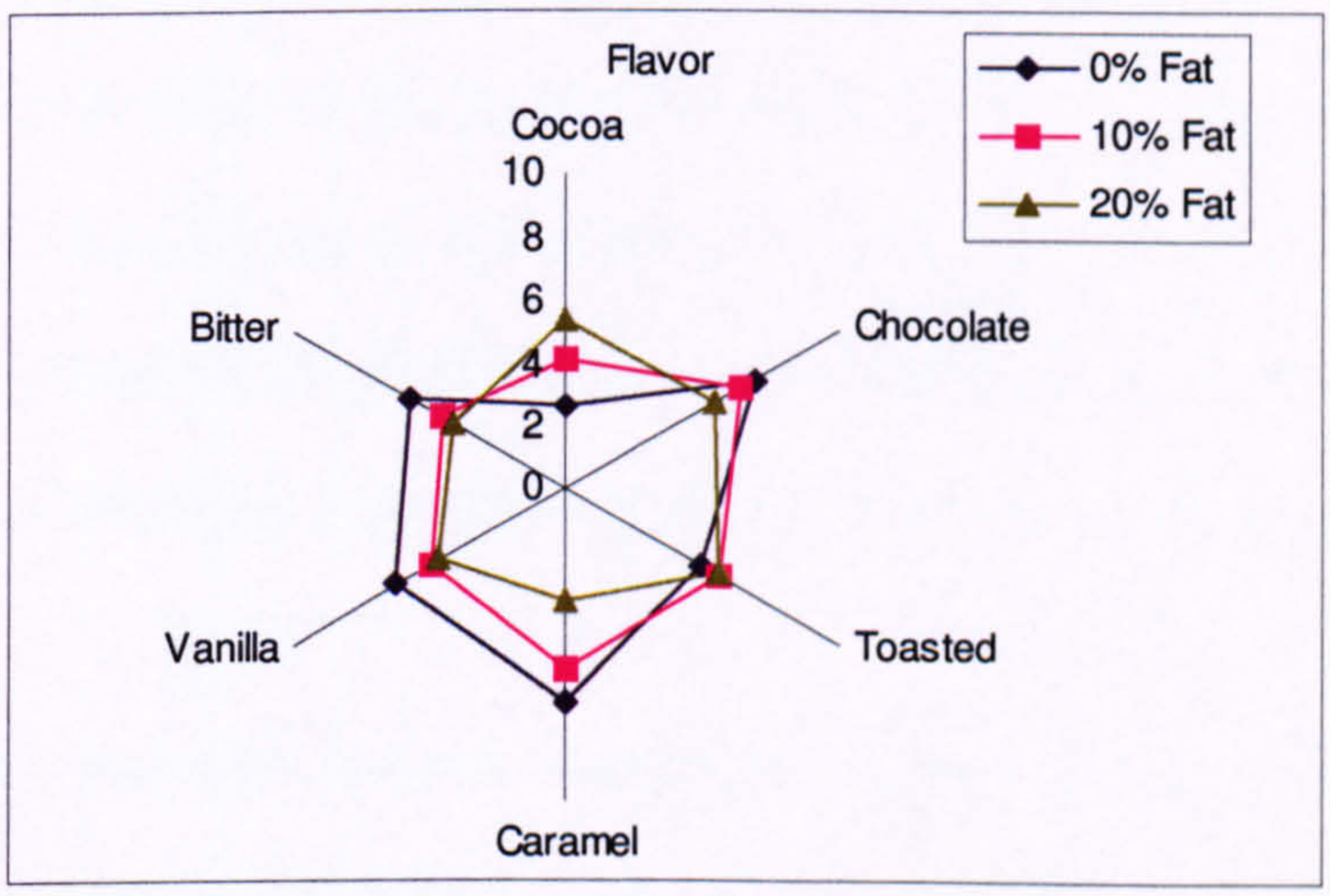


Figure 6.24 Profile representation of the flavour descriptors

The profile test exhibited differences between the three samples obtained using different cocoa fat content (0-1%, 10% and 20%). These differences are illustrated in Table 6.6 and in Figure 6.23 and Figure 6.24. Using Stat Graphics™ software of statistics analysis, significant differences were found between cocoa flavour, caramel

flavour, vanilla aroma and vanilla flavour. Furthermore, clear tendency can be observed even in the descriptors where no statistical significance was found. The representation of the descriptor for the aroma and flavour show that the sample obtained using the non-fat cocoa had the lowest score in the cocoa descriptor and the highest in chocolate for both aroma and flavour graphs while the sample with 20% fat cocoa powder scored the highest in the cocoa descriptor and the lowest in chocolate. The sample obtained with 10% fat cocoa was found to fit in between the other two samples. These results showed a clear trend; the higher the fat content the higher the cocoa descriptor and the lower the chocolate descriptors. The training of the panel was mainly focussed on differentiating between cocoa and chocolate, because the objective of the project was to develop chocolate flavours from cocoa. Therefore, it was essential that the panel was able to differentiate between those two different descriptors.

No other conclusion could be drawn from the others descriptors for the aroma representation. In the case of the flavour, the sample with the non-fat cocoa powder is clearly the most bitter and displays the highest vanilla/caramel descriptor. There was no difference in the toasted flavour descriptor between the samples. In general, the non-fat cocoa powder sample scored the highest in all the descriptors, with the exception of the cocoa.

Comparing with the personal evaluation of the researcher, there was a good correlation with the result obtained from the trained panel, this is reassuring taking into account that the researcher was involved in the set-up and training of the panel.

6.2.3 Conclusion

To obtain a strong chocolate product, the non-fat cocoa powder is recommended; although the evidence shows no guarantee that, the fat content has a significant

influence in the flavour development. However, some interesting findings can be extracted from this study:

- From the chemical analysis of the volatile fraction, the reaction carried out at 90°C for 4 hours resulted in a decrease in aldehydes probably due to the degradation reactions as observed in the previous study. The sugar used in this study was the reducing sugar fructose, no significant Maillard reactions were observed.

- From the sensorial point of view, the samples obtained using non-fat cocoa powder demonstrated more chocolate and less cocoa flavour and aroma than the fat-containing products. This can be attributed to the occurrence of Maillard reactions or flavour development not detected by our analytical methodology. This could also confirm our hypothesis that non-fat cocoa is able to produce more flavour molecules because the cocoa particle are not covered with fat and can therefore react more freely in the reaction mixture.

On the other hand as it was observed in chapter 5 and in the experiments that we carried out, the non-fat cocoa had better molecular diffusion to the volatile phase due to its absence of fat (non-fat experiment had higher volatiles recoveries), and this can have a positive influence in the global chocolaty notes.

We are not able to demonstrate which of the two effects is responsible for the enhancement of the chocolate flavour. Probably both have a contribution but the changes in the matrix have had a key contribution in the volatile profile and from our point of view, we believe this is a leading factor.

6.3 Influence of Sugar

In the time and temperature studies, sucrose was used as the sugar while fructose was used in the study looking at the influence of the cocoa fat content. This led us to study the influence of different sugars. Fructose, glucose, xylose, and a commercial syrup containing a mixture of fructose, glucose and sucrose called Meliose™ 710 (*Roquette*) were employed in the experiments reported in this section (Figure 6.25). The reaction conditions employed in this study were 90°C as reaction temperature and 4 hours as a reaction time.

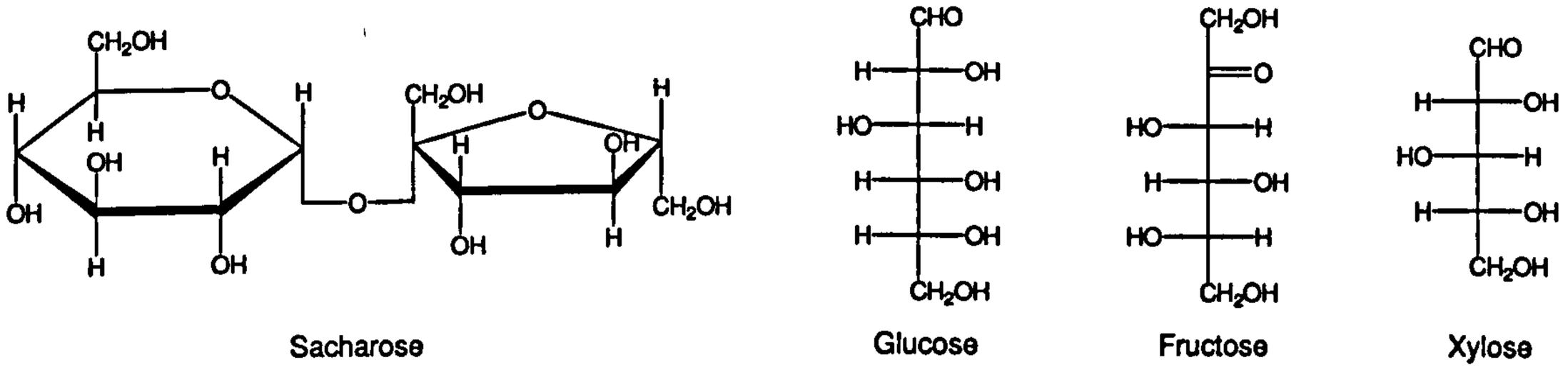


Figure 6.25 Structures of the sugars used in this study

From the non-enzymatic browning point of view, the aldopentose xylose, presents the highest reactivity followed by the ketohexose fructose and aldohexose glucose. All are reducing sugars and should react to give Maillard products themselves, unlike sucrose (disaccharide) which needs to break down into fructose and glucose to be reactive and afford Maillard reactions.

From the sensorial point of view, taking sucrose as reference (sweetness score 100) fructose is around 150, thus 50% sweeter than sucrose. Glucose is about 60, therefore 40% less sweet than sucrose and almost three times less sweet than fructose. On the other hand, xylose presents practically no sweetness and sensorially its contribution to the sweet taste is practically negligible.

The reactions were carried out with 20% fat natural cocoa powder (500 g), mineral water (550 g) and the chosen sugar (500 g). In the case of meliose™, a commercial syrup (70% of dry extract) from *Roquette* (sugar company) composed of 53% of glucose, 42% of fructose and 5 % of disaccharides, 715 g of meliose™ and 335 g of water were used to maintain the ratios (Table 6.7).

Table 6.7 Amounts of the ingredients used in the experiments studying the influence of sugar.

Ingredients				
Cocoa	**500 g** Natural cocoa powder (20% fat)			
Sugars	**500 g** (Fructose)	**500 g** (Glucose)	**500 g** (Xylose)	**715 g** (Meliose™)
Water	**550 g** (mineral water)			**335 g**

6.3.1 Chemical evolution of the volatile profile

The products of these reactions were analysed by HS-SPME-GC-MS (section 5.1.2) which allowed us to follow the evolution of the volatile profile using the different sugars.

The representation of the evolution of the profile against time (Figure 6.26) for the four different sugars used in the experiments shows for all cases, except for xylose, a clear diminution of the concentration of the aldehydes group (dark blue curve) as well as a slight decrease of the pyrazines (pink curve). These results fit with the ones obtained under the same conditions using sucrose. The aromatic aldehydes were responsible for this decrease and a significant increase of the 2-methyl butanal was also recorded. This time, an increase in the furans and pyrroles group (yellow curve), not observed with sucrose, was detected. This could be attributed to the higher reactivity of

the sugars used in these experiments. Thus, at 90ºC using these sugars the caramelisation reactions seemed to have occurred more readily than with sucrose, resulting in an increase of almost 3-fold after 4 hours.

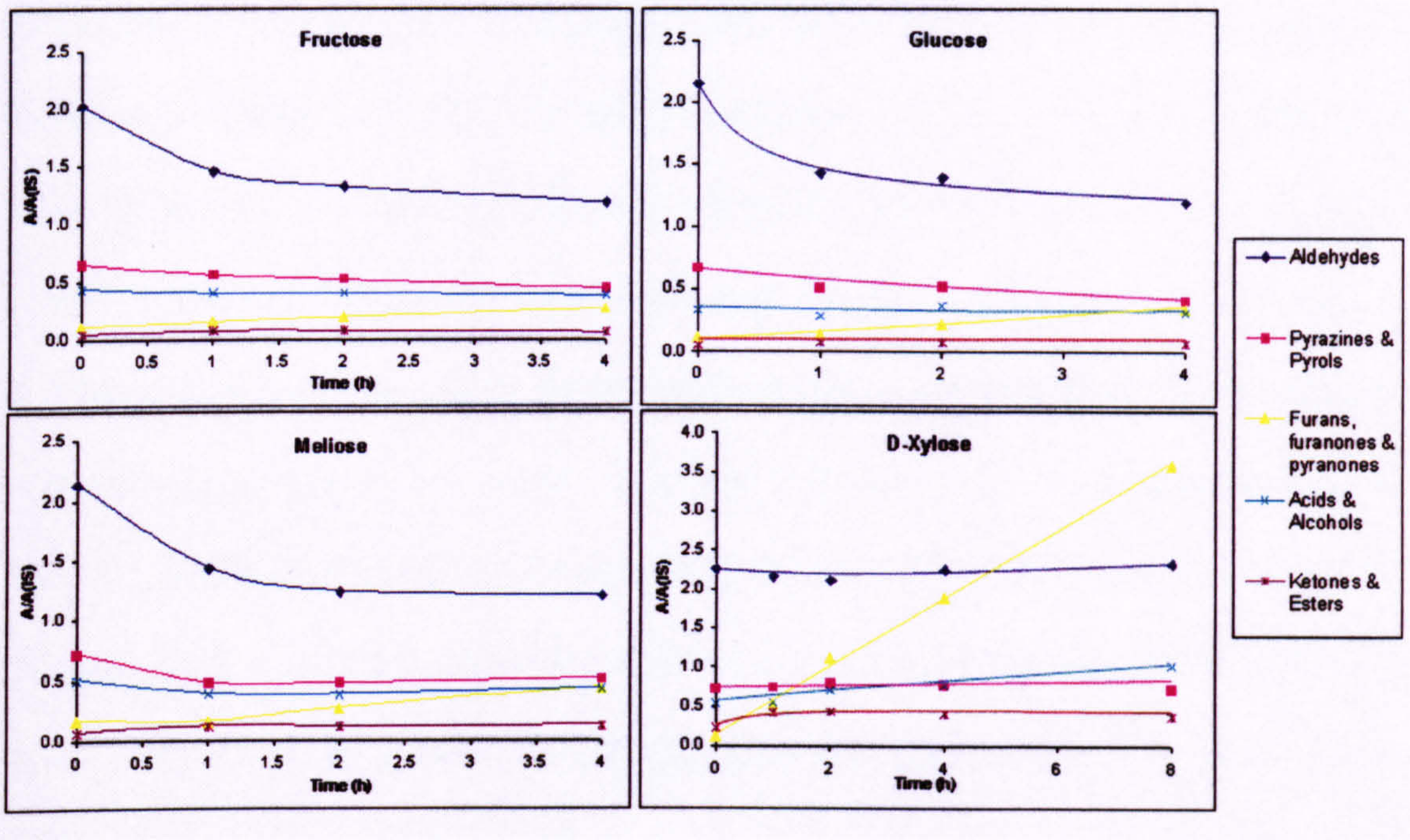


Figure 6.26 Chemical evolution of the volatile profile of the experiments differing in the sugar used.

Comparing these results with those obtained in the fat content study, (section 6.2) where fructose was used; there are similitudes in the diminution of the aldehydes and pyrazines, although no significant increment of the furans was observed. The reason for this lack of concordance in these two experiments where fructose was used could be attributed to the pH of the reaction mixture. In the fat content study, the cocoa powder used was alkalised. The effect of using alkalised cocoa powder (**T** family) increases the pH of the reaction mixture close to 8 while with natural cocoa powder the pH was around 6. The dehydration of the sugars are acid-catalyzed [122] therefore this difference in the pH of the reaction mixture could be the main contribution to the differences observed between both experiments.

In the case of the xylose experiment (Figure 6.26, bottom right), all these arguments are even truer. Not only caramelisation could be detected with a strong increase of the furans, furanones and pyranones group (yellow curve) but even Maillard products were clearly present reflected in the aldehyde group (dark blue curve) which during this experiment did not decrease. The furans, furanones and pyranones group increases constantly becoming the largest volatile group after 4 hours of reaction with again furfural the principal compound of this group. The aldehydes group presented no evolution compared with the other sugar experiments. Looking at the evolution of individual aldehydes, we found compensation between the formations of the aliphatic aldehydes (isopropanal, isobutanal, 3-methyl propanal) and the degradation of the aromatic aldehydes (benzaldehyde, benzacetaldehyde). This rearrangement between the aliphatic against aromatic aldehydes resulted in a constant amount of the aldehydes group. Tables with the individual compound evolution can be found in appendix chapter 10 from Table 10.25 to Table 10.28.

6.3.2 Sensorial evaluation

The sensorial evaluation for these products was carried out by the researcher at Salford (Table 6.8) due to the unavailability of the trained sensorial panel at the sponsoring company.

Table 6.8 Personal sensorial evaluation of the products prepared with different sugars

Sugar used	Fructose	Glucose	Meliose™	Xylose
Aroma	****	****	****	*****
Taste	****	*****	*****	*

Acceptability: * ** *** **** ***** → Bad … Excellent

All products had a very similar sensorial evaluation, except for the one obtained using xylose. Practically no differences were found in the aroma, only the xylose-generated product had a slightly better aroma resulting in a better score. On the other hand, the taste for the xylose experiment was intensely bitter and with clear overcook/burnt shades that were very unpleasant, resulting in the lowest score for the taste. The others products had very close tastes and only the difference in the sweetness make a small difference between them. The fructose experiment, as expected, was the sweetest of all, resulting in the lowest score.

6.3.3 Conclusion

The effect of several reducing sugars was investigated. An increase of the caramelisation products was observed, particularly in the xylose experiment. Unlike the other sugar experiments, no degradation of aldehydes group was observed in the xylose experiment. Sensorially, all the products had a great aroma, especially the xylose product that scored the highest. On the other hand, the flavour for the xylose product had an intense bitterness and overcook/burnt shades.

Chapter 7

7 Biotechnology applied to the flavour development: Enzymatic Technology

In the previous chapter, roasted cocoa powder was submitted to different thermal conditions and the products assessed chemically and sensorially. After the roasting step the levels in cocoa of free $-NH_2$ and amino acids are low and not very reactive.[43] In this part of the PhD project, we will aim to increase the amount of precursors that react to produce flavour molecules in order to achieve higher flavour development.

Coming back to the first steps of the Maillard reaction,[133] sugars react with amino compounds to produce, after several steps, the flavour molecules. If the sugar was used in excess, the amino compounds are the theoretical limiting reactants. Therefore, increasing the amount of amino compounds should lead to a higher flavour development. Keeping in mind that we only want to use high quality natural ingredients, some possibilities such as adding amino compounds or amino acids from an exogenous source were excluded.

The strategy was to free amino acids and short peptides from the cocoa itself. A high percentage of the nitrogen-containing proteins is bound to the matrix unavailable to participate in Maillard reaction. Thus, the cocoa proteins will be used as a source of aroma precursors. Proteins are polymers of amino acids [134], well known as Maillard reactants, therefore the degradation of proteins into their amino acids, peptides or polypeptides should afford precursors that will react in Maillard reactions and enhance the flavour development after a thermal process.

Chemical hydrolysis of proteins into amino acid and peptides is well established, but the conditions needed are strong and the flavour could be compromised. To produce

the degradation of the proteins under soft and natural conditions and preserve the intrinsic aromatic characteristics of the cocoa, enzyme technology was considered.

7.1 Enzymes in food production

Enzymes are naturally occurring proteins which allow all the biochemical processes of life to occur speeding up theses processes that would otherwise take a long time. Enzymes are found in all food raw materials. When purified and used in food preparation, some of these enzymes offer benefits such as improved flavour, texture and digestibility. The application in the industry is obvious. It allows the manufacturer to produce more of a particular product in a shorter amount of time thus increasing product quality and profit.

The use of enzymes in food production has been used from ancient days, for example, the brewing of beer or the production of wine are known before the Egyptians (Table 7.1). They used naturally occurring microorganisms such as bacteria, yeasts and moulds to make food because these microorganisms are the source of the enzymes that produce the desirable changes in the food; many of these processes are still in use nowadays.

Table 7.1 Chronology of enzymes use in food production.[135]

6000 BC	The Egyptians and Sumerians developed fermentation for use in brewing, bread-baking.
2000 BC	Chinese made yogurt and cheese with lactic-acid-producing bacteria
800 BC	Calves' stomachs and the enzyme, chymosin, were used for cheese-making.
1878	The components of yeast cells which cause fermentation were identified and the term "enzyme" was first used, derived from the Greek term meaning "in yeast".
1926	Enzymes were first shown to be proteins.
1980s	Enzyme preparations were developed to improve the digestibility and nutrient-availability of certain animal feeds.
1982	The first food application of a product of gene technology, alpha amylase, took place.
1988	Recombinant chymosin was approved and introduced in Switzerland, marking an early approval of a product of gene technology for a food use.
1990	Two food processing aids obtained using gene technology: - An enzyme for use in cheese-making in the US, and - A yeast used in baking in the UK.

Today, enzymes are used for an increasing range of applications: bakery, cheese making, starch processing and production of fruit juices and other drinks. Here, they can improve texture, appearance and nutritional value, and may generate desirable flavours and aromas. In the contemporary food production, the emerging new enzymes applications have a number of advantages:

- They are welcome as alternatives to traditional chemical-based technology, and can replace synthetic chemicals in many processes. This can allow real advances in the environmental performance of production processes, through lower energy consumption and biodegradability.

- They are more specific in their action than synthetic chemicals. Processes that use enzymes therefore have fewer side reactions and waste by-products, giving higher quality products and reducing the likelihood of pollution.

- They allow some processes to be carried out which would otherwise be impossible. An example is the production of clear apple juice concentrate, which relies on the use of the enzyme, pectinase.

7.1.1 Enzyme production

Where enzymes are produced from microorganisms (the main types include species of *Bacillus, Aspergillus, Streptomyces* and *Kluyveromyces*), these are grown by fermentation in large fermenters with capacities of up to 150,000 litres; here, temperature, nutrients and air supplies are adjusted to suit their optimal development. As in other parts of the food chain, strict rules of hygiene are followed. When the process is complete, the fermenter contains a broth, which includes enzymes, nutrients and microbes. This is purified by passing it through a series of filters to remove impurities and extract the enzyme.

Table 7.2 Enzyme food industrial applications.[136]

Application	Enzymes used	Uses
Baking industry	Fungal alpha amylase enzymes: normally inactivated at about 50 degrees Celsius, destroyed during baking process	Catalyses breakdown of starch in the flour to sugar. Yeast action on sugar produces carbon dioxide. Used in production of white bread, buns, and rolls
	Protease enzymes	Biscuit manufacturers use them to lower the protein level of flour.
Baby foods	Trypsin	To predigest baby foods
Brewing industry	Enzymes from barley are released during the mashing stage of beer production.	They degrade starch and proteins to produce simple sugar, amino acids and peptides that are used by yeast for fermentation.
	Industrially produced barley enzymes.	Widely used in the brewing process to substitute for the natural enzymes found in barley.
	Amylase, glucanases, proteases	Split polysaccharides and proteins in the malt
	Betaglucosidase	Improve the filtration characteristics.
	Amyloglucosidase	Low-calorie beer
	Proteases	Remove cloudiness produced during storage of beers.
Fruit juices	Cellulases, pectinases	Clarify fruit juices
Dairy industry	Rennin, derived from the stomachs of young ruminant animals (calves, lambs)	Manufacture of cheese, used to hydrolyse protein
	Microbially produced enzyme	Now finding increasing use in the dairy industry
	Lipases	Implemented during the production of Roquefort cheese to enhance the ripening of the blue-mould cheese.
	Lactases	Breaks down lactose to glucose and galactose
Starch industry	Amylases, amyloglucosideases and glucoamylases	Converts starch into glucose and various syrups
	Glucose isomerase	Converts glucose into fructose (high fructose syrups derived from starchy materials have enhanced sweetening properties and lower calorific values)

Since the early 1980s, companies that produce enzymes have been using genetic engineering techniques to improve production efficiency and quality and to develop new products (Table 7.2). There are clear advantages here for both, industry and consumers, with major improvements in enzyme production giving better products and processes. However, progress is being slowed down because the debate on some other, more controversial applications of biotechnology - such as genetic engineering in animals - is continuing throughout Europe.

At present, modern biotechnology can be used to give a range of advances in enzymatic production technology improving productivity and cost-effectiveness in existing processes. By producing more efficient enzymes, the amount of raw materials, energy and water needed to make a product can be reduced by as much as one-half by changing from a traditional strain of microbe to a genetically modified one.

Companies can tailor their enzymes more precisely to customer demands for products with specific properties, therefore being able to supply enzymes which otherwise could not be produced in large enough quantities. As a result, the consumers have access to a wider variety of products. An example is the amylase-based product which makes bread stay fresh for longer.[136] Consequently, the market of enzymes has had a significant growth in the last decade. The enzyme manufacturers have increased their portfolio with different enzymes for many applications giving to the food producers a wide range of possibilities combining different mixtures of enzymes to develop their own products. This wide range gives the possibility to obtain a diversification of flavours from the raw material using the same process but different enzymes. The selectivity of this enzymatic technology causes a higher control of the quality of the foods reducing the variation between different batches and even having significant differences with the raw material. This is especially useful in industries such

as the wine industry where the quality of the raw material between harvests can be notably different. Using enzyme technology a similar quality product can be produced.

7.1.2 Enzymes and cocoa products

The studies related to enzymes in cocoa have been focused on the fermentation step that is carried out in the regular and traditional cocoa manufacture.[44, 137, 138] Fermentation of cocoa seeds is carried out in the origin countries where the cocoa is harvested. Traditionally the seeds with some pulp are piled up in heaps or boxes and left to ferment during several days (see section 1.2). During the fermentation, there is a microbial succession of a wide range of yeasts, lactic-acid, and acetic-acid bacteria that cause high temperatures (> 50°C) and microbial products, such as ethanol, lactic acid, and acetic acid. This combination of events causes the death of the beans unleashing the production of flavour precursors. Thus a good fermentation process is crucial to have a good cocoa flavour consequently good chocolate flavour. Studies to understand the natural fermentation determining which enzymes are responsible for the generation of the flavour precursors have been reported in different countries, continents or even genotypes. [34, 44, 139-142] Also a synthetic microbial cocktail has been used to mimic the natural fermentation yielding a good quality chocolate.[47] Misnawi and collaborators activated some enzymes dry beans to generate enough precursors to produce a cocoa with a regular flavour [143], especially useful for those low quality cocoas that have been poorly fermented. Activating these enzymes, the cocoa can be transformed into a higher quality product.

Studies demonstrated that the proteolytic enzyme activity is the mainly responsible for the aroma precursor's formation (amino acids and peptides).[44] Voigt and collaborators reported the *in vitro* generation of aroma precursors using the cocoa

proteins and natural aspartic endoprotease and a carboxypeptidase (proteases) present in ungerminated cocoa seeds.

The use of exogenous enzymes to modify the cocoa flavour has not been extensively studied, only a few studies were found, mainly to reduce the bitterness using exopeptidases. [134]

A recent work has studied the effect of an exogenous protease on fermented cocoa nibs. The formation of precursors and flavour production once roasted was verified. The sensorial evaluation of the chocolate produced with the protease treated cocoa had a stronger perception on the chocolate flavour and a bitter taste.[55]

7.2 Enzymatic hydrolysis of the cocoa proteins

Free cocoa amino acids (**aa**) react during the roasting step with reducing sugars to become flavour molecules in a considerable yield, giving the popular flavour of roasted cocoa. Due to this reaction, the free **aa** decrease in concentration. Serra and Ventura [31] determined the amount of free **aa** in cocoa samples from different origins before and after the roasting using traditional cocoa roasting conditions. They reported an average reduction of 80% with a remaining **aa** concentration around 1.5 mg/kg. The remaining 20% is composed of the least reactive **aa**. The experiments reported in chapter 6 aimed to force these **aa** to react with the intention to increase the aroma production.

The protein content in a regular roasted cocoa powder with 10-12 % fat is close to 25% in weight (Table 7.3). The partial hydrolysis of the protein using exogenous enzymes will increase the amount of **aa** or other reactive $-NH_2$ species, i.e. short peptides, thus increasing the flavour evolution potential as per the objective of this chapter.

Table 7.3 Composition of cocoa powder (10-12%) [11]

Composition	**%**
Moisture	4.5
Protein	23.6
Starch	12.2
Fat (Cocoa Butter)	10.5
Ash (Total)	6.0
Sugars	2.1
Cellulose	18.3
Polyhydroxyphenols	12.5
Methylxantines	2.8

Novozyme™ supplied the enzymes tested in this study. Five food grade enzymes commercially available were assessed to study their hydrolysis activity on cocoa; Flavourzyme™, Protamex™, Papaine (proteases), celluclast (invertase and cellulose) and NS-44055 (complex mixture of carbohydroylases).

The hydrolysis grade was determined using the OPA (*o*-phthaldialdehyde) methodology [111] and reported as % increase in free $-NH_2$ (Table 7.4). This method is based in a specific reaction between *o*-phthaldialdehyde (OPA) and 2-mecaptoethanol with the primary amines of amino acids, peptides and proteins (Figure 7.1). The absorption of OPA adducts of proteins, amino acids and peptides are very similar, with λ_{max} at 334 nm, the quantification is carried out using an asparagine calibration curve (as introduced in section 5.3.2).

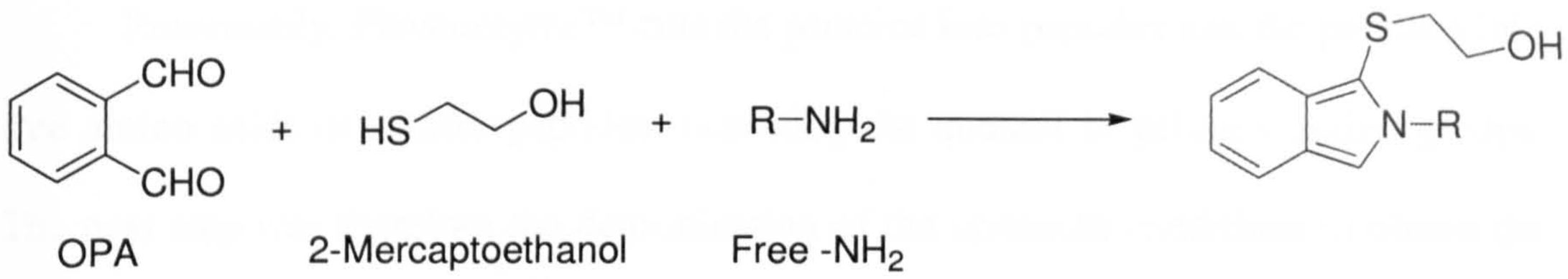


Figure 7.1 Reaction of OPA with a primary amine (R-NH_2,) in the presence of 2-mercaptoethanol to form a fluorescent-labelled complex.

From the point of view of the Maillard reaction, the determination of free amine content in a cocoa mixture can be used as an indicator of the flavour development of the samples. Cocoa-water mixtures were allowed to react with the five enzymes at 50°C for 5 hours (Table 7.4).

Table 7.4 Percentage of hydrolysis caused by each enzyme

Enzyme	% of hydrolysis
Flavourzyme	62.3
Protamex	27.6
Papaine	0.11
Celluclast	5.91
NS-44055	4.02

Conditions : Enzymes (10% v/w) were mixed with cocoa (2 g) and water (6 ml) at 50 °C and incubated during 5 hours.

Flavourzyme™ resulted in the highest hydrolysis rate (62.3 %) with a significant gap with the second highest, Protamex (27.6 %). The other enzymes, NS-44055 and celluclast, only achieved 4.02 and 5.91% hydrolysis respectively, while papaine resulted in almost no increase in the content of free –NH_2. Thus, Flavourzyme was selected to pursue this study.

Presumably, Flavourzyme™ cuts the proteins into peptides and the peptides into free amino acids or shorter peptides increasing the content of primary amino groups. The next step was therefore the determination of the optimum conditions to obtain the highest hydrolysis of the cocoa proteins. Different concentrations and ratios of ingredients were studied. The hydrolysis rate or $-NH_2$ rate ($-NH_2$ content/initial $-NH_2$ content) versus time was followed using the OPA methodology.

The experiments were carried out on a small scale first.

Table 7.5 summarises the conditions used in this study where three different proportions of enzyme (v/w, enzyme/cocoa powder) were evaluated.

Table 7.5 Summary of the conditions tested with Flavourzyme.

	Enzyme	**Cocoa powder**	**Water**	**Temperature**
5 % v/w	0.1 mL	2 g	6 ml	50°C
10 % v/w	0.2 mL	2 g	6 ml	50°C
20 % v/w	0.4 mL	2 g	6 ml	50°C

Figure 7.2 shows the kinetic evolution of three hydrolysis reactions performed using different ratios of Flavourzyme / cocoa powder (Table 7.5). As expected, higher proportion of enzyme resulted in faster hydrolysis rate, the initial speed of the hydrolysis reaction has a direct relationship with the enzyme proportion used.

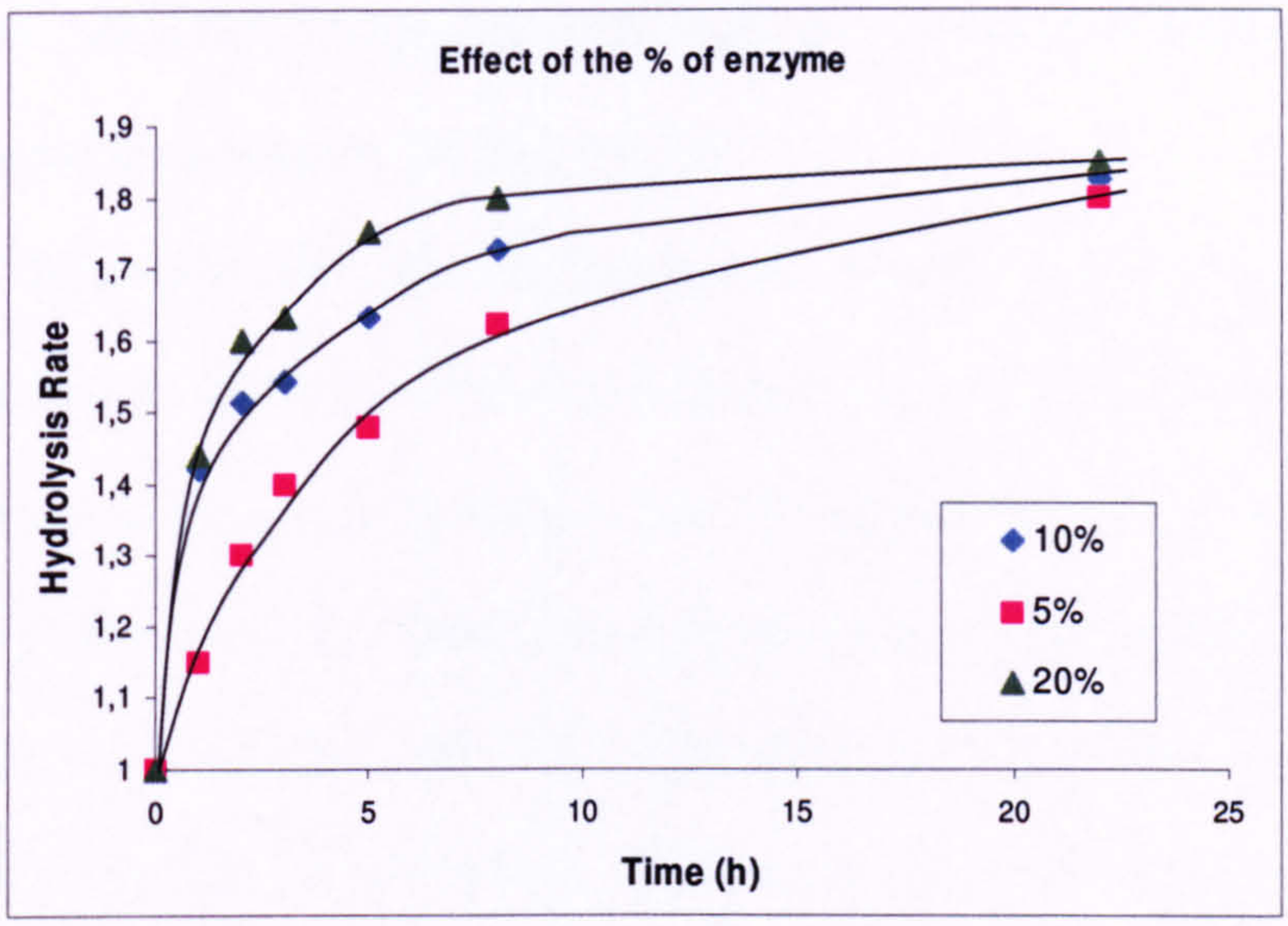


Figure 7.2 Rate of hydrolysis vs. time at three different % of Flavourzyme

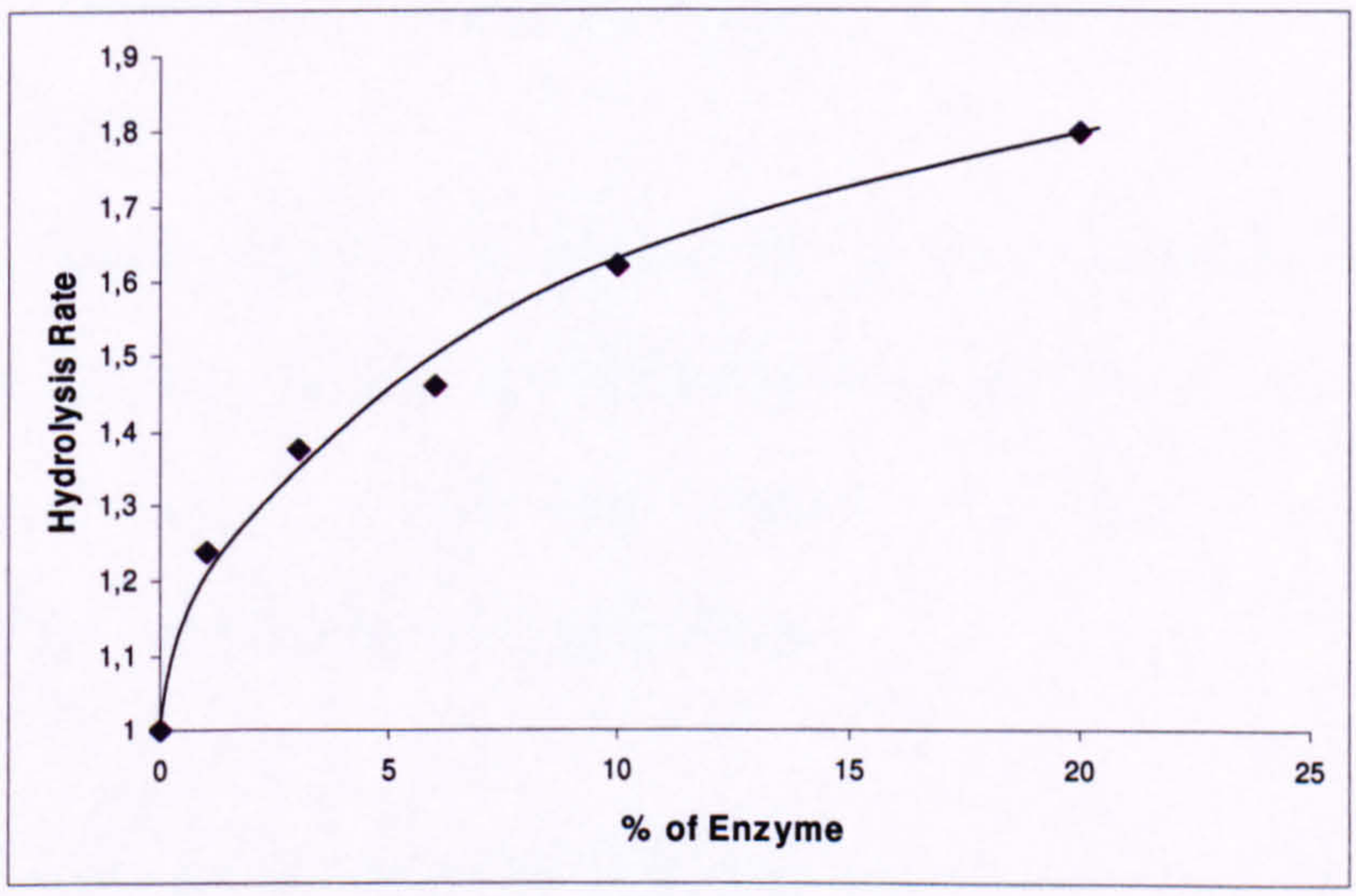


Figure 7.3 Hydrolysis rate vs. % of enzyme after 5 hours of incubation at 50°C

In all three experiments (5%, 10% and 20% enzyme), a plateau was reached and a similar yield (~1.8) was obtained after 24 hours. Reactions with the highest proportion of enzyme reached this yield faster (around 7 h with 20% of enzyme). For reactions with the lowest enzyme proportion, the hydrolysis rate increase was lower and therefore took longer to reach this yield. The hydrolysis proteins from cocoa powder under these

conditions have a maximum when the hydrolysis rate reaches 1.8; the cocoa powder then was exhausted as demonstrated in Figure 7.2.

Figure 7.3 illustrates the effect of the amount of enzyme on the hydrolysis rate at a fixed incubation time (5 hours). These experiments confirmed the direct influence of the enzyme concentration on the hydrolysis rate. This graph shows how the activity of the enzyme decreases when its concentration increase in an like logarithmic manner. Thus, incubating cocoa powder with 10% of enzyme leads to a 60% increase in the content of primary amines. When 20% of enzyme was used, an 80% increase in the content of free amino groups was observed, the extra 10% of enzyme only cause an extra 20% of hydrolysis. Therefore, the range 1%-10% enzyme content was more productive and similar yields with smaller proportions of enzymes can be reached using longer reaction times.

In conclusion, taking into account the possible industrial scale-up, its economical repercussion and the possible off-flavours introduced by the enzyme, the smallest proportion of enzyme **(5%) with 24 hours** of reaction time were chosen as optimum conditions for the enzymatic pre-treatment.

7.3 Volatile development study of enzymatic hydrolysed cocoa powder under thermal processing technology conditions

After selecting the optimum enzymatic pre-treatment conditions, the hydrolysed cocoa was submitted to thermal processing technology with the purpose of studying the generation of volatiles and comparing the results with those found with the thermal technology described in chapter 6.

The experiments were carried out in the two litres IKA reactor, where the cocoa powder and water were mixed and incubated with the enzyme (5%) at 50°C in

continuous mechanical kneading. After 20 hours, the system was sealed and the mixture submitted to different thermal conditions (Figure 7.4). These thermal conditions used temperatures close and over the water boiling point that in sealed reactor produced overpressures around 0.5 to 3 atmospheres.

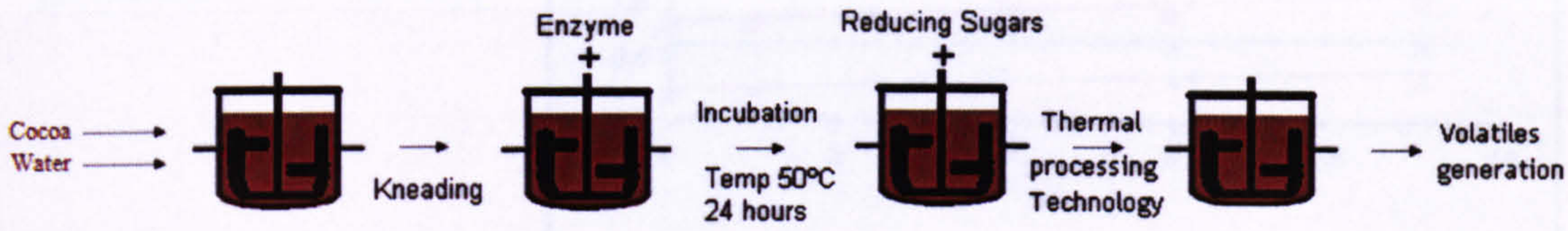


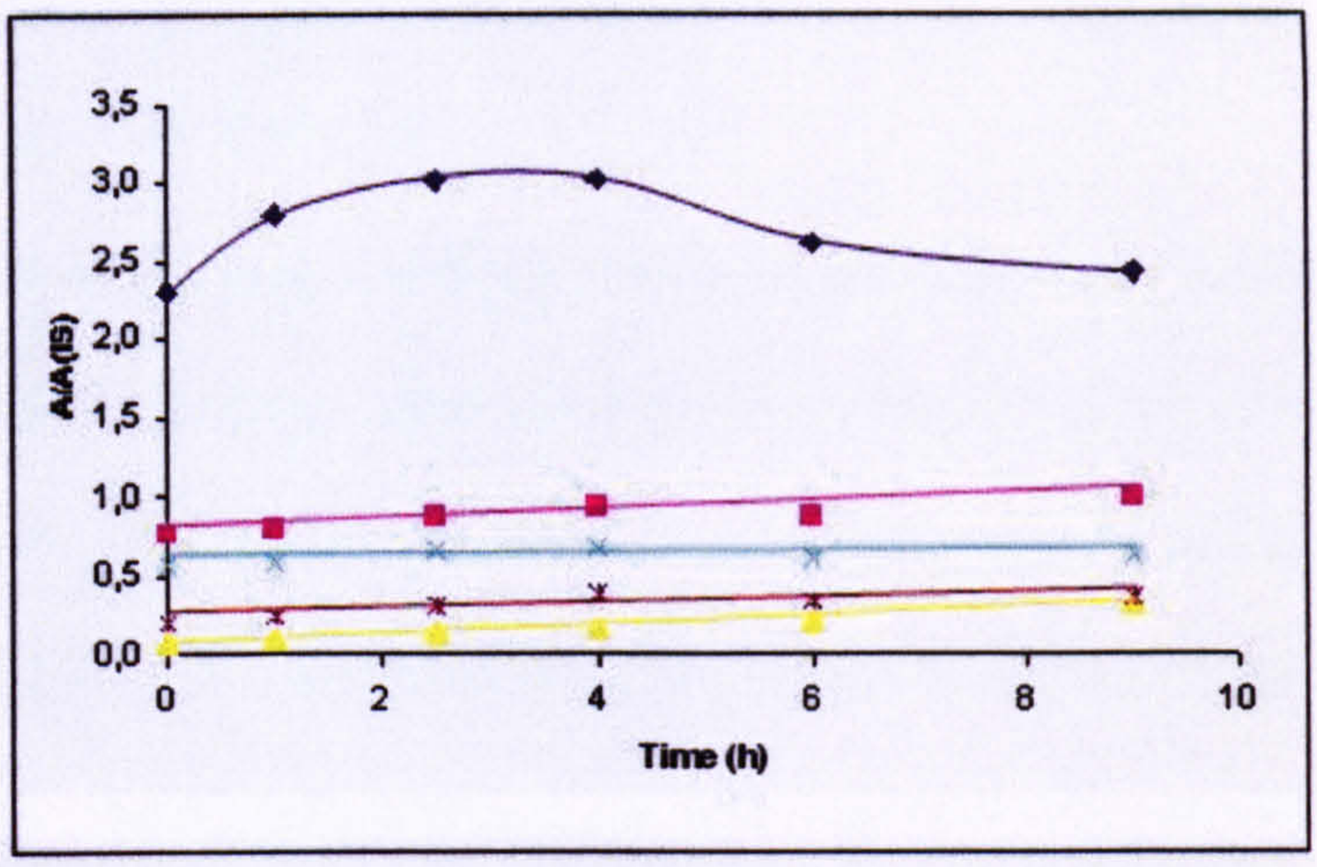
Figure 7.4 Scheme of the experimental process.

7.3.1 Experimental Conditions and Results

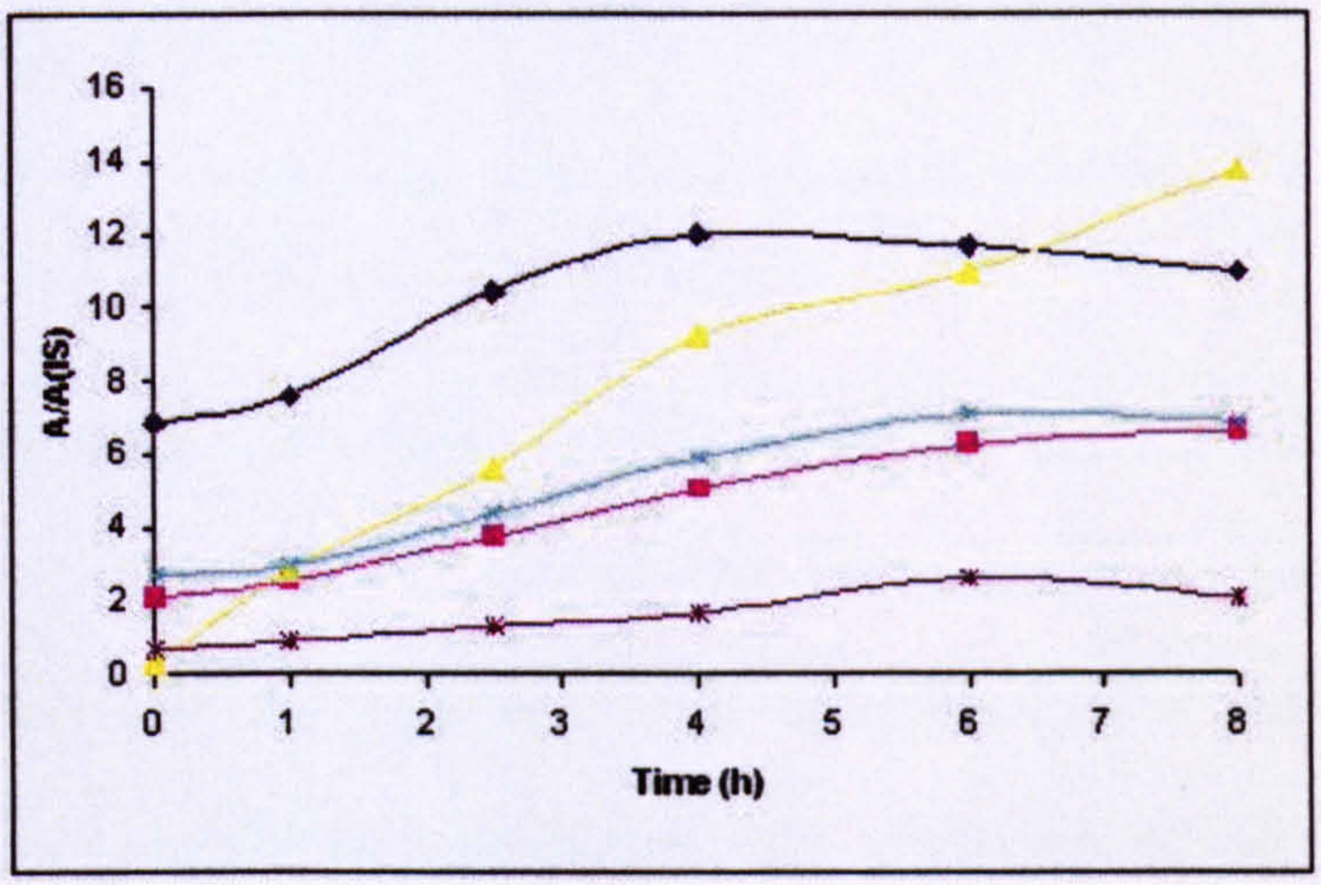
For each batch-experiment, several samples were taken at different times in order to study the evolution of the volatiles. The volatile profile was studied using the SPME methodology, developed in chapter 5 and used to analyse the outcome of the thermal technology in chapter 6. In addition, the content in free $-NH_2$ was measured to study their evolution.

Three sets of experiments were carried out. In all cases, 500 g of natural cocoa powder (20% fat) was mixed with 600 mL of water. The amount of water was increased by 50 mL compared with the "thermal only" experiments to decrease the viscosity. The absence of sugar in the pre-enzymatic step had a great influence on the viscosity of the mixture reaching the limits of the mechanical stirrer of our reactor. This viscosity decreased during the enzymatic reaction probably because of the hydrolytic effect of the enzyme over cocoa powder, reducing the particle size. This was confirmed when samples from these experiments were filtered and difficulties linked to its viscosity

Water	600 gr
Cocoa	500 gr
Enzyme	25 mL
Incubation Temp	50 °C
Incubation Time	20 hours
Sugar	500 gr
Reaction Temp	90 °C
Reaction Time	9 hours



Water	600 gr
Cocoa	500 gr
Enzyme	25 mL
Incubation Temp	50 °C
Incubation Time	20 hours
Sugar	20 gr
Reaction Temp	110 °C
Reaction Time	8 hours



Water	600 gr
Cocoa	500 gr
Enzyme	25 mL
Incubation Temp	50 °C
Incubation Time	20 hours
Sugar	20 gr
Reaction Temp	130 °C
Reaction Time	6 hours

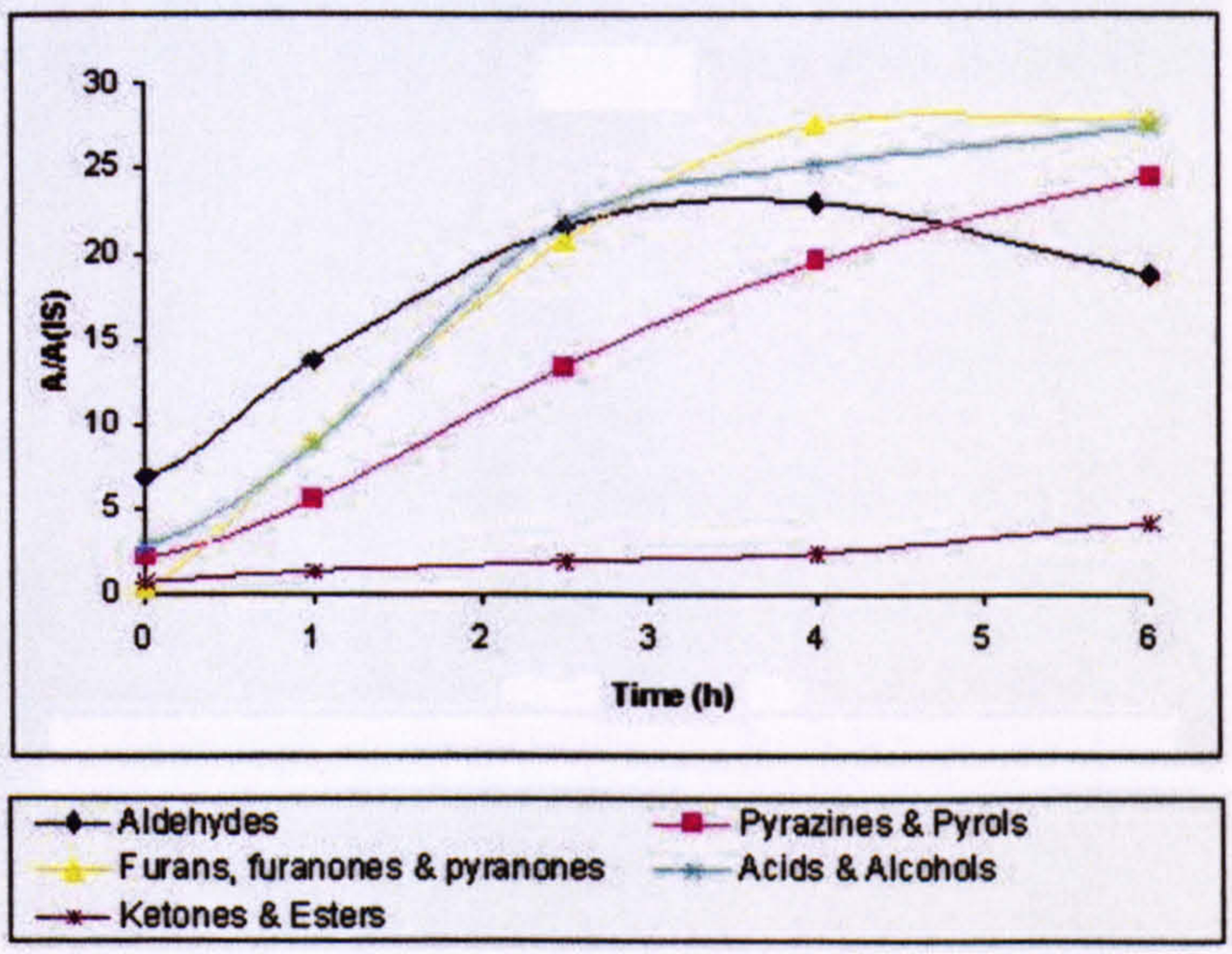


Figure 7.5 Evolution of chemical classes versus time under different thermal conditions. These reactions were carried out with enzymatically pre-treatment of the cocoa powder.

were encountered. Tables with the individual compound evolution can be found in appendix chapter 10 from Table 10.29 to Table 10.31.

The conditions used in these experiments were similar to the ones used in the "thermal only" experiments to allow for comparison and study the influence of the pre-enzymatic treatment on the flavour development. In the case of the experiment carried out at 110°C however, the conditions are slightly different in that the amount of sugar used is different. Only 20 g of sugar was used in the pre-enzymatically treated experiment to avoid the excessive caramelisation observed when 500 g was used.

The evolution of the volatile compounds under these thermal conditions is presented in Figure 7.5.

The $-NH_2$ content (**aa,** peptides, and polypeptides) during the thermal reaction was followed using the OPA method. The hydrolysis rate achieved during the enzymatic step in the reactor was 1.75, an increase of 75% of amino groups was observed, just as predicted by the small-scale experiment. The $-NH_2$ rate, ($-NH_2$ content/initial $-NH_2$), is represented versus time in Figure 7.6 for each of the three reactions carried out at different temperatures.

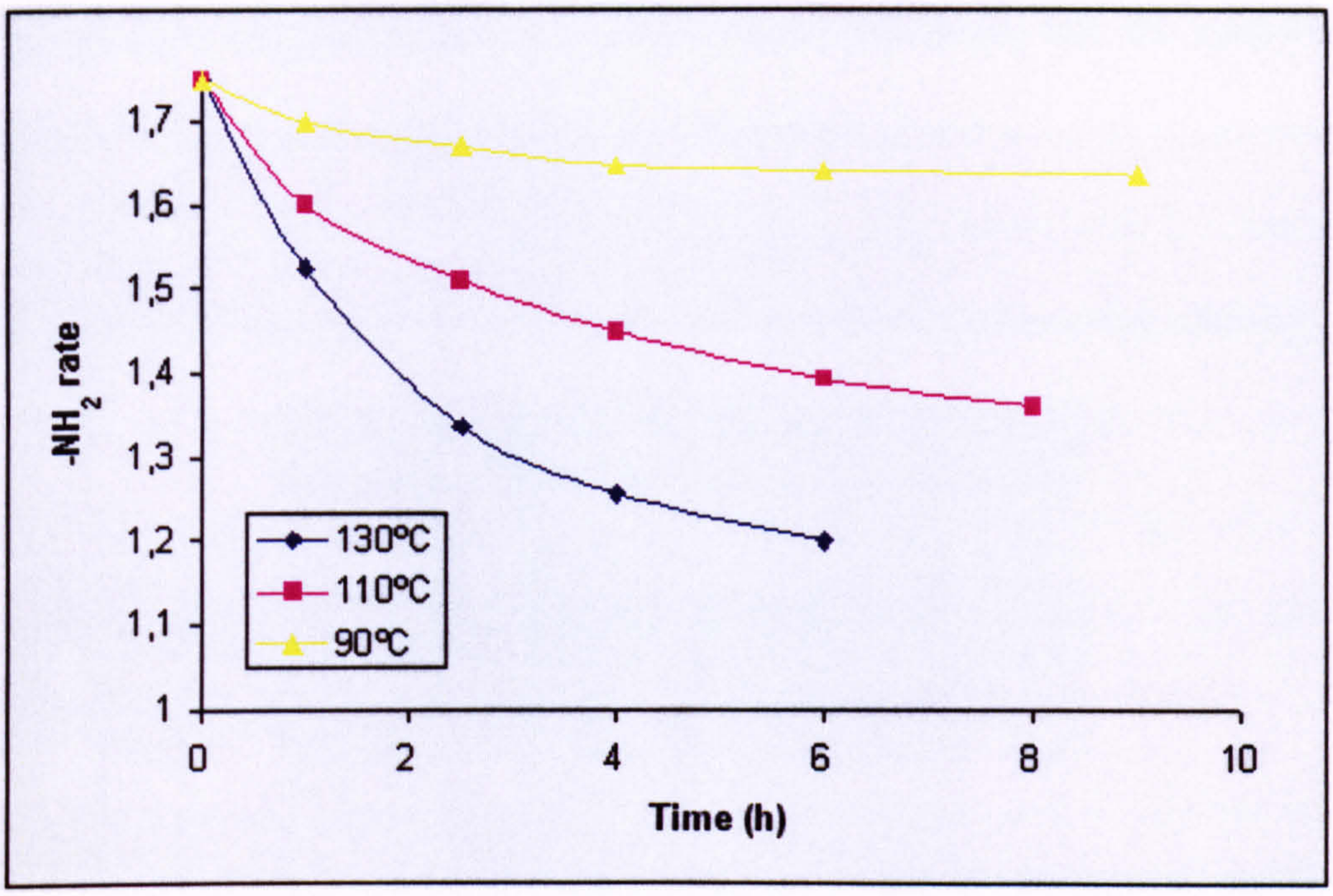


Figure 7.6 –NH_2 content versus reaction time for the enzymatic reaction carried out at three different temperatures (90, 100 and 130°C).

7.3.2 Discussion

For all three experiments (90, 110 and 130ºC), the amount of primary amines decreased (Figure 7.6) more rapidly as the reaction temperature increases. The reaction carried out at 90ºC suffers only a slight decrease, having a reduction close to the 6%. The 110ºC reaction had a stronger reduction (23%) while the 130ºC had the highest decrease reaching 35% reduction in 6 hours. Looking at the free amine evolution for all three reactions, none of them returned to the original amount (-NH_2 rate = 1). This means that some of the primary amines generated in the enzymatic hydrolysis were not able to react.

Looking at the global production of volatiles for all three experiments, the temperature continues being the mandatory parameter (note the different range of the scale in the x-axis between experiments in Figure 7.5). Table 7.6 summarises the rate of volatile formation (amount volatiles / initial amount volatiles) for all three experiments

and reflects the straight relationship between the temperature and the generation of volatiles.

Table 7.6 Rate of Volatiles formation (amount volatiles / initial amount volatiles)

Experiment temperature	Volatiles rate
90ºC	1.1
110ºC	3.2
130ºC	7.0

The results obtained in this study "with enzymatic pre-treatment" and the ones obtained previously in the "thermal only" experiments point to an exponential relationship between the temperature and the generation of volatiles. This relationship can be understood when considering that Maillard reaction and other endothermic reactions such as caramelisation, dehydration, pyrolysis or other degradation reactions depend on energy provided to the system to break down molecules and produce volatiles.

The experiment carried out at 90ºC with the enzymatic pre-treatment showed an interesting evolution of the volatiles (Figure 7.5, top graph). The concentration of aldehydes (dark blue curve) increased in the early stages of the thermal reaction until it reaches a maximum around 4 hours and then declines slightly. Looking at individual aldehydes, the aliphatic aldehydes, 2-methylpropanal and 3-methylbutanal, double in concentration in the first two hours of the reaction and remain constant until the end of the experiment. Aromatic aldehydes, benzaldehyde and benzacetaldehyde had a significant increment until 4 hours then decreased.

There is an important difference between this experiment and the one carried out under the same conditions without the enzymatic pre-treatment (Figure 7.7), where a

general decrease of the volatile fraction was observed, (especially aldehydes). Only degradation reaction were expected under these thermal conditions and no generation of volatiles was observed. On the other hand, in the experiment with enzymatic pre-treatment, a competition between generation and degradation can be observed. The enzymatic hydrolysis is producing new reactive amino acids or peptides that can react at 90°C to create aldehydes, while parallel reactions degrade them as they form. When these reactive amino acids are consumed, only degradation reactions occur decreasing their concentration in the headspace.

The primary amines evolution for the experiment at 90°C shows an initial decrease but after 4 hours remains constant without significant variation (Figure 7.6, yellow curve). This behaviour is in agreement with the hypothesis defended above.

The experiment carried out at 110°C (Figure 7.5, middle graph) with hydrolysed cocoa was not carried out under the same conditions with no enzymatic pre-treatment therefore no direct comparison is possible. Aldehydes (dark blue curve) displayed a similar behaviour as under the "thermal only" conditions but on a larger scale, showing an 80% increase. This experiment demonstrates that temperatures over 100°C are required to produce pyrazines and pyrroles (pink curve). Their increase is constant and after four hours their volatile fraction was multiplied by three. Other groups such as the furans (yellow curve), acids and alcohols (cyan cure) are also generated and their concentration in the headspace increases. Furans (yellow curve) had a linear and constant raise being the group with the greatest increase due to the process of caramelisation of sugars. Alcohols and acid (cyan curve) double in concentration as a result of oxidation products coming from aldehydes mainly.

The evolution of primary amines at 110°C shows a stronger degradation, being 5 times more severe than at 90°C (pink curve). This decrease agrees with the result obtained for the volatile generation that was 3 times more intense than at 90°C.

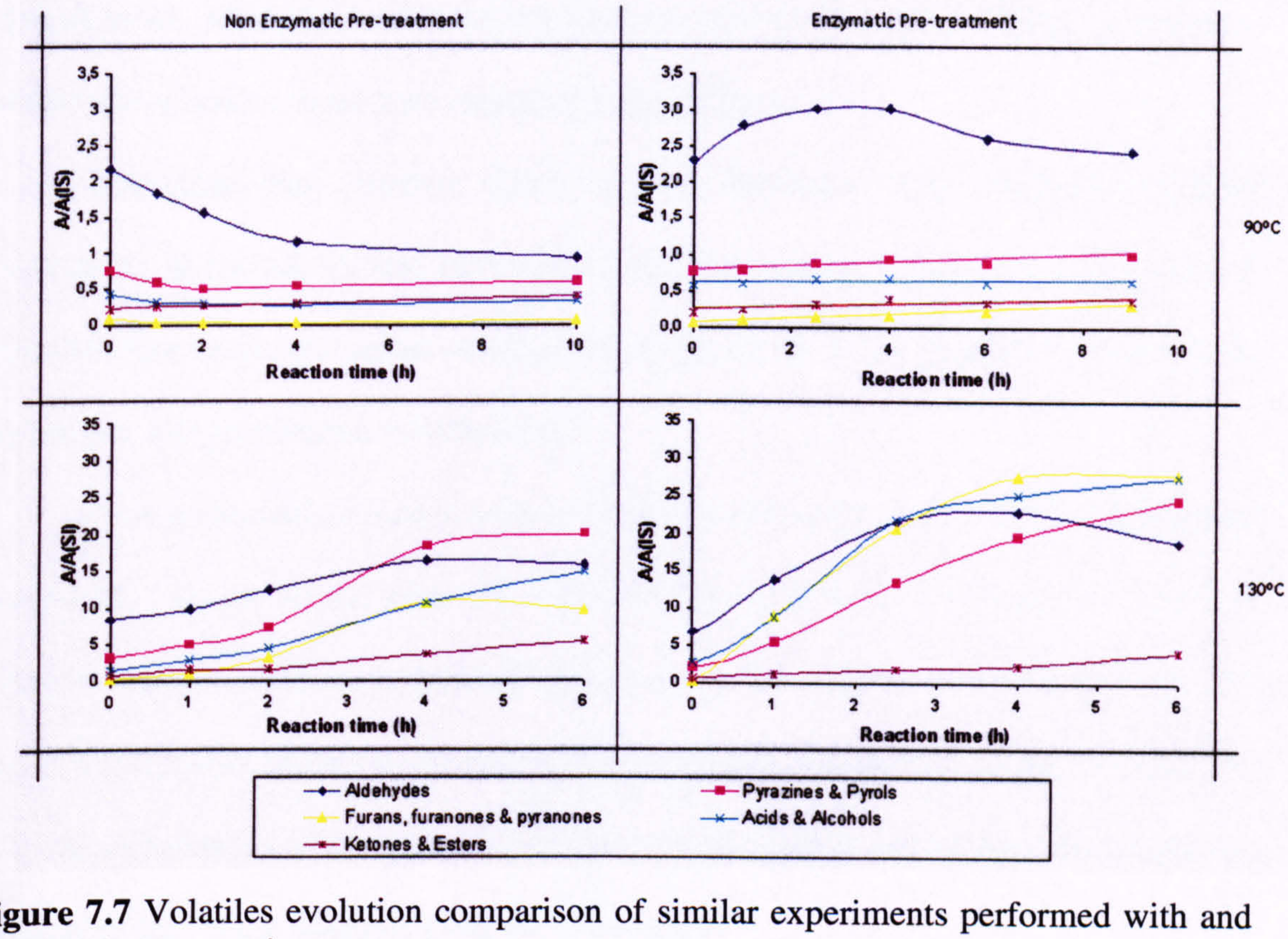


Figure 7.7 Volatiles evolution comparison of similar experiments performed with and without the enzymatic pre-treatment

The experiment at 130°C performed with enzymatically hydrolysed cocoa generated the greatest volatile development. This reaction was carried out under the same conditions without the enzymatic pre-treatment and the outcome of both experiments can be compared (Figure 7.5, bottom graph). Taking into account the global generation of volatile compounds, this reaction yielded 60% more volatiles. Looking at specific groups of volatiles, a 25% increase is observed for the pyrazines and pyrroles (pink curves), aldehydes increased by 20% (dark blue curves), furans, furanones and pyranones (yellow curves) presented the largest difference with a 150% increase. Alcohols and acids (cyan curves) well also generously generated (100% increase) when compared to the "thermal only" experiments (Figure 7.7, bottom graph).

Aldehydes (dark blue curve) had a similar behaviour than observed in the "no enzymatic pre-treated" experiment. The aliphatic and aromatic aldehydes were generated more rapidly and larger (35%), reaching a maximum between 3 and 4 hours. The aliphatic aldehydes remained constant or slightly decreased while the aromatic aldehydes suffered a rapid reduction due to degradation.

Pyrazines and pyrroles (pink curve) displayed their important thermal dependence and at this temperature had a linear and constant increase. No difference in the behaviour of the individual compounds of this group were found, all having similar evolutions and proportions in both cases.

Furans, furanones and pyranones (yellow curve) presented a great enhancement compared with the results obtained under the "no enzyme pre-treatment" conditions. Furfural was the main responsible of this increase. The amount of sugar used was the same in both cases therefore such a big difference must come from another source. The enzyme excipient or collateral effect of the enzyme itself could increase the amount of sugar that causes a higher generation of this group.

Alcohols and acids group (cyan curve) also presented an important difference with the results obtained previously with no enzymatic step. This is in concordance with the increase of furans, furanones and pyranones (caramelisation products) because acetic acid is an important caramelisation by-product. Acetic acid was one of the mainly responsible of the increase helped by the aldehyde degradation product 3-methyl-butanoic acid.

The evolution of the primary amines in the 130°C experiment (Figure 7.6, dark blue curve) showed a vigorous degradation of the primary amines, their degradation was clearly the highest amongst all the experiments. As the temperature increase, faster

reactions occur and this affects directly the reactivity of free $-NH_2$ and the generation of volatiles.

7.3.3 Sensorial Evaluation

The products of the enzymatically and thermally treated reactions were evaluated for their sensorial acceptability and aroma-flavour notes (Table 7.7).

Table 7.7 Sensorial score of the hydrolysed products

	90°C	110°C	130°C
Aroma	****	*****	****
Flavour	***	**	*

Acceptability * ** *** **** *****

Bad → Excellent

Sensorially the main characteristic of these products was a grassy off-flavour detected for all three products, being more clearly identified in the product obtained at 90°C. This off-taste has been attributed to the enzyme used to hydrolyse the cocoa which was probably obtained form a vegetable source.

Strong astringent, bitterness and burn off-flavour were distinguished in the products obtained at temperature above 100°C probably due to the caramelisation and dehydration products, especially intense for the 130°C products.

The aroma was very pleasant with evident and intense chocolate notes for all products, but clearly more intense for the product obtained at 110°C and 130°C. On the other hand, these products had some overcooked and burnt off-aromas a bit unpleasant especially for the 130°C products.

7.4 Conclusion

Enzymatic technology increased the amount of volatiles precursors in the cocoa powder that under thermal technology conditions was translated in an increase generation of volatiles.

The enzymatic hydrolysis carried out with the commercial Flavourzyme™ on the cocoa powder broke down the cocoa proteins into amino acids and peptides or oligopeptides. The increase in free primary amines confirmed this point. The amino compounds were increased close to 80% when 5% of Flavourzyme™ was incubated for 24 hours at 50°C with the cocoa powder.

The generation of volatiles was boosted by the enzymatic technology, when hydrolysed cocoa was submitted to suitable thermal conditions. Better volatile development was achieved (60% increase). Even at low temperatures (90°C), the generation of volatiles was observed. Enzymatic treatment endows cocoa powder with new and reactive forms that roasted cocoa powder lacked, allowing it to produce more volatile compounds.

Sensorially the products had a great aroma with pleasant and deep chocolaty notes but the flavour had a strange grassy off-flavour. Apart form this, significant astringent and bitterness shades were detected as well as some burnt or overcook off-flavours especially when high temperature (>100°C) were used.

Chapter 8

8 Conclusions and achievements

A novel technology to process traditional chocolate-making ingredients was investigated with the main objective to promote the generation of key chocolate odorant volatiles as a part of the commercial interest of the industrial partner to create new line of natural products with intense chocolate flavour.

First, specific chemical and sensorial analyses were set up to asses the generated products and their ingredients. Chemical analyses were performed to determine chemicals such as methylxantines (bitterness), polyphenols (astringency) or free -NH_2 content. But most of the work relied on the development and validation of a methodology to analyse volatiles To analyse the volatiles a laboratory-developed methodology based on solid phase microextraction (SPME) of the headspace (HS) and gas chromatography-mass spectrometry (GC-MS) to analyse the extracted volatiles was optimised for cocoa products. The DVB/CAR-PDMS fibre was found to have the best performance for the extraction of volatiles and semi-volatiles. The incubation temperature and time were optimised and 60°C for 15 minutes were found to afford suitable recovery of these compounds. Moreover, we found that the use of a brine suspension of cocoa powder gave the best recovery of semi-volatiles. The addition of an internal standard (toluene) increased the robustness of the methodology.

Furthermore, quantitative analysis of key aroma compounds was demonstrated to be possible using our methodology.

A parallel study of the evolution of the volatile profile during the industrial chocolate manufacturing process showed the validity of the optimised HS-SPME-GC-MS methodology for the analysis of cocoa products. This study also concluded that the

refined samples produced the largest volatile fraction and the conched samples had the lowest. The refining step reduces particle size of the mixed ingredients enhancing the release of volatiles and creating punctual (< 1 second) high friction temperatures (> 1000°C) while the conching step eliminates volatiles due to the intense kneading, high temperature (70-90°C) and long process times (8-12 hours).

A chemical characterization of several commercial cocoa powders showed that the fat content plays an important role in the ability of volatiles to be extracted efficiently. The coating of the solid cocoa particles with fat led to a general decrease in volatiles extracted. Natural cocoa powders had generally richer volatile profiles than the alkalised cocoas and those that had positive red factor had the lowest volatile profiles. Also was found that natural cocoa powders had higher content in amino acids or amino compounds and in polyphenols compared to the alkalised cocoa powders; the alkalisation step decreases the nutritional value of the cocoa.

Concerning the sensorial evaluation, the researcher was trained at Natraceutical and was responsible for setting up and training a panel of tasters able to evaluate the products and distinguish between several cocoa and chocolate descriptors.

Reaction technology was used to generate volatiles and to enhance the flavours.

First, thermal processing technology was investigated. Reaction temperature and time were studied and although high temperatures (≥ 100°C) were able to generate a significant volatile profile, the products generated at lower temperatures (≤ 90°C) were sensorially more acceptable. The conditions for the optimum acceptability were reaction temperature 90°C and reaction time 4 hours (0.5 bar of overpressure). We also note that the products obtained at high temperature (> 100°C) resulted in the generation of large

quantities of furfural. This is the first report of furfural arising as a main product of the caramelisation of sucrose.

The influence of the fat content on the volatile profile and the flavour development was studied under the optimum conditions (90°C for 4 hours). No differences were found in the evolution of the volatile generation, only degradation of aldehydes was observed. Sensorially, the product obtained from non-fat cocoa achieved the highest chocolate descriptor score and the lowest cocoa descriptor score for aroma and flavour evaluations.

The effect of several reducing sugars was also investigated. Apart from sucrose used in the preliminary studies, glucose, fructose and xylose were submitted to the optimised thermal conditions. An increase of the caramelisation products was observed, particularly in the xylose experiment. Unlike the other sugar experiments, no degradation of aldehyde group was recorded in the xylose experiment. This is probably due to the compensation between formation of aliphatic aldehydes (isopropanal, isobutanal, 3-methyl propanal) and degradation of aromatic aldehydes (benzaldehyde, benzacetaldehyde). Sensorially, all the products had a great aroma, especially the xylose product that scored the highest. On the other hand, the taste of the xylose product had an intense bitterness and overcook/burnt shades that were very unpleasant.

Next, enzymatic technology was investigated as a mean to increase the amount of volatiles precursors in the cocoa powder which would, under thermal technology conditions, lead to an increased generation of volatiles. We tested five commercial enzymes and found that Flavourzyme™ (Novozyme) resulted in the best hydrolysis of the cocoa powders. The $-NH_2$ content was increased by 80% when 5% of Flavourzyme™ was incubated for 24 hours at 50°C with cocoa powder. The generation of volatiles was boosted by the enzymatic technology, when hydrolysed cocoa was

submitted to suitable thermal conditions. Better volatile development was achieved (60% increase). Even at low temperatures (90°C), the generation of volatiles was observed. Sensorially the products had a great aroma with pleasant and deep chocolaty notes but the flavour had a strange grassy off-flavour. Apart from this, significant astringent and bitterness shades were detected as well as some burnt or overcook off-flavours especially when high temperature (>100°C) were used.

In conclusion, we believe that the best product, taking into account the main objective expressed at the beginning of this dissertation, is the one obtained at 130°C with a enzymatically pre-treated cocoa powder. This product showed the best volatile profile, including the most relevant odorant families like pirazines, aldehydes, etc. Comparing the traditional manufacture of chocolate (i.e. conching), where the volatile fraction decreases during the last step, in our case, with the reaction technology (i.e. thermal and enzymatic processing), we have created a product that is richer in typical key chocolate volatiles and which displays an intense chocolate aroma.

If the project were to continue, several changes, ideas and assessments could be introduced and evaluated in order to improve the final product :

- **pH control.** The pH is a key factor in the non-browning reactions in the flavour generation and this was not tackled. It has been reported that high pH (8-11) favours flavour development because it favours the open-chain form of the sugars and the active form of the amino reactant is unprotonated.[118, 144] In the experiments carried out, the pH was set by the ingredients without modification (slightly acidic ~ pH 5). We did notice that the pH decreased versus the reaction time due to the formation of by-products such as acetic acid.

This decrease in pH auto deactivates the non-browning reactions, slowing down the volatiles evolution. We believe that increasing the pH of the natural cocoa powders or even using buffer solutions to maintain the pH at high levels would increase the flavour and volatiles evolutions.

- **Accurate optimisation at high temperature.** The results obtained show that to promote a significant volatile generation under the conditions tested, temperatures above 100°C are needed. Once reached, the evolution is exponential and small temperature changes have a significant effect. Therefore, more accurate temperature and time optimisation in this range is advisable to try and obtain a good flavour development while keeping down other undesirable processes like the over-caramelisation that gives burnt and bitter off-flavours.
- **Product development and sensorial evaluation.** The products generated during the research were sensorially evaluated as ingredients and not as additives. It is well known that additives are used in small amounts in confections and their evaluation as concentrates can result in very unpleasant flavours. Their evaluation in more dilute confections would be very noticeable. It would be necessary to perform a dilute product development, establishing the correct dilution for the sensorial evaluation.
- **Other ingredients.** It may be interesting to extend the study to other sugars, higher temperatures (over 100°C) and/or enzymatic pre-treatment of the cocoa. More reactive sugars could produce Maillard reactions faster and, at softer temperatures, with the new amino acids generated in the enzymatic step, reduce the caramelisation. Use of milk and diary products as ingredients could bring a new source of flavour precursors; lactose and free amino acids could have a positive effect in the volatiles generation.

- **External pressure.** The over-pressure obtained in the experiments was mainly due to the steam created by the water used in the reactions. Thus, at low temperatures (< 100°C) the over-pressure was practically absent. The use of an external source of pressure (eg. cylinder of compressedair) should affect the aromatic processes, speeding up the reactions or promoting some products and decreasing others. Moreover, using different kind of gases like nitrogen N_2 or oxygen O_2, some reactions like oxidations may be enhanced or inhibited.
- **Lower enzyme proportion.** Lower proportions of enzyme could be used to minimise their influence on the taste of the final product (grassy off-flavour). As presented in the chapter 7, the proportion of enzyme only affects the hydrolysis initial speed but with times, all proportion hydrolysis end at the same yield. The same hydrolysis yields and rates would be expected using smaller proportion of enzymes, using similar or maybe longer incubations times. This should not affect the generation of precursors.

Due to the increasing interest of the food industry to claim the use of only natural ingredients, this product presents an excellent potential to be used as natural chocolate and cocoa flavour because holds a large aromatic profile. We fell, it would be necessary to investigate more deeply this technology and to invest more time and resources to obtain a commercially acceptable product. However their numerous possible applications and the fact that the process can be adapted easily to obtain a more suitable product for each application give, to this line of products and to this technology, a high commercial interest and value.

EXPERIMENTAL

Chapter 9

9 Materials and Methods

9.1 Materials

9.1.1 Natural Chocolate-Making Ingredients

Non-alkalised natural cocoa powder (NCP) and conched chocolate powder (CCP), from West African origin, were obtained from The Chocolate Powder Company (Solihull, United Kingdom). The fat content of the NCP was 10-12%. The fat content of the CCP was 10-12% and the cocoa content was 69%. The NCP and CCP were stored in hermetic boxes at room temperature.

Alkalised cocoa powders from West African origin, were obtained from Natra Cacao (Quart de Poblet, Spain) and came in three different fat contents: 0-1%, 10% and 20%. These were stored in hermetic boxes at room temperature.

Sugars (sucrose, fructose, glucose, xylose, meliose™) were obtained from Danisco (Redhill, United Kingdom).

Mineral water was obtained from Nestle Watercoolers UK Ltd.

Natural vanillin, theobromine and caffeine were obtained from Natra Cacao (Valencia, Spain).

Enzymes (Flavourzyme, Protamex, Papaine, Celluclast, NS-44055) were obtained from Novozyme UK Ltd.

9.1.2 Chemicals

Analytical grade 2-methylpropanal, 3-methylbutanal, 2-methylbutanal, 2-phenyl-5-methyl-2-hexanal, 2,3-dimethylpyrazine, trimethylpyrazine, tetramethylpyrazine were purchased from Alfa Aesar (Heysham, United Kingdom) and acetone, benzyl alcohol,

benzaldehyde, vanillin, caffeine and 1-(2-furanylmethyl)-1*H*-pyrrole from Sigma-Aldrich (Dorset, United Kingdom).

9.1.3 Solid-Phase Microextraction (SPME) Fibres

Volatiles from the NCP and CCP samples were extracted using four fibres: 100 µm polydimethylsiloxane coating (PDMS), 65 µm polydimethylsiloxane/divinylbenzene coating (PDMS/ DVB), 75 µm Carboxen/polydimethylsiloxane coating (CAR/PDMS) and 50/30 µm divinylbenzene/carboxen on polydimethylsiloxane on a StableFlex fibre (DVB/CAR-PDMS). These fibres were purchased from Supelco (Bellefonte PA, USA). Before extraction, the fibres were conditioned according to the supplier's instructions. Extractions were carried out in 10 mL PTFE/Silicone septa vials (Supelco, Bellefonte PA, USA). Every septum was pre-drilled with a regular needle before sampling with the SPME fibre.

9.2 Methods

9.2.1 Sample Preparation

9.2.1.1 Solvent Extraction

500 g of conched chocolate powder was added to 3 L of ethanol:water (7:3,v/v), at room temperature. The mixture was stirred until a homogeneous mixture was obtained. The agitation continued for 2 h when the mixture was filtered under suction through a monoplaque filter. The cake was returned to the stirring reactor and a further 2 L of the same extracting solvent was added while continuously stirring. The extraction continued for 2 h, and the mixture was filtered again. The combined filtrates were concentrated *in vacuo* at 55 °C. The concentrate weighed 730 g, and was dried in a vacuum oven at 55 °C and pressure <50 mmHg. The dry product weighed 170 g, representing an extraction yield of 34% in weight. The product was milled in a pin mill, resulting in a fine brown powder, with a strong chocolate flavour. To avoid highly hygroscopic product, 0.5% of aerosol was added. The product was fully soluble in water.

9.2.1.2 Steam Distillation

Where the volatile fraction was recovered by steam distillation, the resulting condensates (200 mL) were adjusted to pH 12 with NaOH, and extracted with dichloromethane (3 x 100 mL) to give the *basic extract.* The aqueous phase was acidified with HCl to pH 5 and extracted with dichloromethane (3 x 100 mL) to give the *acid extract.* Both extracts were concentrated with the help of a Snyder-Kuderna column to preserve the volatile fraction.

9.2.1.3 Static Head Space (SHS)

The sample (1 g) was placed in a 10 mL vial. The vial was hermetically closed with a metallic holed cap and a PTFE septum. The vial was conditionned at 80 – 120 °C for 10 minutes before analysis by GC-MS.

9.2.1.4 Solid-Phase Microextraction (SPME)

Selection of fibre coating. The sample (2.0 g) was placed in the vial and conditioned for 10 min at the extraction temperature, 60 °C. The extraction time was 15 min. The SPME fibre was then exposed to the headspace. The desorption time was 5 min and the temperature in the GC liner was 250 °C.

Selection of extraction time and temperature. The sample (2.0 g) was placed in the vial and conditioned for 10 min at the extraction temperature. The fibre was exposed for 1, 5, 10, 15 and 30 min to the headspace of the vial at four different temperatures, 25, 40, 60 and 80 °C. The desorption time was 5 min and the temperature in the GC liner was 250 °C.

Dry, wet and brine conditions. The sample (1.0 g) was placed in a vial (dry conditions). In a second vial the sample (1.0 g) was mixed with 4 mL of distilled water. The effect of the ionic strength was studied by substituting the water by brine (25% of NaCl in distilled water) in a third vial. In all cases, the headspace extraction was carried out using the optimized conditions (T_{ext} 60 °C, t_{ext} 15 min). The desorption time was 5 min and the temperature in the GC liner was 250 °C.

9.2.2 Sample Analysis

9.2.2.1 Gas Chromatography – Mass Spectrometry (GC-MS)

The analysis of volatiles extracted was carried out using a Varian CP-3800 Gas Chromatograph linked to a Varian 1200L Quadrupole MS/MS. The GC was equipped with a CTC CombiPAL autosampler upgraded with a SPME analysis kit. The injector port has a deactivated glass SPME liner supplied by Supelco. The GC was fitted with a 30 m capillary column with a 0.25 mm ID and 1 µm FD, Factor 4 VF-5ms purchased from Varian. After absorption, the SPME fibres were introduced into the injector port of the GC (in splitless mode at 250 °C for 5 min). The volatiles extracted by the fibres were thermally desorbed and introduced in the capillary column. The GC was setup with a constant flow of 1.0 mL/min (Helium), the oven temperature was programmed to start at 30 °C (5 min) – (10 °C/min) – 200 °C – (25 °C/min) – 280 °C (5 min). The transfer line was at 280°C. The MS was setup with the source at 280 °C, where electronic ionization energy was -70 eV and with a 1200 Volts in the detector. One mass spectra scan every second was acquired. The compounds were identified by a combination the US national Institute of Standards and Technology (NIST) 98 library of mass spectra and gas chromatographic retention indexes reported of standard compounds. Under these conditions, no sample carry-over was observed as confirmed by blank runs conducted between each analysis.

9.2.2.2 Amine ($-NH_2$) content

The amino content was measured with OPA using the modification of the method described by Church. [111] The OPA reagent (160 mg OPA in 4 mL of ethanol, 7.620 g $Na_2B_4O_7 \cdot 10^* H_2O$, 200 mg sodium dodecyl sulphate, 176 mg dithiothreitol in 200 mL distilled water) was prepared freshly when needed.

Cocoa samples (400 µL, 1 g of cocoa extracted in 100 mL of water) were added to the OPA solution (3 mL) in a test tube protected from light. The samples were shaken for 5 sec. kept at room temperature for 2 minutes before the absorbance was measured at λ 340 nm in a Cary 300 Bio Spectrophotometer (Varian). The standard curve was obtained using asparagine (0.25 to 3 mM) as a reference compound (slope : 0.7177, intercept : 0.1717, R^2 : 0.9996, Figure 9.1). The blank was made up from water (400 mL) and OPA (3 mL). The absorbance of the cocoa extract (400 µL in 3 mL of water) was also measured at λ 340 nm to eliminate any interference from the sample.

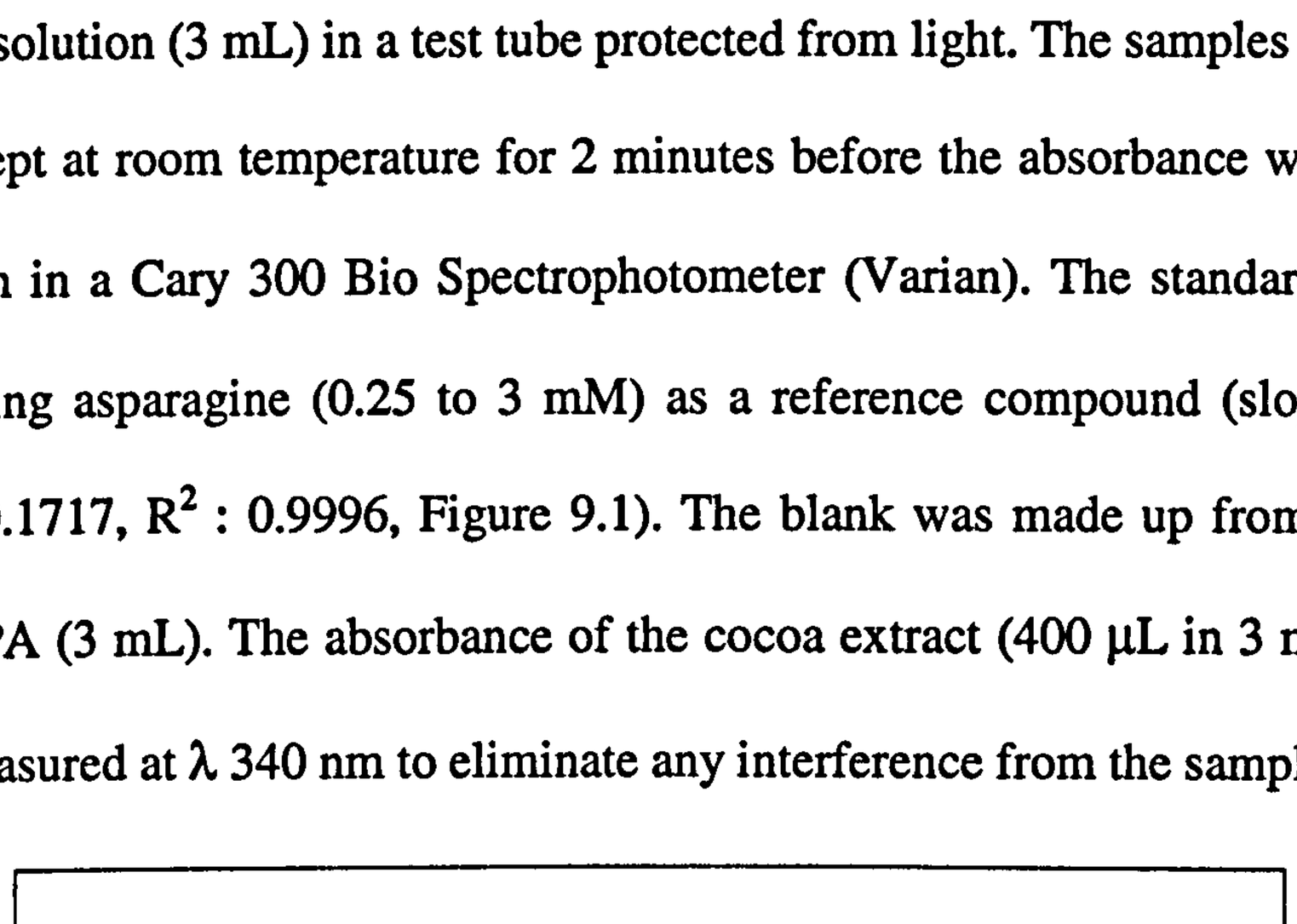


Figure 9.1 OPA Calibration curve for Asparagine

9.2.2.3 Theobromine and caffeine content

Caffeine and theobromine were quantified by HPLC (Gilson system) using an isocratic gradient (water:methanol, 75:25) equipped with a reversed-phase C18 column (Varian Polaris, 3 µm packing, 250 x 4.6 mm id) at room temperature. The flow rate was 0.5 mL/min and the total elution time was 20 minutes. The injected volume was 20 µL (0.25 g of cocoa powder extracted in 100 mL of mobile phase and filtered). Detection was performed using a UV detector set at λ 275 nm. Quantification (Figure 9.2) was carried out by external calibration using a mixture of caffeine (75 ppm) and theobromine (60 ppm).

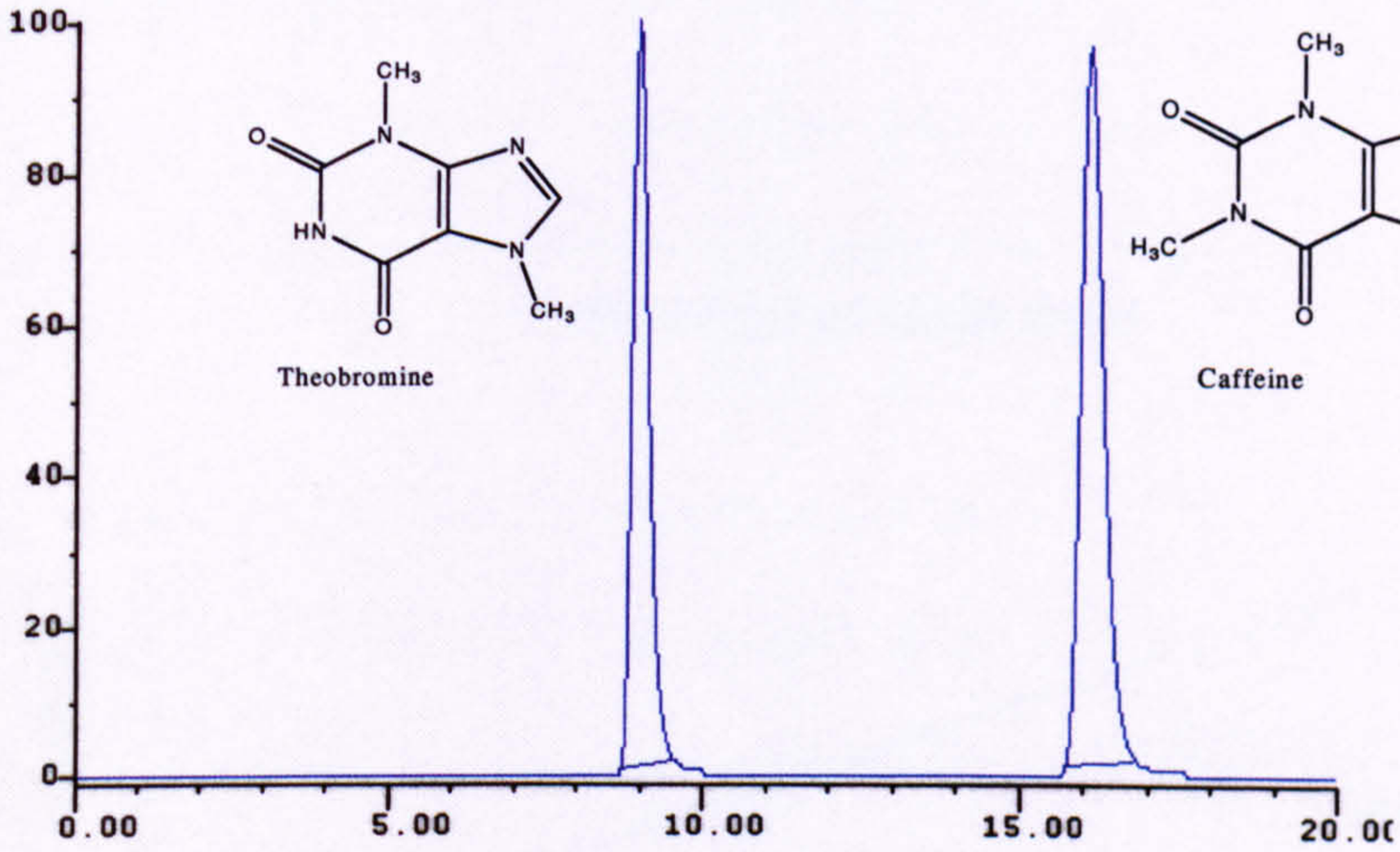


Figure 9.2 Chromatogram obtained for Caffeine and theobromine.

9.2.2.4 Total polyphenol content

The total polyphenol content was measured using the Folin-Ciocalteu assay as described by Georgé. [110] A 2.5 mL sample of water-diluted Folin-Ciocalteu reagent (1/10) was added to the different extracts (1 g cocoa extracted in 10 mL acetone : water : acetic acid, 70:29.8:0.2 and filtered). The mixture was incubated for 2 minutes at room temperature, and 2 mL of sodium carbonate (75 $g.L^{-1}$) was added. The mixture was incubated for 15 minutes at 50°C and finally cooled in an ice-water bath. The specific absorbance at λ 760 nm was immediately measured in a Cary 300 Bio spectrophotometer. The total polyphenol content (TPC) was expressed as mg of gallic acid per g of cocoa (slope : 0.0102, intercept : 0.0503, R^2 : 0.9994) obtained between 12 and 333 mg/L (Figure 9.3).

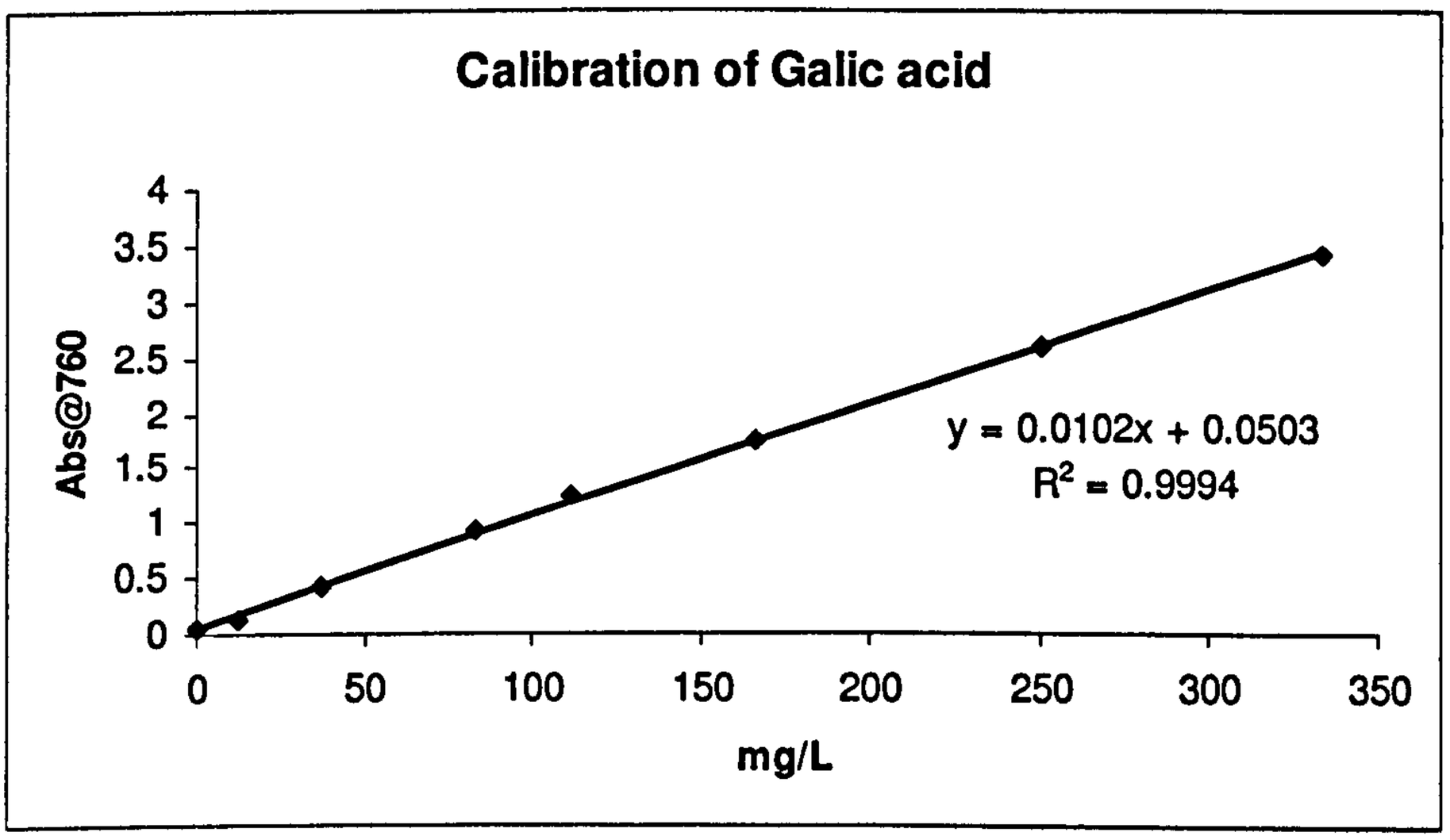


Figure 9.3 Calibration curve for Gallic acid

9.2.3 Reaction Technology

9.2.3.1 Thermal Technology

The reactor (Appendix 10.3) is warmed up to 40 °C and mineral water is added. The temperature is increased to 60 °C then sugar and vanillin are added to the warm water. The reactor is then closed and mechanically stirred until a homogeneous mixture is obtained. Cocoa powder is added in small portions over 25 minutes using a charging system fitted on the reactor when the working temperature is reached. The temperature is maintained at the working temperature and steam generation causes the pressure to increase to 6 bars. When the reaction time has elapsed, the mixture is cooled down to room temperature and the pressure equalized. The products are usually dark brown pastes with very strong chocolate flavour. The paste is dried in a vacuum oven at 40°C and < 10 mmHg for 8 h. The dried product is ground into a fine powder.

Influence of reaction times and temperatures. Experiments were carried out at 70 °C, 90 °C, 110 °C and 130 °C with 20% fat natural cocoa powder (500 g), sucrose (500 g) and mineral water (550 g). During the reaction, the reactor pressure increased to 0.1 bar at 70 °C, 0.5 bar at 90 °C, 1.2 bars at 110 °C and 3 bars at 130 °C. Samples were taken at 0, 1, 2, 4 hours and in the case of temperatures below 100 °C after 10 hours.

Influence of fat content. Experiments were carried out with alkalised cocoa powder with three different fat contents: 0-1 % (400 g), 10% (444 g) and 20% (500 g) in the presence of fructose (250 g) and mineral water (600 g).

Influence of sugar. Experiments were carried out in the presence of different sugars: sucrose (500 g), fructose (500 g), glucose (500 g), xylose (500 g) or meliose™ (715 g) with 20% fat natural cocoa powder (500 g) and water (550 g and 335 g in the case of meliose™).

9.2.3.2 Enzyme Technology

Selection of Enzyme. Experiments were performed using natural cocoa powder (2 g) in mineral water (6 mL) with the chosen enzyme (6% w/v). The mixture was incubated at 50 °C for 5 hours.

Kinetic study with flavourzyme. Experiments were carried out with natural cocoa powder (2 g) in water (6 mL) at 50 °C with different amount of enzyme (5%, 10% and 20%). Samples were taken after 1, 2, 3, 5, 8 and 24 hours and analysed.

Enzyme technology. Natural cocoa powder (500 g), mineral water (600 g) and flavourzyme (25 mL) were incubated at 50 °C with continuous kneading at the IKA reactor. After 20 hours, sucrose (500 g) was added and the thermal reaction was carried out as described above.

Chapter 10

10 Appendices

10.1 Appendix 1 - Training at Natra Cacao (Valencia, Spain)

At the beginning of the project, I spent three months in the cocoa and chocolate factory (Natra Cacao) in Valencia, Spain.

During this period, I developed a formative work of documentation reading on the manufacture of cocoa and chocolate. I tackled from the knowledge of the cocoa, looking at the different classes, producing countries, as well as the processes that take place initially in the country of origin (fermentation and drying), and also the processes that take place in the factory, like the different alkaline treatments, etc.. I also studied all the industrial processes with the machinery adapted for each process, as well as the different possible options. Books "Chocolate, cocoa and confectionery" Bernard W. Minifie and "Industrial chocolate manufactures and uses" S.T. Beckett were used as reference, plus internal documents of quality instructions of the factory were read.

I then moved to the quality laboratory of Natra Cacao. I learned about the different quality points (grain, Cocoa liquor, production, butter of cacao, microbiology, cocoa powder and chocolate) with the different analysts, watching and applying all I had read earlier.

With the heads production of the factory I learn about the different processes carried out in the factory and were explained to me, from the entrance of the grain, to the final packaging of the commercial products (cacao powder, cacao mass and cacao butter, chocolate mass, tablets of chocolate), as well as the different sale formats.

In the time spent in the factory, I trained as a panel taster. I attended tasting sessions carried out in the company in that period. I learned how to do a tasting session. I was trained in the various chocolate descriptors and in the statistic data treatment

This three-month training gave me a very good background to develop the project.

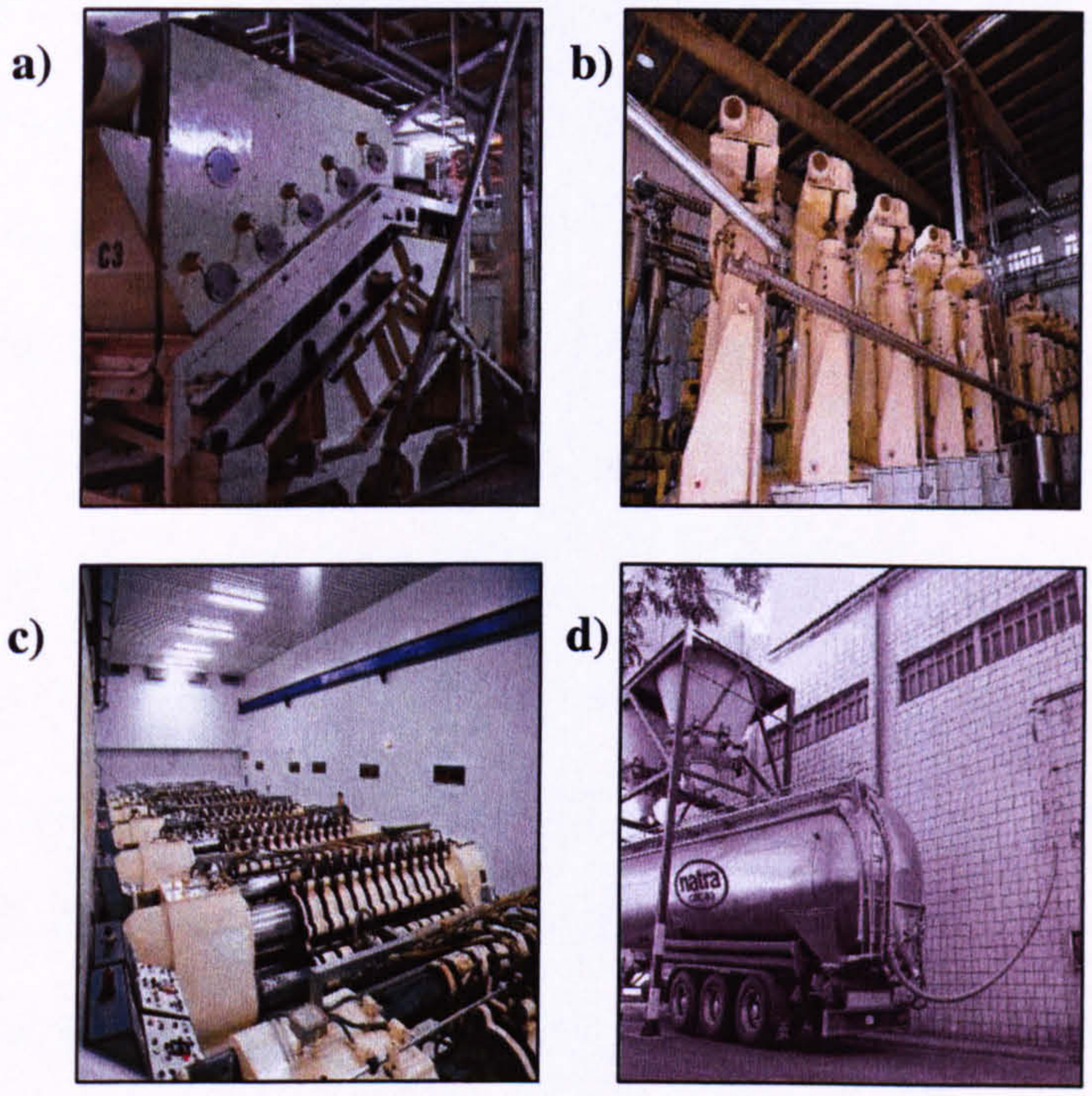


Figure 10.1 Cocoa machineries a) Winnowing b) Grinding c) Pressing d) Charging the final product in tanks.

10.2 Appendix 2. Training session carried before the sensorial fat content evaluation.

In order to improve the results obtained in the sensorial evaluation of the fat content experiment a training session was carried out before each trail using standards for each descriptor. The samples for training were prepared following (Table 10.1 to 10.6). The ingredients were obtained from the following methods. Sugar was purchased from Alcampo. The cocoa powder used was natural cocoa powder (Granada 10-12). The water used was bottled water (Font D'or). The caramel standard was obtained by caramelizing glucose syrup and mixing it with cocoa natural cocoa powder until a caramel aroma was obtained (20%). The standard for toasted flavour was obtained by roasting natural cocoa powder (Granada 10-12) at 150°C for 30 minutes. Vanillin as vanilla standard and Caffeine as bitterness standard were used.

The results of the training are shown in Tables 10.7 to 10.13. Applying the Kramer Table to these results, indicates that the panel was able to distinguish between the aromas of cocoa, chocolate, caramel, toasted and vanilla and the flavours of cocoa, chocolate, and bitter. However, the standards used for two descriptors, caramel and vanilla and they could not the clearly ranked.

Table 10.1 Samples for the training of aroma and taste of cocoa

Ingredients	**653**	**749**	**522**
Cocoa Granada	3 g	18 g	15 g
Sugar	15 g	30 g	15 g
Water	282 ml	552 ml	279 ml

Table 10.2 Samples for the training of aroma and taste of chocolate

Ingredients	**335**	**139**	**667**
Chocolate	3 g	9 g	15 g
Sugar	14 g	12 g	10 g
Water	283 ml	279 ml	275 ml

Table 10.3 Samples for the training of aroma and taste of toasted

Ingredients	**993**	**129**	**372**
Toasted Cocoa	0 g	3 g	6 g
Cocoa	12 g	9 g	6 g
Sugar	15 g	15 g	15 g
Water	273 ml	273 ml	271 ml

Table 10.4 Samples for the training of aroma and taste of caramel

Ingredients	**141**	**363**	**448**
Caramelized Cocoa	0 g	3 g	6 g
Cocoa	12 g	9 g	6 g
Sugar	15 g	13.5 g	12 g
Water	273 ml	274.5 ml	276 ml

Table 10.5 Samples for the training of aroma and taste of vanilla

Ingredients	**414**	**811**	**634**
Vanilla	0.03 g (0.01%)	0.3 g (0.1%)	1.5 g (0.5 %)
Cocoa Granada	12 g (4%)	12g (4%)	12 g (4%)
Sugar	12 g (4%)	12 g (4%)	12 g (4%)
Water	275,97 g (91.99%)	275,7 (91.9%)	274.5 (91.5%)

Table 10.6 Samples for the training of taste of bitter

Ingredients	**874**	**542**	**275**
Caffeine	0.015 g (0.05 g/L)	0.06 g (0.2 g/L)	0.12 g (0.4 g/L)
Water	300 ml	300 ml	300 ml

Table 10.7 Results from training in cocoa aroma

Judge	741	522	653
Beatriz	1	2	3
A Tornero	2	1	3
A. Teruel	2	1	3
César	2	1	3
Marie	2	1	3
Carol	2	1	3
Toni	2	1	3
Raquel	2	1	3
Total and significance	15[b]	9[a]	24[c]

Table 10.8 Results from training in cocoa flavour

Judge	741	522	653
Beatriz	2	1	3
A Tornero	2	1	3
A. Teruel	2	1	3
César	2	1	3
Marie	2	1	3
Carol	2	1	3
Toni	2	1	3
Raquel	2	1	3
Total and significance	16[b]	8[a]	24[c]

Table 10.9 Results from training in chocolate aroma

Judge	667	139	335
Beatriz	1	2	3
A Tornero	1	2	3
A. Teruel	1	2	3
César	1	2	3
Marie	1	2	3
Carol	1	2	3
Toni	1	2	3
Raquel	1	2	3
Total and significance	8[a]	16[b]	24[c]

Table 10.10 Results from training in chocolate flavour

Judge	667	139	335
Beatriz	2	1	3
A Tornero	1	2	3
A. Teruel	1	2	3
César	1	2	3
Marie	1	2	3
Carol	1	2	3
Toni	1	2	3
Raquel	1	2	3
Total and significance	9[a]	15[b]	24[c]

Table 10.11 Results from training in toasted aroma

Judge	372	129	993
Vicent	1	2	3
Agustin	1	2	3
Carol	1	3	2
A. Teruel	1	3	2
Beatriz	1	2	3
Pedro Arenas	1	2	3
Elena	1	2	3
Raquel	2	1	3
Toni	1	3	2
Total and significance	10[a]	20[b]	24[b]

Table 10.12 Results from training in toasted flavour

Judge	372	129	993
Vicent	1	2	3
Agustin	1	3	2
Carol	1	3	2
A. Teruel	3	1	2
Beatriz	1	2	3
Pedro Arena	2	1	3
Elena	2	1	3
Raquel	2	1	3
Marie	2	1	3
Toni	1	3	2
Total and significance	16[a]	18[a]	26[b]

Table 10.13 Results from training in caramel aroma

Judge	141	448	363
Vicent	1	2	3
Agustin	3	2	1
Carol	1	2	3
A. Teruel	2	1	3
Beatriz	2	1	3
Pedro	2	3	1
Elena	2	1	3
Raquel	3	1	2
Toni	3	2	1
Total and significance	19[a]	15[a]	20[a]

Table 10.14 Results from training in caramel flavour

Judge	141	448	363
Vicent	1	2	3
Agustin	3	2	1
Carol	1	2	3
A. Teruel	2	1	3
Beatriz	2	3	1
Pedro	2	1	3
Elena	1	2	3
Raquel	3	1	2
Marie	2	3	1
Toni	2	3	1
Total and significance	19[a]	20[a]	21[a]

Table 10.15 Results from training in bitter taste

Judge	275	542	874
Elena	1	2	3
César	1	2	3
A. Tornero	1	2	3
Vicent	1	2	3
A. Teruel	1	2	3
Toni	1	2	3
Carol	2	1	3
Raquel	1	2	3
Marie	1	2	3
Bea	2	3	1
Total and significance	12[a]	20[b]	28[c]

Table 10.16 Results from training in vanilla aroma

Judge	811	414	634
Elena	1	2	3
César	1	3	2
A. Tornero	1	3	2
Vicent	1	2	3
A. Teruel	1	3	2
Toni	1	2	3
Carol	1	2	3
Raquel	1	2	3
Marie	3	1	2
Bea	1	3	2
Total and significance	12[a]	23[b]	25[b]

10.3 Appendix 3. The reactor

Many reactors are available on the market. We had to choose the one that was most suitable and versatile for our purpose. We chose the IKA LR2000P reactor (Figure 10.2). It is a temperature-controlled reactor, jacketed and linked to a recirculation thermostatic bath with a temperature control device. With an internal thermometric bore, the reaction heat is controlled at any time by the computer. The reactor arrived in our laboratories in June 2003.

This reactor can support 6 bars of overpressure and 220 ºC of temperature. With 2 litres of total volume, the reactor can work with only a ¼ of its capacity. Its stirred system is perfect for our purposes, with an anchor form and with PTFE scrapers renew the mass near the wall of the reactor to avoid any possible overheating and obtain a perfect mix of the ingredients. A flow breaker was added to the reactor to increase the kneaded of the mass.

Figure 10.2 The IKA LR2000P reactor

10.4 Appendix 4. Data associated with chapters 6 and 7

Table 10.17 Compound evolution Temp & time study. 70ºC & 10 h experiment (**Figure 6.1 a**) (A/A(IS))

Compounds	0 h	1 h	2 h	4 h	6,5 h	10 h
2-Methyl-propanal	0,106	0,138	0,134	0,134	0,157	0,142
3-Methyl-butanal	0,381	0,402	0,404	0,371	0,388	0,303
2-Methyl-butanal	0,186	0,260	0,281	0,295	0,379	0,331
Benzaldehyde	0,826	0,820	0,824	0,784	0,815	0,783
Benzene acetaldehyde	0,811	0,705	0,640	0,565	0,493	0,359
Nonanal	0,001	0,001	0,001	0,001	0,001	0,002
3.5-Dimethyl benzaldehyde	0,013	0,014	0,011	0,012	0,009	0,011
5-Methyl-2-phenyl-2-hexenal	0,001	0,001	0,001	0,001	0,001	0,001
Aldehydes	2,325	2,341	2,298	2,163	2,243	1,931
Methyl-pyrazine	0,083	0,077	0,070	0,062	0,064	0,064
2.5-Dimethyl-pyrazine	0,191	0,176	0,160	0,148	0,148	0,144
Ethyl-pyrazine	0,053	0,043	0,037	0,038	0,040	0,036
2.3-Dimethyl-pyrazine	0,039	0,028	0,025	0,025	0,017	0,026
2-Ethyl-6-methy pyrazine	0,047	0,049	0,043	0,040	0,038	0,037
Trimethyl-pyrazine	0,164	0,153	0,129	0,125	0,124	0,113
3-Ethyl-2.5-dimethyl-pyrazine	0,075	0,076	0,064	0,058	0,052	0,044
Tetramethyl-pyrazine	0,043	0,042	0,038	0,034	0,035	0,035
3.5-Diethyl-2-methyl-pyrazine	0,010	0,016	0,010	0,009	0,009	0,010
2-Butyl-3.5-methyl-pyrazine	0,008	0,008	0,007	0,007	0,006	0,007
2.6-Dimethyl-5-isopentylpyrazine	0,010	0,010	0,009	0,008	0,008	0,008
2-Carboxaldehyde-pyrrole	0,013	0,014	0,013	0,013	0,010	0,011
2-Acetylpyrrole	0,069	0,043	0,040	0,046	0,033	0,040
Pyrazines & Pyrroles	0,805	0,735	0,644	0,611	0,586	0,575
Dihydro-2-methyl-3-furanone	0,002	0,002	0,000	0,001	0,000	0,002
Furfural	0,060	0,033	0,022	0,017	0,014	0,012
2-Furanmethanol	0,012	0,009	0,006	0,004	0,006	0,006
2-Acetilfuran	0,012	0,008	0,008	0,008	0,008	0,016
1-Propanone-2-furanyl	0,016	0,015	0,016	0,015	0,006	0,013
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,032	0,022	0,018	0,012	0,011	0,010
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,003	0,002	0,002	0,002	0,002	0,002
Furans. furanones & pyranones	0,137	0,090	0,073	0,059	0,047	0,060
Acetic acid	0,138	0,107	0,111	0,118	0,111	0,099
3-Methyl-butanoic acid	0,194	0,162	0,153	0,143	0,157	0,162
2-Methyl-Butanoic acid	0,040	0,030	0,024	0,025	0,033	0,028
Benzyl Alcohol	0,007	0,005	0,004	0,005	0,004	0,005
Phenylethyl Alcohol	0,137	0,153	0,142	0,117	0,117	0,097
Benzoic Acid	0,006	0,004	0,004	0,004	0,004	0,004
Acids & Alcohols	0,521	0,461	0,438	0,412	0,426	0,396
Acetone	0,106	0,134	0,148	0,172	0,210	0,261
Acetic acid. methyl ester	0,054	0,042	0,038	0,030	0,034	0,033
2.3-Butanedione	0,047	0,047	0,034	0,024	0,015	0,007
2-Butanone	0,091	0,088	0,093	0,089	0,112	0,114
Isoamyl benzoate	0,010	0,009	0,008	0,007	0,006	0,007
Ketones & Esters	0,308	0,320	0,321	0,322	0,377	0,422

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Table 10.18 Compound evolution Temp & time study. 90°C & 10 h experiment (**Figure 6.1** b) (A/A(IS))

Compounds	0 h	1 h	2 h	4 h	10 h
2-Methyl-propanal	0,075	0,121	0,101	0,080	0,070
3-Methyl-butanal	0,322	0,397	0,313	0,193	0,167
2-Methyl-butanal	0,143	0,258	0,239	0,209	0,224
Benzaldehyde	0,824	0,646	0,635	0,421	0,315
Benzene acetaldehyde	0,795	0,396	0,256	0,143	0,131
Nonanal	0,000	0,002	0,001	0,001	0,001
3.5-Dimethyl benzaldehyde	0,011	0,008	0,008	0,008	0,006
5-Methyl-2-phenyl-2-hexenal	0,001	0,001	0,001	0,001	0,001
Aldehydes	2,171	1,828	1,554	1,056	0,916
Methyl-pyrazine	0,086	0,073	0,082	0,075	0,075
2.5-Dimethyl-pyrazine	0,218	0,163	0,096	0,145	0,197
Ethyl-pyrazine	0,030	0,035	0,026	0,039	0,036
2.3-Dimethyl-pyrazine	0,030	0,021	0,023	0,026	0,030
2-Ethyl-6-methy pyrazine	0,050	0,048	0,034	0,029	0,032
Trimethyl-pyrazine	0,139	0,099	0,100	0,080	0,084
3-Ethyl-2.5-dimethyl-pyrazine	0,059	0,046	0,041	0,056	0,060
Tetramethyl-pyrazine	0,046	0,039	0,041	0,047	0,046
3.5-Diethyl-2-methyl-pyrazine	0,014	0,011	0,012	0,011	0,010
2-Butyl-3.5-methyl-pyrazine	0,008	0,006	0,006	0,005	0,005
2.6-Dimethyl-5-isopentylpyrazine	0,011	0,008	0,008	0,006	0,006
2-Carboxaldehyde-pyrrole	0,013	0,008	0,009	0,004	0,006
2-Acetylpyrrole	0,057	0,035	0,033	0,021	0,025
Pyrazines & Pyrroles	0,759	0,592	0,508	0,544	0,613
Dihydro-2-methyl-3-furanone	0,000	0,000	0,000	0,000	0,000
Furfural	0,037	0,005	0,006	0,004	0,013
2-Furanmethanol	0,003	0,004	0,003	0,004	0,009
2-Acetilfuran	0,008	0,007	0,005	0,005	0,011
1-Propanone-2-furanyl	0,011	0,007	0,007	0,003	0,005
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,027	0,013	0,013	0,010	0,025
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,002	0,001	0,001	0,001	0,001
Furans. furanones & pyranones	0,088	0,036	0,037	0,026	0,064
Acetic acid	0,128	0,089	0,082	0,081	0,117
3-Methyl-butanoic acid	0,154	0,115	0,117	0,108	0,127
2-Methyl-Butanoic acid	0,030	0,021	0,021	0,018	0,021
Benzyl Alcohol	0,004	0,004	0,003	0,002	0,002
Phenylethyl Alcohol	0,120	0,095	0,079	0,072	0,067
Benzoic Acid	0,001	0,002	0,001	0,000	0,002
Acids & Alcohols	0,436	0,326	0,303	0,282	0,336
Acetone	0,116	0,176	0,191	0,209	0,290
Acetic acid. methyl ester	0,034	0,022	0,020	0,020	0,028
2.3-Butanedione	0,019	0,008	0,004	0,002	0,003
2-Butanone	0,055	0,060	0,056	0,065	0,086
Isoamyl benzoate	0,006	0,005	0,005	0,004	0,004
Ketones & Esters	0,230	0,270	0,276	0,300	0,411

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Table 10.19 Compound evolution Temp & time study. 110°C & 8 h experiment (**Figure 6.1 c**) (A/A(IS))

Compounds	**0 h**	**1 h**	**2 h**	**4 h**	**8 h**
2-Methyl-propanal	0,090	0,129	0,194	0,347	0,529
3-Methyl-butanal	0,344	0,528	0,612	0,844	1,253
2-Methyl-butanal	0,163	0,314	0,422	0,656	0,813
Benzaldehyde	0,707	0,749	0,676	0,687	0,419
Benzene acetaldehyde	0,701	0,407	0,380	0,390	0,302
Nonanal	0,000	0,002	0,003	0,004	0,004
3.5-Dimethyl benzaldehyde	0,011	0,011	0,010	0,009	0,009
5-Methyl-2-phenyl-2-hexenal	0,001	0,002	0,001	0,002	0,003
Aldehydes	2,017	2,142	2,298	2,939	3,332
Methyl-pyrazine	0,053	0,075	0,073	0,088	0,103
2.5-Dimethyl-pyrazine	0,135	0,183	0,192	0,233	0,268
Ethyl-pyrazine	0,032	0,036	0,041	0,044	0,053
2.3-Dimethyl-pyrazine	0,015	0,032	0,047	0,019	0,019
2-Ethyl-6-methy pyrazine	0,035	0,050	0,041	0,060	0,058
Trimethyl-pyrazine	0,097	0,122	0,104	0,109	0,103
3-Ethyl-2.5-dimethyl-pyrazine	0,048	0,050	0,042	0,047	0,053
Tetramethyl-pyrazine	0,030	0,033	0,038	0,031	0,036
3.5-Diethyl-2-methyl-pyrazine	0,008	0,010	0,011	0,012	0,012
2-Butyl-3.5-methyl-pyrazine	0,006	0,007	0,007	0,007	0,008
2.6-Dimethyl-5-isopentylpyrazine	0,007	0,008	0,007	0,008	0,009
2-Carboxaldehyde-pyrrole	0,011	0,023	0,021	0,025	0,040
2-Acetylpyrrole	0,032	0,053	0,059	0,134	0,379
Pyrazines & Pyrroles	0,506	0,682	0,683	0,815	1,140
Dihydro-2-methyl-3-furanone	0,000	0,003	0,004	0,020	0,038
Furfural	0,030	0,042	0,083	0,854	2,633
2-Furanmethanol	0,008	0,025	0,039	0,078	0,231
2-Acetilfuran	0,005	0,011	0,020	0,113	0,633
1-Propanone-2-furanyl	0,011	0,023	0,021	0,025	0,045
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,021	0,060	0,154	0,576	1,176
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,001	0,002	0,002	0,003	0,002
Furans. furanones & pyranones	0,075	0,167	0,324	1,669	4,758
Acetic acid	0,080	0,131	0,199	0,300	0,437
3-Methyl-butanoic acid	0,111	0,157	0,158	0,365	0,583
2-Methyl-Butanoic acid	0,023	0,029	0,030	0,049	0,038
Benzyl Alcohol	0,003	0,004	0,004	0,003	0,007
Phenylethyl Alcohol	0,075	0,100	0,078	0,094	0,099
Benzoic Acid	0,003	0,005	0,006	0,011	0,018
Acids & Alcohols	0,295	0,425	0,474	0,823	1,183
Acetone	0,055	0,268	0,393	0,438	0,358
Acetic acid. methyl ester	0,029	0,022	0,029	0,048	0,062
2.3-Butanedione	0,028	0,000	0,008	0,018	0,000
2-Butanone	0,050	0,067	0,079	0,106	0,098
Isoamyl benzoate	0,005	0,006	0,006	0,010	0,010
Ketones & Esters	0,168	0,362	0,515	0,620	0,527

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Table 10.20 Compound evolution Temp & time study. 130°C & 4 h experiment (**Figure 6.1** d) (A/A(IS))

Compounds	**0 h**	**1 h**	**2 h**	**4 h**
2-Methyl-propanal	0,136	0,266	0,376	0,635
3-Methyl-butanal	0,276	0,635	0,888	1,340
2-Methyl-butanal	0,201	0,477	0,549	1,224
Benzaldehyde	0,682	0,912	0,974	0,960
Benzene acetaldehyde	0,751	2,144	2,242	0,956
Nonanal	0,000	0,023	0,033	0,095
3.5-Dimethyl benzaldehyde	0,021	0,000	0,049	0,000
5-Methyl-2-phenyl-2-hexenal	0,003	0,013	0,025	0,039
Aldehydes	2,068	4,469	5,136	5,249
Methyl-pyrazine	0,078	0,389	0,631	0,634
2.5-Dimethyl-pyrazine	0,159	0,568	0,985	1,097
Ethyl-pyrazine	0,061	0,095	0,103	0,112
2.3-Dimethyl-pyrazine	0,054	0,078	0,097	0,084
2-Ethyl-6-methy pyrazine	0,098	0,199	0,372	0,458
Trimethyl-pyrazine	0,147	0,348	0,549	0,602
3-Ethyl-2.5-dimethyl-pyrazine	0,075	0,220	0,316	0,399
Tetramethyl-pyrazine	0,043	0,186	0,282	0,294
3.5-Diethyl-2-methyl-pyrazine	0,010	0,102	0,142	0,181
2-Butyl-3.5-methyl-pyrazine	0,021	0,035	0,055	0,088
2.6-Dimethyl-5-isopentylpyrazine	0,029	0,063	0,103	0,175
2-Carboxaldehyde-pyrrole	0,035	0,125	0,385	0,648
2-Acetylpyrrole	0,073	0,687	1,531	2,179
Pyrazines & Pyrroles	0,883	3,096	5,550	6,951
Dihydro-2-methyl-3-furanone	0,000	0,148	0,520	1,846
Furfural	0,055	13,321	42,939	121,494
2-Furanmethanol	0,015	1,398	2,253	0,464
2-Acetylfuran	0,017	1,536	5,201	16,345
1-Propanone-2-furanyl	0,035	0,158	0,715	3,474
5- hydroxymethyl-2-furfural	0,000	0,760	3,836	13,648
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,032	3,327	6,252	5,370
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,009	0,018	0,029	0,060
Furans. furanones & pyranones	0,164	20,666	61,744	162,700
Acetic acid	0,171	1,084	2,702	6,725
3-Methyl-butanoic acid	0,135	0,449	0,705	0,879
2-Methyl-Butanoic acid	0,035	0,110	0,152	0,000
Benzyl Alcohol	0,000	0,013	0,021	0,000
Phenylethyl Alcohol	0,175	0,245	0,341	0,377
Benzoic Acid	0,012	0,107	0,199	0,403
Acids & Alcohols	0,528	2,008	4,121	8,384
Acetone	0,016	0,045	0,088	0,318
Acetic acid. methyl ester	0,010	0,000	0,022	0,095
2.3-Butanedione	0,009	0,000	0,028	0,081
2-Butanone	0,016	0,020	0,037	0,055
Isoamyl benzoate	0,020	0,044	0,076	0,126
Ketones & Esters	0,072	0,109	0,251	0,675

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Table 10.21 Compound evolution Temp & time study. Sugar reduction experiment (130°C & 6 h) (**Figure 6.16**) (A/A(IS))

Compounds	0 h	1 h	2 h	4 h	6 h
2-Methyl-propanal	0,219	0,241	0,257	0,672	0,756
3-Methyl-butanal	0,987	0,950	1,896	1,893	3,096
2-Methyl-butanal	0,565	0,832	1,036	2,226	3,934
Benzaldehyde	3,484	4,119	4,122	4,470	3,400
Benzene acetaldehyde	3,053	3,425	4,934	6,897	4,494
Nonanal	0,021	0,139	0,081	0,106	0,062
3.5-Dimethyl benzaldehyde	0,044	0,055	0,050	0,049	0,000
5-Methyl-2-phenyl-2-hexenal	0,012	0,030	0,037	0,060	0,055
Aldehydes	8,385	9,791	12,414	16,373	15,796
Methyl-pyrazine	0,257	0,413	0,227	1,565	1,684
2.5-Dimethyl-pyrazine	0,738	1,143	2,143	4,157	4,987
Ethyl-pyrazine	0,183	0,271	0,281	0,636	0,752
2.3-Dimethyl-pyrazine	0,187	0,200	0,376	0,387	0,972
2-Ethyl-6-methy pyrazine	0,252	0,414	0,589	1,417	1,541
Trimethyl-pyrazine	0,737	1,004	1,047	1,902	1,878
3-Ethyl-2.5-dimethyl-pyrazine	0,237	0,299	0,288	0,442	0,389
Tetramethyl-pyrazine	0,148	0,212	0,243	0,335	0,311
3.5-Diethyl-2-methyl-pyrazine	0,049	0,080	0,099	0,140	0,112
2-Butyl-3.5-methyl-pyrazine	0,037	0,062	0,080	0,131	0,125
2.6-Dimethyl-5-isopentylpyrazine	0,054	0,098	0,131	0,216	0,215
2-Carboxaldehyde-pyrrole	0,087	0,323	0,787	3,508	3,440
2-Acetylpyrrole	0,269	0,471	0,977	3,605	3,679
Pyrazines & Pyrroles	3,235	4,991	7,268	18,441	20,087
Dihydro-2-methyl-3-furanone	0,000	0,000	0,000	0,210	0,255
Furfural	0,115	0,317	0,877	5,579	5,247
2-Furanmethanol	0,000	0,016	0,000	1,647	0,199
2-Acetylfuran	0,017	0,212	0,611	2,085	3,147
1-Propanone-2-furanyl	0,087	0,323	0,787	0,222	0,362
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,065	0,254	0,859	0,805	0,323
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,010	0,020	0,025	0,038	0,029
Furans. furanones & pyranones	0,293	1,142	3,158	10,586	9,562
Acetic acid	0,497	0,872	2,826	5,738	10,181
3-Methyl-butanoic acid	0,243	0,805	0,000	2,234	2,510
2-Methyl-Butanoic acid	0,069	0,145	0,000	0,347	0,341
Benzyl Alcohol	0,000	0,046	0,048	0,204	0,198
Phenylethyl Alcohol	0,653	1,006	1,427	1,843	1,314
Benzoic Acid	0,028	0,082	0,137	0,305	0,257
Acids & Alcohols	1,491	2,956	4,438	10,671	14,801
Acetone	0,316	1,165	1,235	2,149	2,843
Acetic acid. methyl ester	0,144	0,081	0,254	0,678	1,538
2.3-Butanedione	0,128	0,000	0,000	0,000	0,000
2-Butanone	0,191	0,250	0,243	0,644	0,840
Isoamyl benzoate	0,059	0,103	0,121	0,184	0,159
Ketones & Esters	0,840	1,599	1,853	3,656	5,381

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Table 10.22 Compound evolution fat content study. 0% fat experiment **(Figure 6.20)** (A/A(IS))

Compounds	**0 h**	**1 h**	**2 h**	**4 h**
2-Methyl-propanal	0,059	0,063	0,055	0,047
3-Methyl-butanal	0,145	0,127	0,104	0,075
2-Methyl-butanal	0,089	0,092	0,080	0,071
Benzaldehyde	1,070	0,794	0,622	0,407
Benzene acetaldehyde	1,091	0,800	0,713	0,552
Nonanal	0,009	0,012	0,015	0,010
3.5-Dimethyl benzaldehyde	0,089	0,069	0,058	0,043
5-Methyl-2-phenyl-2-hexenal	0,005	0,004	0,004	0,003
Aldehydes	2,557	1,961	1,650	1,207
Methyl-pyrazine	0,079	0,089	0,098	0,089
2.5-Dimethyl-pyrazine	0,216	0,280	0,321	0,294
Ethyl-pyrazine	0,027	0,024	0,023	0,015
2.3-Dimethyl-pyrazine	0,027	0,015	0,008	0,007
2-Ethyl-6-methy pyrazine	0,054	0,052	0,054	0,050
Trimethyl-pyrazine	0,205	0,177	0,153	0,121
3-Ethyl-2.5-dimethyl-pyrazine	0,121	0,104	0,093	0,072
Tetramethyl-pyrazine	0,106	0,088	0,070	0,057
3.5-Diethyl-2-methyl-pyrazine	0,039	0,034	0,030	0,021
2-Butyl-3.5-methyl-pyrazine	0,011	0,008	0,008	0,006
2.6-Dimethyl-5-isopentylpyrazine	0,019	0,015	0,013	0,010
2-Carboxaldehyde-pyrrole	0,029	0,006	0,021	0,009
2-Acetylpyrrole	0,018	0,017	0,014	0,011
Pyrazines & Pyrroles	0,951	0,907	0,907	0,764
Dihydro-2-methyl-3-furanone	0,000	0,000	0,000	0,002
Furfural	0,009	0,014	0,017	0,022
2-Furanmethanol	0,000	0,025	0,045	0,167
2-Acetylfuran	0,003	0,009	0,007	0,007
1-Propanone-2-furanyl	0,029	0,006	0,011	0,009
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,000	0,015	0,024	0,036
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,009	0,008	0,006	0,005
Furans. furanones & pyranones	0,050	0,077	0,110	0,248
Acetic acid	0,073	0,152	0,119	0,117
3-Methyl-butanoic acid	0,050	0,127	0,165	0,177
2-Methyl-Butanoic acid	0,007	0,017	0,025	0,052
Benzyl Alcohol	0,010	0,003	0,000	0,006
Phenylethyl Alcohol	0,143	0,125	0,131	0,086
Benzoic Acid	0,004	0,003	0,004	0,003
Acids & Alcohols	0,286	0,428	0,444	0,442
Acetone	0,019	0,058	0,051	0,050
Acetic acid. methyl ester	0,017	0,000	0,000	0,000
2.3-Butanedione	0,000	0,000	0,000	0,000
2-Butanone	0,031	0,031	0,029	0,025
Isoamyl benzoate	0,010	0,009	0,008	0,005
Ketones & Esters	0,078	0,098	0,088	0,081

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Table 10.23 Compound evolution fat content study. 10% fat experiment (**Figure 6.20**) (A/A(IS))

Compounds	**0 h**	**1 h**	**2 h**	**4 h**
2-Methyl-propanal	0,033	0,031	0,023	0,026
3-Methyl-butanal	0,088	0,059	0,041	0,040
2-Methyl-butanal	0,061	0,059	0,044	0,047
Benzaldehyde	1,096	0,755	0,499	0,355
Benzene acetaldehyde	0,513	0,419	0,350	0,392
Nonanal	0,010	0,017	0,012	0,008
3.5-Dimethyl benzaldehyde	0,000	0,000	0,000	0,000
5-Methyl-2-phenyl-2-hexenal	0,053	0,034	0,029	0,031
Aldehydes	1,853	1,375	0,998	0,899
Methyl-pyrazine	0,034	0,039	0,030	0,036
2.5-Dimethyl-pyrazine	0,111	0,123	0,105	0,138
Ethyl-pyrazine	0,015	0,015	0,011	0,011
2.3-Dimethyl-pyrazine	0,010	0,009	0,007	0,014
2-Ethyl-6-methy pyrazine	0,048	0,050	0,046	0,056
Trimethyl-pyrazine	0,122	0,115	0,090	0,102
3-Ethyl-2.5-dimethyl-pyrazine	0,153	0,141	0,118	0,129
Tetramethyl-pyrazine	0,045	0,043	0,033	0,038
3.5-Diethyl-2-methyl-pyrazine	0,088	0,076	0,060	0,067
2-Butyl-3.5-methyl-pyrazine	0,045	0,035	0,028	0,033
2.6-Dimethyl-5-isopentylpyrazine	0,134	0,102	0,085	0,103
2-Carboxaldehyde-pyrrole	0,006	0,006	0,005	0,005
2-Acetylpyrrole	0,008	0,011	0,007	0,007
Pyrazines & Pyrroles	0,820	0,763	0,625	0,738
Dihydro-2-methyl-3-furanone	0,000	0,000	0,000	0,000
Furfural	0,046	0,113	0,091	0,049
2-Furanmethanol	0,000	0,026	0,019	0,017
2-Acetylfuran	0,020	0,024	0,024	0,019
1-Propanone-2-furanyl	0,047	0,020	0,015	0,018
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,000	0,027	0,031	0,070
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,014	0,006	0,006	0,007
Furans. furanones & pyranones	0,127	0,216	0,186	0,180
Acetic acid	0,101	0,155	0,154	0,211
3-Methyl-butanoic acid	0,043	0,131	0,114	0,078
2-Methyl-Butanoic acid	0,009	0,018	0,015	0,015
Benzyl Alcohol	0,008	0,008	0,011	0,004
Phenylethyl Alcohol	0,149	0,019	0,086	0,085
Benzoic Acid	0,000	0,012	0,000	0,003
Acids & Alcohols	0,310	0,343	0,380	0,395
Acetone	0,014	0,029	0,041	0,057
Acetic acid. methyl ester	0,006	0,004	0,003	0,004
2.3-Butanedione	0,000	0,000	0,000	0,000
2-Butanone	0,016	0,017	0,016	0,018
Isoamyl benzoate	0,009	0,008	0,007	0,007
Ketones & Esters	0,045	0,059	0,067	0,086

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Table 10.24 Compound evolution fat content study. 20% fat experiment **(Figure 6.20)** (A/A(IS))

Compounds	**0 h**	**1 h**	**2 h**	**4 h**
2-Methyl-propanal	0,056	0,054	0,043	0,052
3-Methyl-butanal	0,103	0,096	0,066	0,072
2-Methyl-butanal	0,081	0,086	0,074	0,080
Benzaldehyde	1,150	1,036	0,746	0,651
Benzene acetaldehyde	0,432	0,301	0,427	0,256
Nonanal	0,011	0,029	0,019	0,023
3.5-Dimethyl benzaldehyde	0,000	0,000	0,000	0,000
5-Methyl-2-phenyl-2-hexenal	0,020	0,019	0,033	0,015
Aldehydes	1,853	1,621	1,408	1,149
Methyl-pyrazine	0,065	0,074	0,042	0,061
2.5-Dimethyl-pyrazine	0,135	0,146	0,124	0,153
Ethyl-pyrazine	0,032	0,026	0,015	0,017
2.3-Dimethyl-pyrazine	0,023	0,012	0,011	0,009
2-Ethyl-6-methy pyrazine	0,056	0,060	0,051	0,051
Trimethyl-pyrazine	0,116	0,081	0,096	0,058
3-Ethyl-2.5-dimethyl-pyrazine	0,104	0,092	0,110	0,068
Tetramethyl-pyrazine	0,040	0,039	0,032	0,027
3.5-Diethyl-2-methyl-pyrazine	0,038	0,034	0,055	0,021
2-Butyl-3.5-methyl-pyrazine	0,016	0,014	0,026	0,009
2.6-Dimethyl-5-isopentylpyrazine	0,024	0,022	0,076	0,014
2-Carboxaldehyde-pyrrole	0,028	0,014	0,006	0,019
2-Acetylpyrrole	0,016	0,015	0,008	0,010
Pyrazines & Pyrroles	0,693	0,630	0,651	0,518
Dihydro-2-methyl-3-furanone	0,000	0,000	0,000	0,000
Furfural	0,005	0,033	0,041	0,050
2-Furanmethanol	0,000	0,006	0,028	0,022
2-Acetylfuran	0,002	0,009	0,011	0,010
1-Propanone-2-furanyl	0,032	0,016	0,008	0,023
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,000	0,013	0,015	0,030
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,009	0,010	0,005	0,006
Furans. furanones & pyranones	0,048	0,088	0,107	0,142
Acetic acid	0,117	0,146	0,068	0,121
3-Methyl-butanoic acid	0,029	0,087	0,079	0,102
2-Methyl-Butanoic acid	0,003	0,011	0,010	0,026
Benzyl Alcohol	0,010	0,001	0,000	0,000
Phenylethyl Alcohol	0,227	0,100	0,050	0,065
Benzoic Acid	0,001	0,006	0,000	0,003
Acids & Alcohols	0,388	0,353	0,206	0,316
Acetone	0,032	0,070	0,049	0,065
Acetic acid. methyl ester	0,007	0,000	0,004	0,000
2.3-Butanedione	0,000	0,000	0,000	0,000
2-Butanone	0,044	0,044	0,024	0,033
Isoamyl benzoate	0,010	0,009	0,005	0,006
Ketones & Esters	0,093	0,122	0,082	0,104

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Table 10.25 Compound evolution sugar study. Fructose experiment **(Figure 6.26)**

Compounds	**0 h**	**1 h**	**2 h**	**4 h**
2-Methyl-propanal	0,044	0,056	0,056	0,052
3-Methyl-butanal	0,106	0,116	0,108	0,098
2-Methyl-butanal	0,054	0,069	0,076	0,078
Benzaldehyde	0,680	0,682	0,577	0,491
Benzene acetaldehyde	1,108	0,491	0,471	0,431
Nonanal	0,003	0,017	0,015	0,016
3.5-Dimethyl benzaldehyde	0,029	0,026	0,024	0,022
5-Methyl-2-phenyl-2-hexenal	0,000	0,000	0,000	0,000
Aldehydes	2,024	1,457	1,325	1,188
Methyl-pyrazine	0,071	0,062	0,059	0,049
2.5-Dimethyl-pyrazine	0,131	0,124	0,119	0,107
Ethyl-pyrazine	0,026	0,021	0,020	0,017
2.3-Dimethyl-pyrazine	0,020	0,017	0,018	0,013
2-Ethyl-6-methy pyrazine	0,044	0,041	0,038	0,034
Trimethyl-pyrazine	0,134	0,115	0,108	0,090
3-Ethyl-2.5-dimethyl-pyrazine	0,060	0,051	0,045	0,039
Tetramethyl-pyrazine	0,064	0,052	0,047	0,038
3.5-Diethyl-2-methyl-pyrazine	0,028	0,023	0,020	0,016
2-Butyl-3.5-methyl-pyrazine	0,009	0,008	0,007	0,006
2.6-Dimethyl-5-isopentylpyrazine	0,012	0,011	0,010	0,008
2-Carboxaldehyde-pyrrole	0,017	0,013	0,012	0,010
2-Acetylpyrrole	0,032	0,028	0,025	0,022
Pyrazines & Pyrroles	0,647	0,566	0,526	0,449
Dihydro-2-methyl-3-furanone	0,004	0,004	0,005	0,005
Furfural	0,036	0,054	0,065	0,092
2-Furanmethanol	0,017	0,017	0,021	0,039
2-Acetylfuran	0,010	0,011	0,013	0,016
1-Propanone-2-furanyl	0,024	0,018	0,017	0,014
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,020	0,051	0,077	0,101
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,005	0,005	0,002	0,004
Furans. furanones & pyranones	0,116	0,159	0,201	0,272
Acetic acid	0,143	0,150	0,158	0,136
3-Methyl-butanoic acid	0,143	0,131	0,135	0,132
2-Methyl-Butanoic acid	0,048	0,044	0,045	0,045
Benzyl Alcohol	0,000	0,000	0,000	0,000
Phenylethyl Alcohol	0,105	0,088	0,067	0,070
Benzoic Acid	0,005	0,004	0,006	0,005
Acids & Alcohols	0,445	0,417	0,411	0,389
Acetone	0,015	0,049	0,050	0,052
Acetic acid. methyl ester	0,012	0,006	0,007	0,000
2.3-Butanedione	0,000	0,000	0,000	0,000
2-Butanone	0,016	0,016	0,017	0,013
Isoamyl benzoate	0,013	0,010	0,009	0,008
Ketones & Esters	0,056	0,081	0,083	0,072

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Table 10.26 Compound evolution sugar study. Glucose experiment **(Figure 6.26)**

Compounds	**0 h**	**1 h**	**2 h**	**4 h**
2-Methyl-propanal	0,043	0,061	0,075	0,062
3-Methyl-butanal	0,108	0,160	0,163	0,155
2-Methyl-butanal	0,054	0,079	0,089	0,092
Benzaldehyde	0,687	0,734	0,712	0,571
Benzene acetaldehyde	1,232	0,360	0,317	0,285
Nonanal	0,000	0,011	0,011	0,010
3.5-Dimethyl benzaldehyde	0,028	0,023	0,025	0,020
5-Methyl-2-phenyl-2-hexenal	0,000	0,000	0,000	0,000
Aldehydes	2,151	1,429	1,393	1,195
Methyl-pyrazine	0,069	0,057	0,057	0,043
2.5-Dimethyl-pyrazine	0,130	0,103	0,101	0,077
Ethyl-pyrazine	0,028	0,021	0,020	0,014
2.3-Dimethyl-pyrazine	0,026	0,017	0,015	0,014
2-Ethyl-6-methy pyrazine	0,045	0,035	0,036	0,028
Trimethyl-pyrazine	0,144	0,108	0,107	0,088
3-Ethyl-2.5-dimethyl-pyrazine	0,059	0,046	0,048	0,034
Tetramethyl-pyrazine	0,063	0,052	0,048	0,040
3.5-Diethyl-2-methyl-pyrazine	0,028	0,022	0,022	0,016
2-Butyl-3.5-methyl-pyrazine	0,009	0,003	0,006	0,006
2.6-Dimethyl-5-isopentylpyrazine	0,013	0,009	0,009	0,008
2-Carboxaldehyde-pyrrole	0,018	0,012	0,015	0,012
2-Acetylpyrrole	0,035	0,026	0,030	0,029
Pyrazines & Pyrroles	0,666	0,511	0,515	0,408
Dihydro-2-methyl-3-furanone	0,003	0,000	0,005	0,005
Furfural	0,038	0,069	0,108	0,174
2-Furanmethanol	0,017	0,011	0,010	0,014
2-Acetylfuran	0,011	0,012	0,015	0,016
1-Propanone-2-furanyl	0,024	0,017	0,020	0,018
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,021	0,034	0,061	0,108
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,005	0,000	0,005	0,005
Furans. furanones & pyranones	0,119	0,143	0,225	0,341
Acetic acid	0,113	0,110	0,149	0,125
3-Methyl-butanoic acid	0,116	0,095	0,122	0,105
2-Methyl-Butanoic acid	0,041	0,031	0,043	0,037
Benzyl Alcohol	0,000	0,000	0,000	0,000
Phenylethyl Alcohol	0,066	0,053	0,056	0,041
Benzoic Acid	0,002	0,000	0,002	0,004
Acids & Alcohols	0,337	0,289	0,371	0,312
Acetone	0,017	0,049	0,048	0,049
Acetic acid. methyl ester	0,005	0,000	0,000	0,000
2.3-Butanedione	0,000	0,000	0,000	0,000
2-Butanone	0,018	0,018	0,021	0,015
Isoamyl benzoate	0,011	0,008	0,010	0,007
Ketones & Esters	0,050	0,076	0,078	0,071

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Table 10.27 Compound evolution sugar study. Meliose™ experiment (**Figure 6.26**)

Compounds	0 h	1 h	2 h	4 h
2-Methyl-propanal	0,054	0,077	0,065	0,056
3-Methyl-butanal	0,115	0,154	0,149	0,137
2-Methyl-butanal	0,063	0,083	0,079	0,096
Benzaldehyde	0,702	0,647	0,583	0,533
Benzene acetaldehyde	1,177	0,448	0,330	0,350
Nonanal	0,000	0,000	0,005	0,005
3.5-Dimethyl benzaldehyde	0,026	0,024	0,025	0,022
5-Methyl-2-phenyl-2-hexenal	0,000	0,000	0,000	0,000
Aldehydes	2,137	1,432	1,235	1,201
Methyl-pyrazine	0,074	0,053	0,045	0,047
2.5-Dimethyl-pyrazine	0,142	0,099	0,097	0,098
Ethyl-pyrazine	0,028	0,019	0,015	0,021
2.3-Dimethyl-pyrazine	0,021	0,020	0,030	0,023
2-Ethyl-6-methy pyrazine	0,048	0,034	0,034	0,036
Trimethyl-pyrazine	0,166	0,111	0,105	0,113
3-Ethyl-2.5-dimethyl-pyrazine	0,064	0,044	0,040	0,050
Tetramethyl-pyrazine	0,070	0,048	0,044	0,054
3.5-Diethyl-2-methyl-pyrazine	0,026	0,019	0,019	0,020
2-Butyl-3.5-methyl-pyrazine	0,010	0,000	0,006	0,000
2.6-Dimethyl-5-isopentylpyrazine	0,014	0,009	0,009	0,009
2-Carboxaldehyde-pyrrole	0,018	0,004	0,005	0,006
2-Acetylpyrrole	0,033	0,019	0,023	0,028
Pyrazines & Pyrroles	0,716	0,480	0,472	0,505
Dihydro-2-methyl-3-furanone	0,000	0,000	0,000	0,000
Furfural	0,069	0,070	0,128	0,226
2-Furanmethanol	0,019	0,016	0,025	0,049
2-Acetylfuran	0,024	0,022	0,023	0,035
1-Propanone-2-furanyl	0,028	0,008	0,010	0,013
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,025	0,039	0,074	0,107
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,000	0,000	0,000	0,000
Furans. furanones & pyranones	0,165	0,154	0,260	0,430
Acetic acid	0,178	0,159	0,143	0,175
3-Methyl-butanoic acid	0,176	0,125	0,131	0,142
2-Methyl-Butanoic acid	0,060	0,046	0,051	0,052
Benzyl Alcohol	0,000	0,000	0,000	0,004
Phenylethyl Alcohol	0,089	0,058	0,055	0,049
Benzoic Acid	0,000	0,000	0,000	0,000
Acids & Alcohols	0,503	0,388	0,380	0,423
Acetone	0,024	0,080	0,081	0,083
Acetic acid. methyl ester	0,000	0,000	0,000	0,000
2.3-Butanedione	0,000	0,000	0,000	0,000
2-Butanone	0,025	0,025	0,022	0,025
Isoamyl benzoate	0,016	0,016	0,012	0,012
Ketones & Esters	0,064	0,120	0,115	0,120

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Table 10.28 Compound evolution sugar study. Xilose experiment **(Figure 6.26)**

Compounds	0 h	1 h	2 h	4 h	8 h
2-Methyl-propanal	0,097	0,143	0,162	0,158	0,189
3-Methyl-butanal	0,348	0,449	0,445	0,544	0,481
2-Methyl-butanal	0,168	0,282	0,289	0,331	0,386
Benzaldehyde	0,818	0,787	0,715	0,629	0,562
Benzene acetaldehyde	0,809	0,481	0,475	0,565	0,576
Nonanal	0,003	0,008	0,010	0,009	0,012
3.5-Dimethyl benzaldehyde	0,011	0,008	0,009	0,007	0,009
5-Methyl-2-phenyl-2-hexenal	0,003	0,003	0,004	0,003	0,004
Aldehydes	2,256	2,161	2,108	2,246	2,220
Methyl-pyrazine	0,074	0,074	0,076	0,072	0,051
2.5-Dimethyl-pyrazine	0,163	0,182	0,256	0,236	0,216
Ethyl-pyrazine	0,043	0,047	0,039	0,036	0,032
2.3-Dimethyl-pyrazine	0,026	0,037	0,036	0,022	0,023
2-Ethyl-6-methy pyrazine	0,051	0,056	0,058	0,065	0,062
Trimethyl-pyrazine	0,150	0,146	0,135	0,133	0,117
3-Ethyl-2.5-dimethyl-pyrazine	0,057	0,051	0,046	0,046	0,032
Tetramethyl-pyrazine	0,037	0,034	0,030	0,031	0,024
3.5-Diethyl-2-methyl-pyrazine	0,011	0,011	0,011	0,012	0,012
2-Butyl-3.5-methyl-pyrazine	0,007	0,007	0,008	0,008	0,009
2.6-Dimethyl-5-isopentylpyrazine	0,011	0,010	0,012	0,010	0,014
2-Carboxaldehyde-pyrrole	0,020	0,022	0,025	0,025	0,026
2-Acetylpyrrole	0,071	0,062	0,064	0,076	0,092
Pyrazines & Pyrroles	0,721	0,739	0,796	0,772	0,711
Dihydro-2-methyl-3-furanone	0,000	0,005	0,006	0,006	0,016
Furfural	0,050	0,168	0,385	0,704	1,578
2-Furanmethanol	0,011	0,039	0,093	0,204	0,182
2-Acetylfuran	0,008	0,036	0,078	0,190	0,508
1-Propanone-2-furanyl	0,025	0,027	0,029	0,011	0,048
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,033	0,242	0,514	0,779	1,239
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,003	0,002	0,003	0,002	0,004
Furans. furanones & pyranones	0,129	0,520	1,109	1,895	3,576
Acetic acid	0,103	0,130	0,180	0,204	0,244
3-Methyl-butanoic acid	0,169	0,179	0,259	0,309	0,502
2-Methyl-Butanoic acid	0,035	0,037	0,043	0,030	0,059
Benzyl Alcohol	0,005	0,005	0,008	0,009	0,014
Phenylethyl Alcohol	0,219	0,188	0,198	0,190	0,163
Benzoic Acid	0,010	0,011	0,017	0,017	0,029
Acids & Alcohols	0,542	0,549	0,705	0,759	1,011
Acetone	0,078	0,314	0,324	0,300	0,257
Acetic acid. methyl ester	0,039	0,026	0,026	0,025	0,026
2.3-Butanedione	0,041	0,000	0,000	0,000	0,000
2-Butanone	0,059	0,075	0,074	0,072	0,075
Isoamyl benzoate	0,016	0,014	0,018	0,015	0,020
Ketones & Esters	0,233	0,429	0,442	0,412	0,378

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Table 10.29 Enzyme effect study. 90°C & 9 hours experiment (**Figure 7.5** top graph)

Compounds	Pre-enz	0 h	1 h	2'5 h	4 h	6 h	9 h
2-Methyl-propanal	0,101	0,058	0,091	0,113	0,134	0,105	0,109
3-Methyl-butanal	0,455	0,158	0,385	0,400	0,430	0,355	0,341
2-Methyl-butanal	0,259	0,202	0,299	0,378	0,380	0,365	0,367
Benzaldehyde	1,759	1,230	1,360	1,419	1,417	1,236	1,141
Benzene acetaldehyde	1,301	0,642	0,644	0,685	0,639	0,530	0,451
Nonanal	0,007	0,004	0,007	0,010	0,010	0,008	0,008
3.5-Dimethyl benzaldehyde	0,014	0,006	0,006	0,006	0,007	0,006	0,007
5-Methyl-2-phenyl-2-hexenal	0,003	0,002	0,003	0,003	0,004	0,003	0,004
Aldehydes	3,900	2,302	2,795	3,014	3,021	2,607	2,428
Methyl-pyrazine	0,127	0,060	0,066	0,066	0,071	0,064	0,059
2.5-Dimethyl-pyrazine	0,207	0,131	0,142	0,160	0,160	0,145	0,149
Ethyl-pyrazine	0,054	0,030	0,023	0,030	0,028	0,027	0,027
2.3-Dimethyl-pyrazine	0,053	0,031	0,030	0,032	0,032	0,033	0,030
2-Ethyl-6-methy pyrazine	0,055	0,032	0,035	0,041	0,042	0,036	0,040
Trimethyl-pyrazine	0,166	0,090	0,092	0,107	0,110	0,094	0,096
3-Ethyl-2.5-dimethyl-pyrazine	0,060	0,033	0,036	0,043	0,039	0,036	0,034
Tetramethyl-pyrazine	0,068	0,040	0,040	0,046	0,049	0,042	0,040
3.5-Diethyl-2-methyl-pyrazine	0,034	0,012	0,016	0,016	0,014	0,012	0,011
2-Butyl-3.5-methyl-pyrazine	0,011	0,005	0,005	0,005	0,006	0,005	0,005
2.6-Dimethyl-5-isopentylpyrazine	0,014	0,006	0,008	0,008	0,008	0,008	0,008
2-Carboxaldehyde-pyrrole	0,065	0,035	0,035	0,035	0,036	0,030	0,033
2-Acetylpyrrole	0,110	0,064	0,064	0,073	0,078	0,075	0,094
Pyrazines & Pyrroles	1,025	0,570	0,592	0,664	0,674	0,606	0,626
Dihydro-2-methyl-3-furanone	0,003	0,001	0,001	0,001	0,002	0,001	0,002
Furfural	0,069	0,009	0,032	0,051	0,067	0,101	0,156
2-Furanmethanol	0,020	0,011	0,012	0,017	0,020	0,026	0,044
2-Acetylfuran	0,024	0,011	0,014	0,013	0,019	0,019	0,024
1-Propanone-2-furanyl	0,065	0,035	0,035	0,035	0,036	0,030	0,033
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,028	0,002	0,000	0,016	0,021	0,031	0,057
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,003	0,002	0,002	0,001	0,003	0,001	0,002
Furans. furanones & pyranones	0,212	0,071	0,097	0,134	0,166	0,210	0,318
Acetic acid	0,202	0,096	0,129	0,156	0,172	0,144	0,182
3-Methyl-butanoic acid	0,368	0,317	0,329	0,338	0,369	0,367	0,415
2-Methyl-Butanoic acid	0,082	0,087	0,086	0,085	0,089	0,097	0,102
Benzyl Alcohol	0,039	0,041	0,038	0,042	0,043	0,038	0,042
Phenylethyl Alcohol	0,330	0,222	0,208	0,249	0,249	0,225	0,239
Benzoic Acid	0,009	0,006	0,007	0,008	0,012	0,008	0,012
Acids & Alcohols	1,030	0,770	0,796	0,877	0,934	0,877	0,991
Acetone	0,064	0,128	0,165	0,230	0,292	0,260	0,285
Acetic acid. methyl ester	0,028	0,009	0,007	0,010	0,011	0,009	0,011
2.3-Butanedione	0,031	0,009	0,007	0,004	0,006	0,003	0,004
2-Butanone	0,061	0,045	0,051	0,057	0,059	0,052	0,049
Isoamyl benzoate	0,023	0,011	0,014	0,014	0,016	0,014	0,015
Ketones & Esters	0,206	0,203	0,244	0,314	0,384	0,339	0,363

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Table 10.30 Enzyme effect study. 110°C & 8 hours experiment (**Figure 7.5** middle)

Compounds	Pre-enz	0 h	1 h	2'5 h	4 h	6 h	8 h
2-Methyl-propanal	0,180	0,179	0,113	0,260	0,235	0,485	0,573
3-Methyl-butanal	0,869	0,776	0,600	0,940	0,981	1,605	1,924
2-Methyl-butanal	0,499	0,452	0,519	1,093	1,222	2,524	2,746
Benzaldehyde	3,548	3,262	3,803	4,712	4,638	3,100	2,467
Benzene acetaldehyde	2,754	2,137	2,517	3,295	4,943	3,889	2,942
Nonanal	0,016	0,027	0,037	0,040	0,045	0,037	0,042
3.5-Dimethyl benzaldehyde	0,060	0,033	0,020	0,025	0,032	0,015	0,028
5-Methyl-2-phenyl-2-hexenal	0,011	0,004	0,011	0,020	0,027	0,025	0,031
Aldehydes	7,938	6,869	7,619	10,384	12,122	11,680	10,753
Methyl-pyrazine	0,217	0,240	0,269	0,396	0,764	1,037	1,156
2.5-Dimethyl-pyrazine	0,542	0,449	0,487	0,860	1,238	1,828	1,946
Ethyl-pyrazine	0,145	0,105	0,110	0,140	0,216	0,250	0,281
2.3-Dimethyl-pyrazine	0,221	0,127	0,121	0,168	0,181	0,133	0,157
2-Ethyl-6-methy pyrazine	0,164	0,129	0,168	0,206	0,280	0,344	0,416
Trimethyl-pyrazine	0,509	0,360	0,404	0,413	0,445	0,436	0,412
3-Ethyl-2.5-dimethyl-pyrazine	0,162	0,145	0,113	0,138	0,098	0,098	0,087
Tetramethyl-pyrazine	0,160	0,158	0,151	0,138	0,110	0,088	0,091
3.5-Diethyl-2-methyl-pyrazine	0,128	0,063	0,072	0,032	0,030	0,029	0,026
2-Butyl-3.5-methyl-pyrazine	0,038	0,019	0,015	0,022	0,023	0,026	0,024
2.6-Dimethyl-5-isopentylpyrazine	0,045	0,022	0,023	0,031	0,035	0,038	0,034
2-Carboxaldehyde-pyrrole	0,165	0,091	0,389	0,869	1,055	1,147	1,187
2-Acetylpyrrole	0,314	0,183	0,238	0,341	0,582	0,819	0,912
Pyrazines & Pyrroles	2,810	2,091	2,559	3,753	5,057	6,273	6,729
Dihydro-2-methyl-3-furanone	0,000	0,005	0,000	0,026	0,079	0,100	0,184
Furfural	0,170	0,083	0,603	1,466	3,190	3,688	4,681
2-Furanmethanol	0,000	0,045	0,928	2,743	2,994	3,712	4,374
2-Acetylfuran	0,058	0,035	0,320	0,325	1,074	1,807	2,687
1-Propanone-2-furanyl	0,165	0,089	0,714	0,753	1,195	1,022	1,147
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,120	0,019	0,220	0,295	0,630	0,524	0,671
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,013	0,004	0,029	0,021	0,043	0,025	0,054
Furans. furanones & pyranones	0,526	0,280	2,813	5,630	9,204	10,878	13,798
Acetic acid	0,949	0,173	0,991	1,145	2,727	3,027	3,124
3-Methyl-butanoic acid	0,942	0,347	0,805	1,441	1,713	2,236	2,147
2-Methyl-Butanoic acid	0,149	0,073	0,132	0,323	0,360	0,524	0,612
Benzyl Alcohol	0,053	0,226	0,131	0,227	0,181	0,230	0,187
Phenylethyl Alcohol	0,923	1,901	0,893	1,216	0,891	0,980	0,751
Benzoic Acid	0,025	0,013	0,022	0,028	0,047	0,081	0,071
Acids & Alcohols	3,041	2,734	2,975	4,381	5,919	7,078	6,892
Acetone	0,096	0,175	0,461	0,749	0,862	1,549	1,513
Acetic acid. methyl ester	0,067	0,196	0,125	0,194	0,435	0,533	0,357
2.3-Butanedione	0,073	0,098	0,043	0,049	0,043	0,037	0,042
2-Butanone	0,123	0,152	0,171	0,206	0,223	0,389	0,185
Isoamyl benzoate	0,070	0,055	0,107	0,115	0,125	0,142	0,112
Ketones & Esters	0,429	0,676	0,906	1,313	1,689	2,650	2,209

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Table 10.31 Enzyme effect study. 130°C & 6 hours experiment (**Figure 7.5** bottom)

Compounds	Pre-enzy	0 h	1 h	2'5 h	4 h	6 h
2-Methyl-propanal	0,180	0,312	0,429	0,925	1,312	1,395
3-Methyl-butanal	0,869	0,610	1,810	3,186	3,285	2,867
2-Methyl-butanal	0,499	0,776	1,707	3,217	3,963	3,664
Benzaldehyde	3,548	3,217	5,409	6,846	6,134	5,323
Benzene acetaldehyde	2,754	1,867	4,286	7,114	7,951	5,288
Nonanal	0,016	0,028	0,082	0,121	0,101	0,084
3.5-Dimethyl benzaldehyde	0,060	0,049	0,061	0,087	0,070	0,000
5-Methyl-2-phenyl-2-hexenal	0,011	0,010	0,032	0,069	0,088	0,113
Aldehydes	7,938	6,869	13,816	21,565	22,905	18,733
Methyl-pyrazine	0,217	0,234	0,753	1,510	2,034	3,227
2.5-Dimethyl-pyrazine	0,542	0,432	0,998	2,945	4,134	4,857
Ethyl-pyrazine	0,145	0,103	0,275	0,478	0,693	0,866
2.3-Dimethyl-pyrazine	0,221	0,106	0,183	0,401	0,408	0,419
2-Ethyl-6-methy pyrazine	0,164	0,112	0,254	0,529	0,891	1,346
Trimethyl-pyrazine	0,509	0,340	0,523	0,825	1,088	1,283
3-Ethyl-2.5-dimethyl-pyrazine	0,162	0,116	0,205	0,336	0,429	0,520
Tetramethyl-pyrazine	0,160	0,130	0,297	0,419	0,420	0,455
3.5-Diethyl-2-methyl-pyrazine	0,128	0,085	0,089	0,163	0,153	0,178
2-Butyl-3.5-methyl-pyrazine	0,038	0,024	0,049	0,083	0,104	0,125
2.6-Dimethyl-5-isopentylpyrazine	0,045	0,033	0,070	0,127	0,156	0,177
2-Carboxaldehyde-pyrrole	0,165	0,138	0,670	2,158	3,836	5,179
2-Acetylpyrrole	0,314	0,238	1,136	3,451	5,206	5,831
Pyrazines & Pyrroles	2,810	2,093	5,502	13,425	19,552	24,463
Dihydro-2-methyl-3-furanone	0,000	0,005	0,000	0,150	0,238	0,351
Furfural	0,170	0,031	2,791	8,349	10,723	11,847
2-Furanmethanol	0,000	0,041	3,360	6,110	8,673	6,197
2-Acetylfuran	0,058	0,040	1,203	4,078	6,011	7,441
1-Propanone-2-furanyl	0,165	0,138	0,670	0,427	0,862	1,370
2.3-dihydro-3.5-dihydroxy-6-methyl-4H-pyran-4-one	0,120	0,016	0,939	1,555	0,995	0,444
3.4-dihydro-8-hydroxy-3-methyl-1H-2Benzopyran-1-one	0,013	0,008	0,013	0,033	0,025	0,029
Furans. furanones & pyranones	0,526	0,280	8,976	20,703	27,528	27,678
Acetic acid	0,949	0,444	2,113	9,206	10,798	12,614
3-Methyl-butanoic acid	0,942	1,065	4,079	8,159	8,917	9,388
2-Methyl-Butanoic acid	0,149	0,271	0,843	1,371	1,852	1,827
Benzyl Alcohol	0,053	0,115	0,231	0,445	0,677	0,749
Phenylethyl Alcohol	0,923	0,813	1,413	2,319	2,539	2,439
Benzoic Acid	0,025	0,026	0,111	0,303	0,362	0,401
Acids & Alcohols	3,041	2,734	8,789	21,803	25,145	27,417
Acetone	0,096	0,382	0,880	1,139	1,379	2,485
Acetic acid. methyl ester	0,067	0,043	0,060	0,136	0,249	0,524
2.3-Butanedione	0,073	0,027	0,045	0,069	0,101	0,105
2-Butanone	0,123	0,175	0,210	0,352	0,480	0,851
Isoamyl benzoate	0,070	0,048	0,098	0,179	0,203	0,218
Ketones & Esters	0,429	0,676	1,294	1,876	2,413	4,184

All runs preformed extracting a headspace of vial with 4g of the reaction mixture at 60°C for 15 min. Column 30m x 0.25mm ID 1 µm IF Varian Factor 4

Chapter 11

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