2015

OPTIMIZATION OF AN ANALYTICAL APPROACH BASED ON HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE CHARACTERIZATION OF A NATURAL VANILLA EXTRACT

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Graduation Memory
Class 012

Year
2014-2015

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"Life gives you opportunities; your life is on your hands.
So it's your decision to take the risk or to ask yourself all of your life as would have been.
It's the moment to take charge of your destiny.
I will begin building my future."

Acknowledges

I would like to thank and express my sincere gratitude to all the following individuals and organisations that help me to success in this report. I appreciate them for giving me generously of their time and expertise, to assist, advise, encourage and guide me through this process.

I would acknowledge Colciencias who is the Administrative Department of Science, Technology and Innovation in Colombia. It supports fundamental and applied research through many programs as the **Young Engineering France internship.** It is a program between Colciencias, the Education Minister of Colombia and the French Embassy that gives to Colombian students of superior education the opportunity to do a master degree in France.

I would grateful them to give me the opportunity to improve my knowledge in this amazing country. Without this internship, I would never have the occasion to be involved in French culture, to meet awesome people of the entire world and to live an unforgettable experience.

I am grateful to Nactis Gourmet and specifically with **Sevarome** society for funding my graduate. In particular, I would like to acknowledge **Mr. Gerard Trivier** who opens the doors of his company to let me learn and finish my education program.

I would like to express my gratitude to **Mr. Petru Jitaru**, who was my academic tutor, for his ideas and guidance on this entire dissertation. I would also like to thank my tutor **Dr. Luz Maria Alzate**, who guide me during this process and gives me her academic support during this period.

I would like to thank **Dr. Julian Londoño** who gives me the opportunity to participate in research, specifically in Young Research program at La Corporación Universitaria LaSallista and encourage me during my career at the University.

I can't forget Christian Martin, who instructed me during my internship period in Sevarome and without whom, I would not have learned about this job. To my co-workers Carine and Isabelle who bring me their help when I need it. In general all of Sevarome team, I am grateful to them.

To the most important people in my life my mother Irma Suaza and my sister Sara Vélez. They are the engines that drive me towards better horizons, which encourage me to pursue my dreams, thanks to being there always when I need you.

Abstract, Résumé & Resumen

Abstract

An overview of flavour industry and specifically vanilla flavour has been realised to understand its importance. Vanilla, as the second most expensive spice in the world, has an important value for flavour industries, but currently falsifications are a big problem, so regulations are increasingly stringent. To this fact, a chromatographic method has been developed to the characterisation of natural vanilla extracts. The method development included a standardisation of the extraction method and a validation of. An extraction method of a natural vanilla extract has been developed, as part of the analytical method, using an emergent technology called Ultrasonic Assisted Extraction (UAE). This study has enabled to probe the role of UAE in enhancing the evaluated responses by a comparison study with a conventional extraction. A fast, precise and accurate RP-HPLC method has been developed for the working conditions of Sevarome laboratory, by the stabilised regulations of method standardization of ICH. The method was validated for linearity, selectivity, LOD, LOQ, precision and accuracy. The method is useful for the identification and quantification of the main compounds in natural vanilla extracts and other vanilla products.

Keywords: *Vanilla flavour, Natural vanilla extracts, Vanilla analysis, HPLC, Ultrasonic Assisted Extraction, Vanilla extraction.*

Résumé

Une vision générale de l'industrie aromatique et spécifiquement de l'arôme vanille a été réalisée afin de comprendre son importance. La vanille, est la deuxième épice la plus chère dans le monde, elle a une valeur très importante pour les industries aromatiques. D'ici qu'actuellement les falsifications dans l'étiquetage ont devenu un grand problème, pour cela les réglementations sont de plus en plus exigeantes. Au but de faire face à cette problématique une méthode d'analyse par chromatographie a été développée. La méthode permet la caractérisation des extraits naturels de vanille. On présents d'abord une standardisation de la méthode d'extraction de la vanille, le développement de la méthode analytique suivi d'une validation. Une méthode d'extraction de l'arôme vanille a été développée, comme partie du procédé analytique, en utilisant l'extraction assistée par ultrasons. Une étude comparative entre l'extraction par ultrasons et une méthode conventionnelle permet de prouver l'efficience des ultrasons lors de l'extraction de l'arôme vanille. Une méthode chromatographique RP-HPLC, rapide, précise e exacte a été développée pour les conditions de travail du laboratoire de Sevarome. Le développement de la méthode a été faite d'après les règles établies par l'ICH. La méthode a été validée pour la linéarité, sélectivité, LDD, LDQ, précision et exactitude. La méthode est utile pour l'identification et la quantification des composants principaux des extraits naturels de vanille et des autres produits provenant de la vanille.

Mots clés : *Arôme vanille, Extraits naturels de vanille, Analyse de la vanille, HPLC, extraction assistée par ultrasons, extraction de la vanille.*

Resumen

En este trabajo se presenta una visión general de la industria aromática, específicamente del aroma de vainilla, con el fin de comprender su importancia. La vainilla es la segunda especia más costosa del mundo y tiene un valor muy importante para la industria aromática, sin embargo, las falsificaciones en el etiquetado de este producto se han convertido en un problema, por lo tanto las reglamentaciones son cada vez más exigentes. Debido a todo lo anterior, un método cromatográfico ha sido desarrollado con el objetivo de caracterizar los extractos naturales de vainilla. El desarrollo del método incluye una primera parte donde se estandariza el método de extracción del aroma, seguido del desarrollo y la validación del método analítico. Se desarrollo un método de extracción del aroma, usando para ello la tecnología de extracción asistida por ultrasonidos. Un estudio comparativo entre el ultrasonido y un método convencional ha sido útil para probar la eficiencia de esta tecnología para la extracción del aroma de vainilla. Se ha desarrollado un método de RP-HPLC rápido, preciso y exacto para las condiciones de trabajo del laboratorio de Sevarome, de acuerdo a los parámetros establecidos por la ICH, para la estandarización de métodos. El método fue validado según los criterios de linealidad, selectividad, LDD, LDC, precisión y exactitud. EL método es útil para la identificación y cuantificación de los compuestos principales de los extractos naturales de vainilla y otros productos de la vainilla.

Palabras clave: *Aroma de vainilla, Extracto natural de vainilla, Análisis de la vainilla, HPLC, Extracción asistida por ultrasonido, extracción de la vainilla.*

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Glossary and Abbreviations

Aroma: an aroma is a mixture of different volatile molecules enough to reach the olfactory organ, is every odoriferous substance whose objective is to bring a flavour.

Bourbon (Vanilla): Is a name that is referred to the curing process of vanilla originates from the Indian Ocean region including Madagascar, Comoros, Mayotte and Reunion Island.

Curing: Is the process that transforms green vanilla beans into vanilla flavourers' beans. During this process, the entire specific and characteristic aroma is developed, specifically vanillin.

Flavour: a flavour is the result of the presence, within complex matrices, of many volatiles and non-volatiles compounds possessing different chemical and physicochemical proprieties.

Vanillin: Is the principal compound developed during curing, which gives the specific aroma of vanilla.

Abbreviations

ACN	Acetonitrile
ANOVA	Analysis of Variance
CRM	Certified Reference Material
DAD	Diode Array Detector
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization for United Nations
FDA	Food and Drug Administration
FEMA	Flavour and Extract Manufactures Association
HPLC	High-Performance Liquid Chromatography
ICH	International conference of Harmonization
IUPAC	International Union of Pure and Applied Chemistry
UAE	Ultrasonic assisted extraction
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
OP	Orthogonal power
RSD	Relative standard deviation
RSS	Really simple syndication
R&D	Research and Development
SD	Standard deviation
SNIAA	National Syndicate of Food Aromatic Industry Syndicat National des Industries Aromatiques Alimentaires
TFA	Triflouroacetic acid
USA	United States of America

CHAPTER 1: BACKGROUND AND JUSTIFICATION

1.1. Introduction

Vanilla beans are the fruits of Vanilla planifolia Andrews that belongs to Orchidaceae group. The curing of green vanilla beans to obtain the well-appreciated vanilla aroma is a very laborious process. Natural vanilla is considered as one of the most widely used and important flavoring materials worldwide (GHAZALI, 2006). The source of vanilla flavour is the bean or pod of the tropical Vanilla orchid that is principally cultivated in Madagascar and is correctly called as "Bourbon" Vanilla Planifolia, the best quality.

In the last years, an increasing demand for natural molecules has been identified due to growing preference by consumers for high-quality flavouring agents (A. SHARMA ET AL., 2006). Vanilla has a very high demand and prices in the world for its popular uses in flavouring ice creams, soft drinks, pastry. Even if the natural vanilla flavour is pretty expensive, the extraction methods result in an extended period of extraction, high solvent yield and a hard work. Food flavouring industries, specifically vanilla flavour producers, have a big potential to grow up in this worldwide market.

Many flavouring industries as Sevarome, a Society, which produces natural vanilla extracts, are currently improving vanilla extraction methods. The aim is to reduce extraction time, solvent consummation and increase the yield of the most important compounds contributing to the aroma in the final product. Nevertheless, it is very important to have a validated method to characterize their products, ensure their quality and guaranty their conformity with regulations.

Because of the vast price difference between natural vanillin, vanilla's major ingredient, and its synthetic counterpart, the risk of adulteration is significant (A.G. HUESGEN (AGILENT TECHNOLOGIES), 2011; CICCHETTI & CHAINTREAU, 2009a). In 1999, the French administration ran a series of analysis on products supposed to contain a natural flavour. Moreover, synthetic vanilla's flavour was detected (CICCHETTI & CHAINTREAU, 2009a). To contrast adulterations, a several analytical techniques consisting in High-Performance Liquid Chromatography have been developed.

Ultrasonic assisted extraction (UAE), and conventional extraction of vanilla are described in subsequent chapters. Also, the development and validation of an analytical method by HPLC to the characterization of natural vanilla extracts are explained. Before that, this chapter shows a brief description of the reasons that led to the development of this project in Sevarome.

1.2. Brief description of NACTIS Company

Nactis is a familial company founded in 1996, specialised in the production of flavours, aromatic and functional ingredients (LESCENE, 2013).

They have experts in aromas and flavouring ingredients, also is a company highly recognized by its innovation technology.

Nactis is a company that is always at the forefront of regulations and sociological challenges, anticipating changes and client's expectations.

Key figures:

- **250** employees, with 175 in France and 75 internationally
- **Six** production sites in France (Bondoufle, Chartres, Yssingeaux, Furdenheim and Illkirch) and Belgium (Schoten)
- **4** research and development centres in France (i.e. R&D centre in Sevarome)
- **4** international commercial subsidiaries: Bulgaria, Poland, Tunisia, USA
- **€55 million** in revenues, so **25%** from international (exportation) distributing in almost 50 countries of Europe Union, North America, Asia and the Maghreb region.
- **6%** of revenues devoted to R&D, to offer consumers high-value products.

To provide more service and proximity for these two types of customers, Nactis has built its offer around two ranges, industrial and gourmet.

Nactis gourmet, a subsidiary of Nactis, is specialized in the production of ingredients for bakers, pastry makers, confectioners, ice-cream makers, butchers, charcuterie makers, gourmet caterers and the catering trade in general. They develop a range of high-end aromatic and functional ingredients and flavours. Sevarome Is a society conformed by Prodeal, Prochar and Sevarome, which installation is at Yssingeaux (France).

1.3. Sevarome

Sevarome is a society which originates from Nactis Gourmet. It is placed at the artisanal zone of Yssingeaux, at L'haute Loire department, in France. It produces food flavours and ingredients and is involved in the pastry and sweet products industry of.

Their production is ensured with specific quality controls in agreement with the legal requirements. The main objectives of Sevarome is to ensure high quality products, satisfaction and confidence of their clients, being in the same time transparent professionally ethic. The certification ISO 9001 shows its engagement.

Sevarome has a research laboratory totally dedicated to developing specific products used to create and make tailor-made flavouring solutions. Sevarome has experts in flavouring area, which's with their expertise and creativity meet the specifics demands of each client. The main tasks are summarized in research, creation and innovation. Also, they develop formulas, adapt the formulation to the type of application and create applications in order to analyze and validate behaviour in the end product.

Sevarome is a brand with a recognized experience that puts all its know-how in the service of their clients. Is then, a company that offer functional solutions, improving customer satisfaction, and keeping technical, financial, and regulatory environment.

1.4. Internship Presentation

The worldwide interest in natural vanilla flavour is currently growing and consequently the interest to standardize a product of high value. This aim was the starting point of a project in Sevarome producing for several years' natural and artificial vanilla flavours. The project aims at the

development and the validation of an analytical method to characterize natural vanilla extracts (with flavour properties). The method development implies the standardization of the extraction method of vanilla extract that is also produced by Sevarome.

Currently, there is a growing importance to consumers to obtaining natural and high-quality products, and the only way that they have to check the naturalness of is the labelling, which is controlled by regulations organisms. However, in food flavouring industry, and specifically with vanilla flavour, there are many products that have been labelling as naturals and they are not. So that fraud in this industry is evident and to solve this problem, regulations are increasingly stringent. As a response, several methods have been developed for controlling of the authenticity of vanilla extracts.

To Sevarome, the standardized analytical method will serve to characterize vanilla pods, but the higher importance is the characterisation of vanilla extracts as a quality control. Such an approach is important to ensure that the vanilla flavours price been based on a well-defined composition that define its quality (PACKER, 2008), which, therefore, could reduce the fraud in this industrial field.

In the context described above developing an analytical method in Sevarome is an ongoing project that would ensure the accurate definition of the vanilla extracts, both for quality industrial controls and regulations (FERNANDEZ & CABROL-BASS, 2015). A complete description of the product will allow Sevarome to guarantee the purity and naturalness of the vanilla extract produced to their clients.

Nowadays natural's products are ahead of the national and international market. Many industries continued with progress and to be competitive with the growing demand for this kind of products, it is necessary to ensure and guaranty its quality, because of consumers.

To make this possible, the first step is the extraction of all the aroma constituents, which contributes to specific flavour; it is the extraction of a natural vanilla extract. However, this extract should be rich in vanillin, to improve its characteristic aroma. For this fact, conventional and UAE were compared (Chapter III), then the extracts were characterized by HPLC.

To be a useful method in Sevarome as a routine quality control or routine analysis procedure performed criteria of analytical HPLC method were examined (Chapter IV). So a final validation step (Chapter V) is also important to guaranteed accuracy and precision of the analytical method.

1.5. Objectives

Several chromatography methods have been developed to characterize vanilla extracts. The already existing methods described in the literature have as advantages the determination of vanillin, but the disadvantage is that they never give a quality approach to food flavouring industries.

Sevarome is a company for which keep up with all regulations concerning natural vanilla extracts is very important. The general objective of this work is:

Develop and validate an analytical method for the characterization of natural vanilla extracts.

Two parts are important during the internship, the first one consists in the extraction method of vanilla flavour, and the second one consists in the development and validation of the analytical method to characterization. So the specifics objectives of this work are as follows:

- Determine the best chromatographic parameters to the separation of the main constituents of vanilla extract.
- Develop an analytical HPLC method to determinate the quantity of vanillin on the obtained extract.
- Evaluate the effectiveness of two extraction methods of vanilla extract; traditional maceration and UAE.
- Validate the analytical method in agreement with the International Union of Pure and Applied Chemistry (IUPAC) specifications, to evaluate the quality and naturalness of vanilla extracts.
- Identify and quantify the main constituents of natural vanilla flavour produced by Sevarome.

1.6. Planning

The planning of this project was made in the software Project 2010 of the office pack. A GANT diagram was realised for a period considered between February and July for the whole part made at Sevarome. ***Fig. 1*** summarized the principal's stages of the project.

Phase of project (month/ week)		Feb				Mar				Apr				May				Jun				Jul			
		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Research																									
Optimization of the extraction	Experimental																								
	Standardisation																								
	Analytical																								
	Operating balance																								
Development and Validation of HPLC Method	Exploratory																								
	Plan																								
	Experimental																								
	Validation																								
	Analytical																								
	Testing																								
Final Analysis - Control Process																									

Figure 1: Overall project planning

CHAPTER 2: CHARACTERIZATION OF VANILLA FLAVOUR AND ITS INDUSTRY

2.1. Introduction

The development of industrial supply processes has led to several new requirements. Food flavouring industry has had an excellent progress around 50's when the necessity to obtain standard products, always available, consistent in quality and concentration was increasingly important.

Flavours are used as additives in foods and in most cases can define consumers' preferences. In fact, the aroma makes appetizing and enjoyable to eat food; from here the importance of innovation in this domain.

There are two different types of flavours: naturals and synthetic, obtained by various extraction routes or compounds by artificial materials. The quantity of natural flavours is limited, its extraction is always more difficult, expensive and represents a significant work hand, so its price becomes considerably higher compared to synthetic flavours. Nevertheless, currently consumers have an increasing preference for natural products.

Vanilla spice that has always been coveted is now the most common flavour in the world is one of the most appreciated natural flavours and has a high commercial value and importance due to their particular organoleptic proprieties. The beverage industry, pastry, ice-creams are only a few products that use vanilla flavour as a tradition, and it is highly used in food industry.

Vanilla beans contain more than 200 volatile compounds (A.G. HUESGEN (AGILENT TECHNOLOGIES), 2011). The main chemical constituent of vanilla is vanillin (4-Hydroxy-3-Methoxy benzaldehyde). Vanillin is the most characteristic component of vanilla flavour. Vanillin content (in percent) is used as a measure of vanilla beans quality, and it ranges can change by the origin of (KUMAR & BALAMOHAN, 2013).

An overview of this flavour is presented. How vanilla has become an outstanding flavour over the world, how cultivation and taxonomy can change its composition and aromatic profile, etc. This chapter will show how this unusual plant has aroused much enthusiasm and led to the imitation of her scent for several processes, over the years, also how regulations have allowed adulteration control. In the same way, it will show as costs also vary widely and play a significant role in vanilla's market.

2.2. Food Flavouring Industry

Food flavouring industry can be characterized as highly technical, specialized and innovative. It is also complex and different in every country, because of regulations, culture and price. Today's food flavouring industry has continually evolved. In fact, the expanding knowledge of natural raw materials, gastronomy, cooking techniques and procedures adopted for the food industry has played a crucial role in this process (EFFA, 2015). Therefore, it's necessary to adapt flavours to local consumers' preferences that impose a significant challenge to this growing industry (INTERNATIONAUX, 2011).

Food flavour manufacturers are usually flavourist, they are specialists that known and recognise the specifics molecules responsible for a particular flavour. They are competent to formulate a broad variety of flavouring essence, both natural and synthetic.

The safety of a flavour is guaranteed by the Flavour and Extract Manufactures Association (FEMA), an industry trade group. The European Union (EU) has created a definitive list of food flavourings, which are approved for use in food manufacturing across the EU, during this process almost seven flavourings has been banning. Since 2003 the European Commission (EC), along with the European Food Safety Authority (EFSA), have been evaluating the safety of food flavouring substances. The result is a list with 2500 flavouring substances being declared fit for human consumption (FEMA Flavour, 2014).

In general, food flavouring industry always has been the aim of satisfy consumers. Therefore, it involves a lot of critical factors as regulations, economy factors, market, and expertise in the area. So the importance of developing tendencies and improving processes in this industry.

2.2.1. Global overview of food flavouring industry

Food flavouring industry is very different in each country and each region. The formulations, preparations and extraction methods change in function of the local traditions, weather conditions and the other external and internal factors. In fact, the preferences in the flavour industry are usually a reflex to food and beverage industry (MARKETS, 2014).

Currently, flavouring companies must adapt their products to an international market. In fact, the flavour perception is different in every country. The purchasing power change, the regulations change and there are more society factors forcing this industry to be at the forefront of the international flavour conditions to face an extremely competitive market (INTERNATIONAUX, 2011; MARKETS, 2014)

In recent years, the interconnection between the scientific work with the market requirements is a crucial activity. Research and development (R&D) has become imperative in industries. It gives the opportunity to introduce new products and process and to improve their quality. R&D workers take the current demands studied by the marketing group; the idea is understood, transformed into solutions then an experimental and analytical phase is realised. Finally, the need becomes a product (INTERNATIONAUX, 2011).

In worldwide the market of this industry is about 5 to 6 billion of euro's, of which 400 million are for France (SNIAA, 2011). The food flavour market in the food industry is estimated to reach 12.07 billion of euro's by 2018 signifying a moderate compound annual growth rate of 5.3% (MARKETS, 2014). This potential growth market also depends on R&D activities.

The European flavouring industry is one of the major players in the European market, its contribution to the economy of EU in terms of employment and production is remarkable (EFFA, 2015). In France, food flavouring industry is a little part of food industry revenue, it represent only 0.25%, that to say 157 billions of euro's (ANIA, 2011). Moreover, France has international recognition as flavour producer (XERFI, 2014); this production stands for different kind of activities as:

- Production and purification of extracts from aromatic natural resources

- The extraction of isolates and their chemical modification
- Defined chemical synthesis of aromatic molecules

The good recognition of France as a flavour producer is justified as the importance that the flavouring companies give to the innovation and research of new products. They invest much of their capital in R&D activities that explain the augmentation of the products range. Currently, a strong growth in demand of flavours has been identified, specifically in natural products (LONGO & SANROMÁN, 2006; XERFI, 2014), from here the interest to innovate with this kind of products.

2.2.2. What a flavour is?

Flavor or flavour can be defined as the sensory impression of a food or other substance and is determined mainly by the chemical senses of taste and smell (GUERRA, DE LARA, MALIZIA, & DÍAZ, 2009).

The aroma of food is a mixture of different volatile molecules enough to reach the olfactory organ. It is every odoriferous substance whose objective is to bring a flavour; it is a nasal and retro nasal perception of any flavoured food like ice creams, chewing gum, beverages, etc. In effect, the number of compounds that may contribute to flavour is large (M.A. DRAKE, R.E. MIRACLE, A.D. CAUDLE, 2007). Aromas have not nutritive properties, but they have an essential role in foods. The aroma perception can determinate the consumers selectivity and food acceptability, the reason is the volatility of flavours that stimulate the appetite before we start eating (FERNANDEZ & CABROL-BASS, 2015).

The quantity of compounds responsible for the aroma of food are complex substances that usually have been extracted from plant sources (LONGO & SANROMÁN, 2006), for consumers that means "natural and healthy foods". Today the interest of consumers by this kind of products has been growing and can explain their increasing price.

A flavour can be natural or synthetic. The first one is obtained by extraction of vegetal or animal raw material, but also it can be obtained using biotechnology (microorganisms or enzymes). Hence, a natural flavour is always produced with natural's raw material. The second one is divided in two: identical to naturals and artificial. Their production is an issue of a chemical process that allows obtaining molecules, subsequently well mixed up the particular scent (MAINGUET & DGCCR, 2006).

2.2.3. Tendencies of food flavouring industry

Natural food flavour have attracted the attention of food ingredients manufacturers with increasing consumer demand for fresh and natural products (MARKETS, 2014), from here that many food ingredient manufacturers are producing natural food flavours. Nevertheless, the production of natural flavours by direct extraction from plants is also a subject of various problems. Those which can stand are highs cost of raw material and the low concentration of the desired compounds (LONGO & SANROMÁN, 2006), resulting in expensive extractions.

Natural flavours are considerably expensive compared to artificial and their stability is also a problem. However, they are being used in high-end premium bakeries and other products where price is not a concern because consumers are ready to pay this difference.

Amongst the variety of natural flavours in use today, vanilla occupies a prominent market (ARUN K SINHA, SHARMA, & SHARMA, 2008). Flavours as vanilla, chocolate and strawberry were still the most

popular flavours in 2010, and enter into the composition of 59% of all new products (INTERNATIONAUX, 2011) like beverages, ice creams, cakes, etc.

Today, vanilla use became much diversified, similarly many consumers are demanding to food and beverage industry use more natural flavours because the return to the naturalness. However, the problem whit this demand is that natural's flavours are more expensive to produce. Although, with the continuing increase in their cost numerous efforts of blending and adulteration in natural vanilla extracts have been reported (FERNANDEZ & CABROL-BASS, 2015). In fact, not every company follows the rules. So the choice of a great vanilla extract just by looking at the label information makes this a little tricky.

Due to the interest in natural vanilla flavours, it is vital that reliable and practical analytical techniques for determined chemical compounds in vanilla are found. However, nowadays the development of a quantifying method cannot be considered as being complete without a validation step. Validation is especially important when a method is intended to be used for the purpose of quality assurance, as in the case of vanilla natural products (CICCHETTI & CHAINTREAU, 2009b).

2.3. Vanilla Plant

Vanilla is a tropical vine of the orchid family (*See: **fig. 2***), grown for its fruit and originates from Mexico. Vanilla is the world most popular spice (CID-PEREZ, 2011; ARUN K SINHA ET AL., 2008), its characteristic aroma is the result of a number of biochemical and chemical transformations, it contains approximately 200 substances (A.G. HUESGEN (AGILENT TECHNOLOGIES), 2011; CICCHETTI & CHAINTREAU, 2009a; RANADIVE, 1992) constituting whole sensory sensations perceived in the aroma.

Figure 2: Vanilla orchid

Vanilla beans have a best growing in a hot and humid climate from sea level to an elevation of 1500 m. The optimal growing temperature is between 15 – 20°C during the day and 15 – 20°C during the night. Its aroma and flavour depend on different parameters like geographical origin, maturity at harvest, curing conditions, curing methods, etc (KUMAR & BALAMOHAN, 2013).

The vanilla species of commerce, Vanilla planifolia, known as ‘‘Mexican’’ or ‘‘Bourbon’’ vanilla, is native to tropical forests of southeastern Mesoamerica. Vanilla is cultivated in many countries as Madagascar, Indonesia, Comoros, India, and others.

2.3.1. History

Vanilla is the most important spice in the world, is a fruit-bearing member of the orchid family which originates from Mexico; it was discovered in Mesoamerica during the 1300s. Vanilla cultivation has

its origin in Mexico, where was traditionally done by the Totonec Indians on small plants. Nevertheless, in the fifteen century when Aztecs conquered the Totonec they soon developed a taste for the vanilla bean. For this fact, Aztecs were the first people who used vanilla beans as a flavour, a tonic and antidote (CHARLES, 2013).

The genus name *Vanilla* is from the Spanish *"vaina"* meaning little pod, the vanilla beans are also referred to as pods of "black flower", after the mature bean. When the Spaniards arrived to Mexico and discovered the vanilla plant, they began to use the bean as flavouring for chocolate. One of the reasons was Hernando Cortez, the first person who returned to Spain with vanilla beans combined with cacao to make an unusual drink, that later helped to the spread of this scent in the old continent (CID-PEREZ, 2011).

Until the mid 19th century, Mexico was the chief producer of vanilla. In fact, a lot of efforts were made to cultivate vanilla outside Mexico, but its cultivation was not possible outside Mexico due to the absent of a natural pollinator (ARUN K SINHA ET AL., 2008). However, Charles Morren (1836) discovered the secret of vanilla's reluctance to bear fruit outside Mexico, which has led to the discovery of artificial pollination of vanilla flowers (CID-PEREZ, 2011; ARUN K SINHA ET AL., 2008). After this, the French developed large vanilla plantation on reunion, soon the orchids went sent from Reunion Islands to Madagascar by French colonist in the 1980s.

Nowadays, vanilla can grow in five main areas of the world; each region produces vanilla beans with distinctive characteristics and attributes. Vanilla cultivation in Madagascar is concentrated along the northeast coast around Antalaha, Andapa, Sambava and Vohemar (CHARLES, 2013). Today the principal vanilla producers are Indonesia and Madagascar; nevertheless Mexico remains considered the historical centre of vanilla cultivation.

2.3.2. Principal Producers

The composition of processed vanilla beans is quite variable and complex due to the number of variables such as species, growth conditions, soil composition, fruit maturity and mainly, the type of processing (JAVIER DE LA CRUZ MEDINA, GUADALUPE C. RODRIGUEZ JIMÉNES, 2009).

Vanilla production reached about 10 thousand tons in green. Due to its high demand, production increased by 150% between 2000 and 2012 (HIDALGO, 2014). However, the existing statistics are no exact, and they are based on estimations made by the producers countries. They can contain assessment and reporting errors; also there are significant variations that made difficult to obtain accurate production data. A database with this statistics is a disposition by the Food and Agriculture Organization for United Nations (FAO) (FAOSTAT, 2015).

Among the countries that produce vanilla beans Madagascar and Indonesia are the higher producers (TOTH, 2012). Some statistics from FAO (Country/Territorial Notes, 2015) show that Madagascar was the largest producer, with 6200 Tons per year, production that has decreased over the last years. Indonesia is the second producer, which has just expanded its production to 2399 - 3700 Tons in late 2006. The third-largest producer is China, with an average production of 1000 Tons per year. Then the smaller producers include Mexico (306 Tons), Turkey (192 Tons), Tonga (144 Tons), and Uganda (195 Tons) (JAVIER DE LA CRUZ MEDINA, GUADALUPE C. RODRIGUEZ JIMÉNES, 2009). ***Fig. 3*** shows the principal vanilla producer in the world.

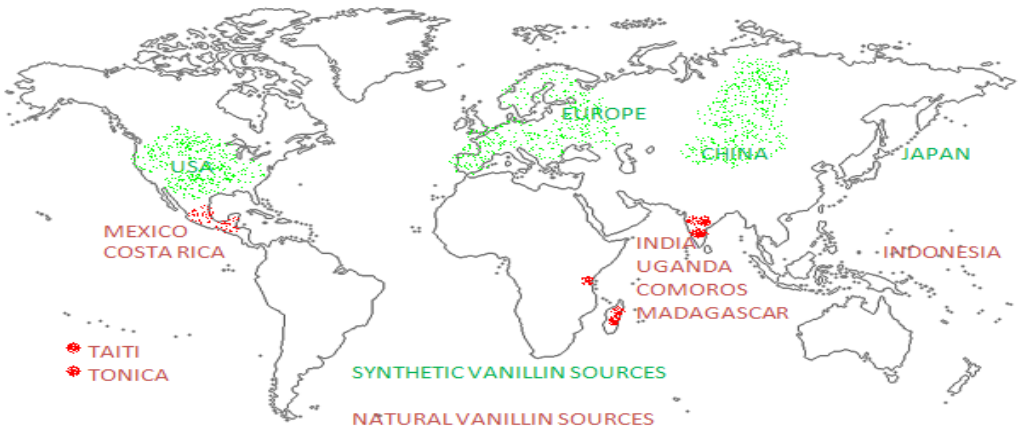

Figure 3: Natural and synthetic vanilla sources around the world

Indonesia produces Java vanilla (from Java island) and Bourbon-like vanilla (from Bali island). Both Java and Bourbon-like can be used to producing fragrances, but Bourbon-like has the best quality, similarly to Bourbon vanilla beans. In the last years, Indonesia has become the first rival of Madagascar, the quality of their vanilla pods highly recognized in the world. In fact, Indonesian production is increasing every year. China has become a good producer and a significant exporter of vanilla; there is not specific information about cultivation or quality of vanilla from China.

2.3.3. Cultivation and Taxonomy

There are different species of vanilla in the world, about 110 have been catalogued, and most of the species are found in tropical America. By far the most important is *V. planifolia* (SUJALMI & SUPRIYANTO, 2005). Vanilla species are distributed throughout America, Africa and Asia-Oceania between the 27th north and south parallels (ERIC ODOUX, 2010).

The Vanilla genus belongs to the Orchidaceae family, one of the largest families of flowering plants in the world. There are more than 800 genera in the family Orchidaceae and approximately 25000 species. The main aromatic species of plant genus *Vanilla* are *vanilla planifolia* probably endemic from eastern Mexico tropical forest, *Vanilla tahitensis* cultivated in several Pacific countries or *Vanilla pompona* from the west of Indies (BORY, GRISONI, DUVAL, & BESSE, 2007; MARUENDA ET AL., 2013; ARUN K SINHA ET AL., 2008).

The cultivation consists in propagating new plants by stem cuttings from vigorous plants in the field. Cuttings are taken along the stem of a producing factory, in optimum lengths of no less than 30cm. Vanilla beans vines are grown on established trees, to ensure support and filtered sun. In plantations, known as vanillaries, vanilla begins to produce flowers at approximately two to three years. Plants reach maturity at seven or eight years (EXLEY, 2011).

Vanilla beans stem tops are cut about six months before fruiting season to encourage an increase in the number of inflorescences. Only flowers on the lower side of the raceme are pollinated so that following fruit may hang down, producing a long straight bean (HAVKIN-FRENKEL & BELANGER, 2011).

Hand pollination is carried out daily until the number of successfully pollinated flowers is reached on each plant. Pollination allows pollen to fertilize the ovules. Vanilla beans appear one month after pollination, it is necessary to wait seven or eight months for the beans are ripe. When the beans are

fully developed and beginning to ripen - the tips begin to turn yellow – they are picked (EXLEY & ISS, 2010).

2.3.4. Preparation of vanilla beans

Vanilla beans should have a specific preparation before their utilisation as raw material for vanilla flavour extraction. After collection of vanilla pods, they do not have any characteristic flavour. But they have the chemical characteristics to develop their particular aroma. It is by this that vanilla beans should be treated with the best conditions to obtain a very good quality of the product. In fact, all preparation steps improve enzymatic reactions leading to the formation of aroma.

All vanilla orchids are hand pollinated, except in Mexico when the bees make the work. The pods are hand-harvested and cured for at least six months. The "Bourbon" method preparation used at The Reunion, Madagascar and Comoro, is one of the most important method knows nowadays and it allows obtaining an excellent quality bean, a brief description of this method is shown in **Annex A.** (ELKE ANKLAM, 1993).

2.4. Vanilla Flavour

Most of what is perceived as flavors in foods is actually the aromas and odors that are being detected by our sense of smell. In terms of food, flavor is perhaps the most important attribute of a product that leads us to decide whether to consume a particular product or not (BUTNER, n.d.).

The chemical constituent responsible of aroma in vanilla pods are acids, ethers, alcohols, acetals, heterocyclics, phenolics, hydrocarbons, esters and carbonyls (ARUN K SINHA ET AL., 2008). Also the main constituents of vanilla flavour is vanillin (4-hydroxy-3methoxybenzaldehyde), among the other volatile constituents of vanilla aroma are p-hydroxybenzoic acid, p-hydroxybenzaldehyde and vanillic acid (RANADIVE, 1992). It is possible to evaluate the quality of vanilla extracts in agreement with the levels and ratios of concentration of the main constituents of vanilla flavour. The ratio of p-hydroxybenzaldehyde and vanillin, five to twenty times vanillin, is an initial parameter to determine the quality of vanilla and also to identify its source, natural or synthetic extract.

2.4.1. The main constituents of vanilla extract/Flavour

Natural vanilla extract is contains components in a concentration greater than 1mg/Kg, vanillin is the major phenolic component (ARUN KUMAR SINHA, VERMA, & SHARMA, 2007). Vanilla contains other flavouring agents which characterise the aroma, as 4-hydroxybenzaldehyde, vanillic acid and 4-hydroxybenzoic acid.

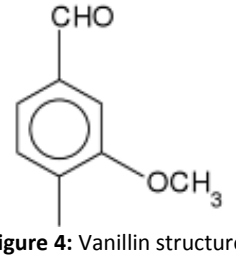

Figure 4: Vanillin structure

Vanillin (4-hydroxy 3-methoxy benzaldehyde) (***fig.4***) is the most important compound in vanilla extracts and flavour. It is probably the most widely used flavouring agent. Vanillin is a plant secondary metabolite and is produced from ground black vanilla bean pods. The annual market for vanillin exceeds 16,000 tonnes, although only 0.25% of this originates from cured seed pods of the vanilla orchid, Vanilla *planifolia* (BROCHADO ET AL., 2010).

Vanillin can also be obtained through various methods like chemical synthesis, biotransformation, from degradation of waste sulphite liquors apart from the extraction of natural vanilla pods (JADHAV,

B.N., GOGATE, & RATHOD, 2009). Vanillin pure is more effective that vanilla extracts (i.e. 1g of vanillin is equivalent to 150g of vanilla extract or 400g of vanilla beans (Ortuéno, M.F., 2006)).

4-Hydroxybenzaldehyde in food flavouring industry is used for the synthesis of vanillin. It is a light yellow or white crystal micro-aromatic. It can be found naturally in vanilla beans.

Vanillic Acid (4-hydroxy-3-methoxybeanzoic acid) is a phenolic acid, the oxidation form of vanillin. Vanillic acid is a constituent of vanilla pods and many other plants but can be obtained synthetically. It is a flavouring and scent agent that produces a pleasant. It is widely used for the preparation of synthetic vanilla flavour.

4-Hydroxybenzoic acid is a phenolic derivative of benzoic acid, it can be found naturally in vanilla beans.

2.4.2. Natural vanilla extract/flavour

"A flavouring substance is considered to be 'natural' when it is obtained from material of vegetable, animal or microbiological origin, by natural processes, and has been "identified in nature"" (EFFA, 2011).

Table 1: General specification to identification of natural vanilla extracts. ([1]p-Hydroxybenzoic)

	Range
Vanillin/pHB[1] Aldehyde	Between 10 and 20
Vanillin/pHB Acid	Between 40 and 110
Vanillin/vanillic Acid	Between 12 and 29
Vanillic Acid/ pHB Aldehyde	Between 0.53 and 1.5
pHB Acid/pHB Aldehyde	Between 0.15 and 0.35

Natural vanilla extracts is a mixture of flavour components extracted from the cured beans of the vanilla plant. It has a pure, delicate spicy flavour that cannot be duplicated exactly by synthetic products, hence its production is limited and prices are high (RAMACHANDRA RAO & RAVISHANKAR, 2000). However, natural vanilla flavour has a high commercial value on the international market and regular progress from 3 to 5% per year in a stable way (MARUENDA ET AL., 2013).

The desirable characteristic flavour and aroma proprieties of vanilla have lead to its widespread use in the food. To satisfy increasing global demand for natural vanilla flavour many industries produces natural vanilla extracts, but they also produce artificial vanilla flavours. The quality improvement of natural vanilla flavour is an important measure of protection, assuring to costumers that the vanilla products they order contains the exact vanilla type and quantity specified on the label with no exceptions. In fact, the United States of America (USA) standard for natural vanilla extract requires the finished product to contain the extractable matter from 13,35 oz of vanilla beans per gallon in 35% of ethanol (BELAY & POOLE, 1993). In France, the National Syndicate of Food Aromatic Industry (SNIAA) has resumed in a text all regulations about natural vanilla flavour. There are some defined proportions that can be used as quality control parameter (***Table 1***) (SNIAA, 2014).

With these values it is possible the identification and the quality control of vanilla pods, also natural vanilla extracts. Because it is so costly, has a limited supply, his economy motivate frauds and as

synthetic vanillin is readily available, efforts are being made to detecting adulteration and to characterised natural vanilla flavours (SHARP, 2009).

2.4.3. Synthetic vanilla flavour

Synthetic or artificial vanilla flavour is produced without using vanilla beans. Synthetic vanillin is the only compound on, and It can be produced by biochemically or chemically. The vanillin compound utilized in these artificial flavorings is usually synthe¬sized from cheap raw material, such as guaiacol, eugenol, or lignin (A.G. HUESGEN (AGILENT TECHNOLOGIES), 2011).

Due to the high prices of vanilla beans and the hard work that its transformation represents, many flavouring industries have resorted to using synthetic chemical vanilla (vanillin). Vanillin that has been synthesized for the first time by Erlenmeyer in 1876 from eugenol, and from guaiacol by Reimer, is almost 20 times cheaper than natural vanilla extracts and the principal ingredient to produce vanilla flavour. Ethyl vanillin is another artificial pro¬duced vanilla compound that is three times more flavoring than vanillin (A.G. HUESGEN (AGILENT TECHNOLOGIES), 2011; HONG, JONES, & MCCONVILLE, 2013).

Chemical synthetic vanilla represents 97 percent of all vanilla flavour used commercially; nevertheless the specific aroma of natural vanilla is complex, and synthetic vanilla has not the specifics notes of this aroma (ETC GROUP, 2013). A list of names for artificial, synthetic, and imitation vanilla is presented in **Annex B.** (in accordance with regulations of EU); also the other chemicals names for vanillin are shown.

2.5. Regulations

The Food and Drug Administration (FDA) is the main regulatory body for the USA, and the EFSA for Europe. Regulations are created by many other organs private and governmental, they principal aim is to control the safety and quality in food and beverage industries through standards (GROCHOLL & SMITH, 2015).

There are not remarkable differences on definitions of "natural" between EU and USA:

- EU: "*Source material must be vegetable, animal, or microbiological. Must be produced by a traditional food preparation process*." (GROCHOLL & SMITH, 2015)
- USA: "*a material is deemed natural when it is derived from a product such as a spice, fruit, extract, oleoresin, or from a group of materials that the FDA recognizes as natural starting materials…*" (GROCHOLL & SMITH, 2015)

The most significant regulation in food flavouring industry in France is the Regulation (EC) n°1334/2008. This regulation is about "flavourings and certain food ingredients with flavouring properties for use in and on foods." (EUROPEAN PARLAMENT, 2008)

With regard to natural vanilla, Article 16.2 of this regulation says: *"The term "natural" for the description of flavouring may only be used if the flavouring component comprises only flavouring preparations and/or natural flavouring substances."* (EUROPEAN PARLAMENT & EUROPEAN COUNCIL, 2008) Hence, a natural vanilla flavour should only contain natural flavouring substances and/or flavouring preparations. In that context, vanilla extracts are flavouring preparations. Nevertheless, natural

vanilla extracts should have general exigencies stipulated for the SNIAA (*See also: Article 16.4 of Regulation (EC) n°1334/2008*), to avoid confusion of interpretation.

Vanilla preparations potency are measured by "folds", in accordance whit FDA's regulation. One gallon of a pure "single-fold" of vanilla extract, should be prepared with a minimum of 13.35 ounces of vanilla pods (ARUN K SINHA ET AL., 2008). Extracts can be concentrated, after that process, it is possible to obtain 2-fold until 40-fold vanilla extracts, which name is vanilla oleoresin (40X).

Considering that Sevarome exports vanilla extracts/flavours. It is always important to be at the forefront of international regulations that in most cases are adapted to FDA. Some regulations for vanilla flavour and vanilla extracts in the EU and the world, are summarized in **Annex C.** that is only a synthesis of interpretations of different texts.

2.6. Economic Aspects

Vanilla beans production is pretty expensive which explains the price of the natural vanilla extract, three times higher than artificial vanilla. Vanilla price, in the entire world, has experienced a massive spike, because of cyclones that has devastated much of the east of Asia. International vanilla market is completely opaque, year-to-year prices are nearly impossible to predict, importations and exportation's have become contradictory (LOEILLET, 2003).

Vanilla beans have a highly market price; it can be estimated from 150 to 200 thousand of dollars by a ton (COMITÉ ESTATAL SISTEMA PRODUCTO VAINILLA DE PUEBLA, 2010). The worldwide production of vanilla has change from 2,795 to 5,352 metric tons per year, between 1982 – 1999 years, with an average annual growth of 3.83%. Nevertheless, vanilla markets prices have always tended to fluctuate due to the existence of different factors as the producer and consumer.

Currently there are two important economic factors' which monopolies the market:

- A **dominant vanilla producer** is Madagascar. It leads both in terms of quality and quantity. The is so market vulnerable to political events, weather conditions or speculation that regularly affects the country; such as supply determined by small productions that are unfamiliar with developments in global demand.
- A **dominant consumer** is the United States, which plays a critical role in price settings.

Vanilla is widely appreciated on the European market for its versatility, from here the term "more than just" vanilla. France is the principal importer country from the Europe Union (EU) (258 tones) followed by the United Kingdom (151 tones) and Germany (144 tones). These countries represent three quarts of vanilla importation in EU (CBI, n.d.). Exportations from Madagascar, Indonesia and Comoros, are the most important international markets of vanilla (see ***fig. 5***).

Vanilla is an imperative ingredient in food industry. The worldwide vanilla market both natural and synthetic is considered around 650 millions of dollars. Two sectors are the largest users of vanilla, beverage and ice-creams. Demand in Europe remains strong, as vanilla (extract) is a very versatile ingredient with many applications. Consequently, many food and beverage manufacturers like to use it in their formulations including New Product Development. Nevertheless, because of the high demand situation and the high price level, synthetics vanilla flavours are often used instead of

naturals. In fact, 97% of vanilla applied as a flavour or fragrance is synthetic, making synthetic vanilla the largest substitute for natural vanilla in global contexts.

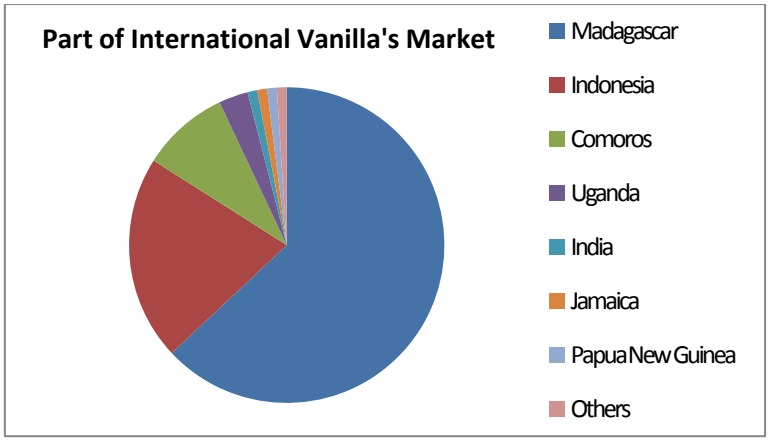

Figure 5: Distribution of International vanilla's market (Country/Territorial Notes, 2015)

Vanillin consumption is about 12000 – 15000 tons per year. Worldwide production of natural vanilla is about 2300 tons per year that only represents 50 tons of natural vanillin; it means 0.4% of vanillin global consumption. However, vanillin is not the only compound of vanilla, and all the other volatile aromatic compounds make that vanilla and, therefore, natural vanilla extracts, very important ingredients on the market.

2.7. Conclusions

This chapter has shown why vanilla is the flavour that people consume more in the world. It is the reason because food flavouring industries are improving quality of this product to **add value**. Vanilla flavour is a complex mixture of volatiles and non-volatiles compounds, is a flavour that has enthralled consumers and whose industry will continue to grow.

Vanilla production requires tropical conditions; which is probably the reason almost 80% of world production is made in Madagascar. The wheatear and hard work during vanilla production make of **vanilla an expensive raw material for food flavouring industries.**

The name "**natural flavour**" is even a **source of consumer confusion**, because of definitions and contemplations in regulation text. Hence, while vanilla flavour experiences strong and growing demand, within the larger category real vanilla is increasingly being substituted with synthetic vanillin. The actual situation is that some producers continue to use the name "natural vanilla" even if, they use synthetic vanillin, which is a **fraud**.

Currently, an urgent area of concern is the broad disparity between the global demand and the actual supply of natural vanilla flavour. There is a need to have a multi-pronged strategy in this direction to decrease fraud. Because the widespread adulteration of natural vanilla flavour is an area of concern, it is important aimed studies at devising effective authentication tools. Also, regulations should be stricter. High-quality vanilla flavouring will still be expensive because it uses an excellent quality vanilla beans and in considerable concentration. In fact, the demand for the natural vanilla is probably only decisive in the gourmet segment but is also important for nowadays consumers.

CHAPTER 3: VANILLA EXTRACTION

3.1. Introduction

Vanilla extract is defined as the solution in aqueous ethyl alcohol of the sapid and odorous principles extractable from vanilla pods (RASOAMANDRARY, FERNANDES, & BASHARI, 2013). It is widely used in food industry and the largest component of vanilla aroma is vanillin. It is mainly important in vanilla extracts and its quantity, in the final product, dependent on the nature of compounds, raw material to be processed, and extraction method.

The importance of vanilla is growing, especially in the food industry. It is important to improve the extraction method to a faster and economic extraction but also increasing flavour through vanillin extraction (A. SHARMA ET AL., 2006). Extraction and concentration are the previous steps before a chromatographic characterization and as the aim of this work is important to improve the extraction method. There are different extraction methods as distillation, maceration, percolation, supercritical fluids; each with various advantages, in this work ultrasonic and maceration extraction methods will be studied.

Ultrasound-assisted extraction (UAE) is an interesting process to obtain high-value compounds, it is considered as a “green extraction technology”. The main benefices of UAE are a more efficient extraction, thus saving energy, faster mass transfer, selective extraction, faster start-up and also the use of moderate temperatures which is beneficial for heat sensitive compounds.

UAE is the application of high-intensity, high-frequency sound waves and their interaction with materials, which improves material extraction. UAE has a mechanical effect in raw material, i.e. cavitations destroy plant material surface and molecules ending by one process faster extraction (RASOAMANDRARY ET AL., 2013). Different studies have shown that UAE can accelerate some biochemical process improving vanillin amount in vanilla extracts and oleoresins.

The current chapter shows a study carried out to optimise the extraction of vanilla extracts with the best yield of vanillin from the cured vanilla beans using UAE. Optimisation of the different operating extraction parameters, namely solvent concentration, frequency and time of extraction was made. Furthermore, the comparison of UAE with a conventional extraction method (maceration) was also investigated.

3.2. Extraction methods of natural vanilla Flavour

The term "aroma extraction" indicates the extraction of the specific aromatic compounds from raw materials, using to this fact conventional or emerging technologies. The etymology of the obtained product depends on its composition, specifically the ratio of the aromatic compound, which describes the aroma.

Vanilla extract is obtained by solid-liquid extraction (maceration, percolation, oleoresin, soxhlet) of vanilla pods in water-ethanol solution. The traditional extraction methods are long and, energy and solvent consuming. However, there is limited research on vanilla extraction and the main vanilla extraction methods namely the percolation method and the oleoresin method are the two most used extraction categories in the world.

Conventional's extraction process takes about 48 to 72 hours. During this process, vanilla beans are in contact with an organic solvent such as ethanol, methanol, chloroform, hexane, acetonitrile (ACN). *Etc*. Solvents improve the vanillin and other aromatic compounds extraction. Temperature is also important, the extraction is usually performed between 45-60°C, which can destroy some aromatic compounds.

Some of the conventional extraction methods are explained:

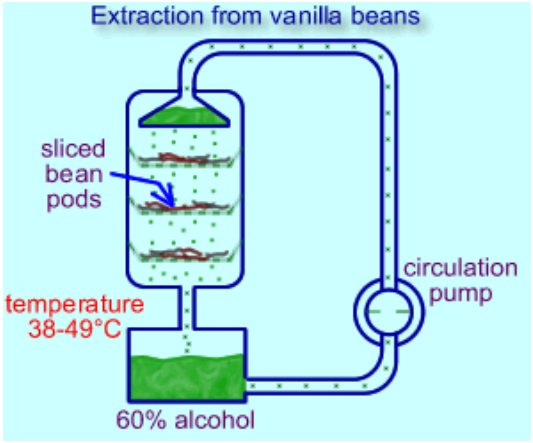

Figure 6: Extraction from vanilla beans by percolation method (GREENER-INDUSTRY)

Maceration: Is a process where the whole raw material is placed in a stoppered container with the solvent and allowed to stand a room temperature for at least three days with agitation until the soluble matter has dissolved (SWAMI, SINGH, LONGO, & RAKESH, 2008).

Another method is the hot water batch extraction, which is a maceration extraction with higher temperatures.

Infusions (another extraction method or approach) are obtained by this method.

When the process is finished, the mixture is strained, the cake is pressed and the liquid follows different process until obtaining the desired product.

Percolation (*see* ***fig.6):*** This is the most frequently process used in industries, because of its simplicity and efficiency. The method consists of a circulating mixture of ethanol and water (35 – 50% of alcohol during 48- 72 hours) which should result in a four-fold vanilla extract (ARUN K SINHA ET AL., 2008).

When the extraction is complete, the outlet of the percolated is opened and the liquid contained is allowed to drip slowly.

Green technologies

Currently, there are relatively few sources for vanilla beans; the price of the beans is affected dramatically by its biggest producer Madagascar. In light of this, a method of producing a natural vanilla extract that increases production, reduces processing costs (time) and/or provides a stronger vanilla aroma would be quite attractive to the flavoring industry (BARTNICK DANIEL D, THOMAS H, & MARK, 2005; RASOAMANDRARY ET AL., 2013), UAE could be a solution.

Over a period of many years ultrasound has be used as assisted extraction to improve extraction of specifics vegetables compounds for laboratory analysis. Several authors have investigated the effect of ultrasonic's during vanillin extraction in water-ethanol solutions. Nevertheless, there are not investigations about the effect of ultrasonic's in the other aromatic compounds presents in vanilla pods and the relation with the sensory quality of the final product.

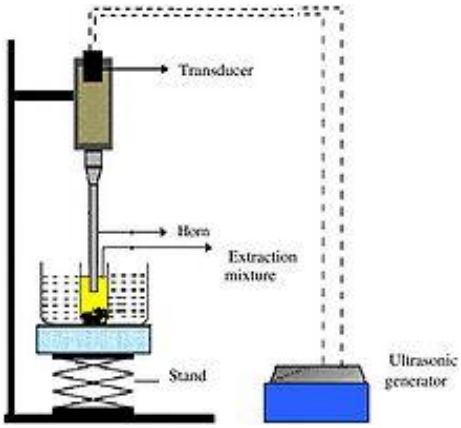

Figure 7: Typical Ultrasonic sound generator. (JADHAV ET AL., 2009)

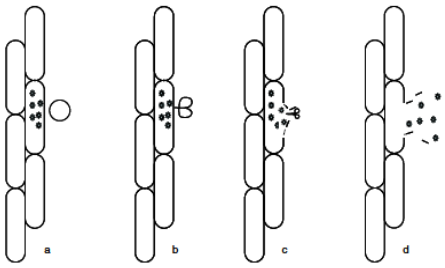

Figure 8: Cavitation effect on raw material surface.

Ultrasonic Assisted Extraction: UAE is a potential emergent technology that can accelerate heat and mass transfer, increase the rate of extraction, increase the yield of extraction components, Etc. It has been successively used in extraction applications include herbal, oil, protein and bioactive from plant materials (TOMAO & CHEMAT, 2010; VILKHU, MAWSON, SIMONS, & BATES, 2008) ***fig. 7*** shows a typical ultrasound system.

The fundamental effect of ultrasound on a continuum fluid is to impose an acoustic pressure (Pa) (PATIST & BATES, 2008). The acoustic pressure is dependent on amplitude (intensity), at higher intensities the local pressure cause tiny bubbles in the liquid. The bubbles begging to grow, until implosion during a single compression cycle, this phenomena is called cavitation and is the most significant effect during UAE process ***fig. 8*** *and* ***fig. 9*** shows the cavitation phenomena.

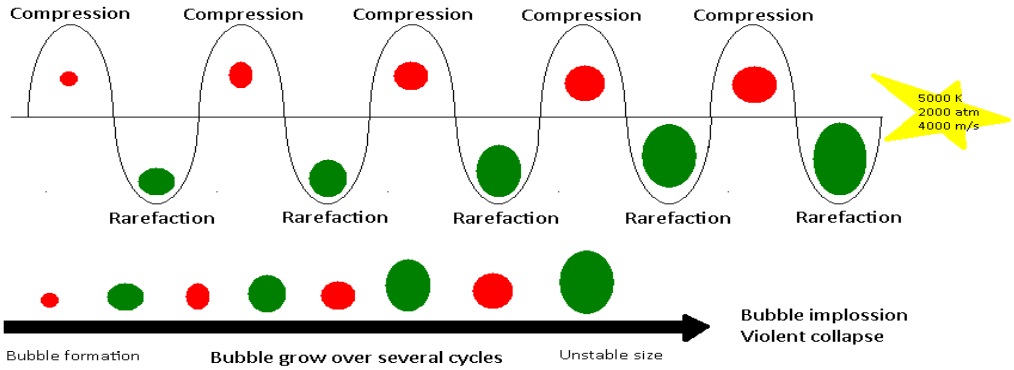

Figure 9: Cavitation phenomena. Grow up of bubble and implosion during a compression cycle. (HELSCHER, 2015)

Temperatures of 5000 K and pressures of up to 1000 atmospheres are the conditions within these imploding bubbles can be dramatic (HIELSCHER, 2015). They produce very high shear energy waves and turbulence in the cavitation zone, causing cellular destruction on the surface of the vegetal raw material. Thus, it is possible to extract the compound of interest (see ***fig. 9***).

The correct combination of the most important factors affecting UAE (Intensity, temperature, organic solvent concentration, and the proportion raw material: solvent) improves solvent penetration into the plant body. It can break down cell walls by itself, so a faster extraction and best yields of extracted compounds can be made.

Due to the quantity of parameters affecting UAE it takes time and effort to develop and fine-tune the extraction process (specifically of vanilla aroma) by this technology. The main objective is to achieve the maximum result (yield of the mean compounds, sensory profile, and extraction efficiency) and minimize the amount of energy required during the process for the commercial applications (faster process).

3.3. Optimization of the extraction method

Different approaches were applied to evaluate the efficiency of UAE for vanilla flavor (aroma) extraction:

1. The first one consisting in the evaluation and comprehension of the most important factors affecting UAE (specifically vanillin extraction from vanilla pods), so an experiment design was performed.
2. The second one, a comparison between UAE (using the best parameters of extraction found in the first approach) and maceration extraction.
3. The third approach is an optimization of the extraction method using UAE. In this one, the sensory profile is one of the additional objectives, because of its importance in the global quality of vanilla extracts.

First approach: *Experiment design*

Response Surface Methodology was used to explain the effects of the most important factors affecting UAE. The intensity (% Amplitude), pulsations, temps of sonication, solvent concentration, and raw material/solvent proportion, on the instrumental measurements, were analyzed. A central composite design was achieved and 50 experiments were realized during three weeks.

The responses measured were vanillin percentage, 4-Hydroxybenzaldehyde percentage, extraction rate, speed extraction and extraction yield (*see* ***Table. 2***). They were analyzed with ***Design-Expert 6***.

Table 2: Conditions for Experiment Design

	MINIMUM	MAXIMUM
AMPLITUD	50%	100%
PULSATIONS	50%	70%
TIME	10 min	90 min
ALCOHOL PERCENTAGE	35%	95%
g VANILLA/50ml OF SOLVENT	10g	15g

The alcohol used for all experiments were food grade (96.2%) and it was purchased from SAD Alcogroup (Société des alcool Dénaturalisés, France (28)). Fresh vanilla pods were used to extraction (Bourbon quality, vanilla beans from Madagascar). Vanilla pods were cut to obtain little pieces of 1 cm X 0.5 cm X 0.2 cm.

An extractor equipped with ultrasonic horn transducer (Ultrasound UP200St of Hielscher company (Germany)), working at 26KHz and 200Watts input power with amplitude range and sample temperature being monitored up to 60°C was employed for UAE.

Second approach: *Comparison between UAE and Maceration*

UAE and maceration extraction were compared. The comparison criteria were the speed of the extraction of vanillin, as the most important aromatic component in the vanilla flavour. The extraction methods are summarized in ***Table 3***.

Table 3: UAE method and maceration method: Extraction conditions.

UAE	Maceration
12g sample of fresh vanilla pods were diluted in 22g of water: ethanol solution (67% ethanol) and treated.	
Amplitude: 95% Pulsations: 50% Maximal temperature: 55°C Time:1.25h or (1h15min)	Temperature: 50°C Agitation Time: 47.26h or (47h15min)
The final product is called first juice The extract is filtered and separate of the moisture. 17g of water: ethanol solution (60% ethanol) is added and the extractions process continues.	
Amplitude: 95% Pulsations: 50% Maximal temperature: 55°C Time:1.50h or (1h30min)	Temperature: 50°C Agitation Time: 21.83h or (21h50min)
The final product is called second juice The extract is filtered and separate of the moisture. 20g of water: ethanol solution (40% ethanol) is added, and the extractions process continues.	
Amplitude: 95% Pulsations: 50% Maximal temperature: 55°C Time:1.25h or (1h15min)	Temperature: 50°C Agitation Time: 22.50 or (22h30min)
The final product is called third juice. In theory with the mixture of 3 juices, we should obtain about 60g of extract that correspond a two-fold [1] extract. The final product should contain 0.20g (0.33%) of vanillin.	

The operation conditions of UAE were selected as the best results obtained in the first approach. Also, the results of the first approach were necessary to understand the factors which influence UAE.

Third approach: *Optimization of UAE (sensorial importance on the final product)*

Vanilla flavour is a complex mixture of many molecules which gives the specific aroma. First and second approaches have as principal objective demonstrate that UAE is an effective emergent technology in terms of reduction of time and energy during the extraction process. Nevertheless, vanilla flavour for the industry has another interest more than the extraction of a significant yield of vanillin. It is the reason is important to optimize the extraction method, to obtain an exquisite sensory quality product with all the advantages that give the emergent technology. This approach won't be deepened in this work.

[1] One fold of vanilla extract should contain the extractable material of 13.35 ounces of vanilla pods at not more than 25% of moisture content, for a gallon of extract.

CHAPTER 4: Method development for vanilla flavor analysis

4.1. Introduction

The identification of the chemical components of vanilla is increasingly important (CICCHETTI & CHAINTREAU, 2009b). The main compounds that have been identified are vanillin (1), 4-hydroxy-benzaldehyde (2), vanillic acid (3) and 4-hydroxybenzoic acid (4) (*See **fig. 7***) (A.G. HUESGEN (AGILENT TECHNOLOGIES), 2011; MARUENDA ET AL., 2013; RAMACHANDRA RAO & RAVISHANKAR, 2000). Vanillin (4-hydroxy-3-methoxybenzaldehyde), is the main chemical compound in vanilla pods, and that has been attributed a third of the flavour and aroma (CID-PEREZ, 2011).

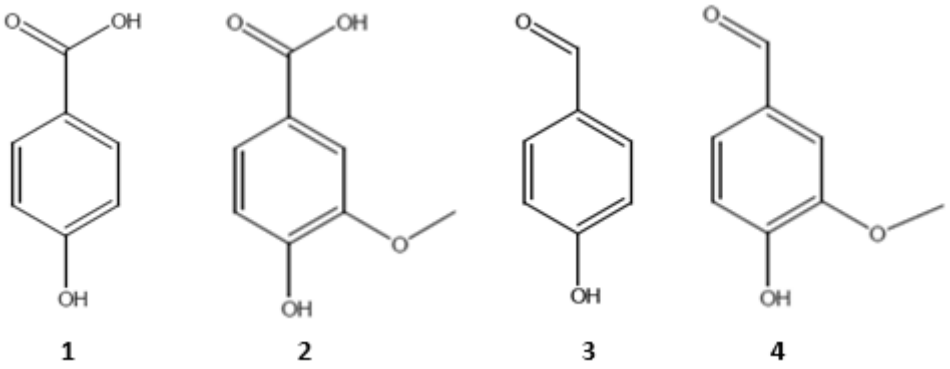

Figure 7: Molecules of the main compounds of vanilla flavour (MAHASSEN, SANDRINE, & LAURA, 2004)

Several chromatographic methods for identification and quantification of chemical compounds have been used in vanilla. HPLC is one of the most powerful and preferred techniques for quantifying organic molecules because of its simplicity, sensitivity, precision and selectivity (SHARP, 2009).

Chromatographic methods are distended of the other separation techniques because the contact of two immiscible solvents. The stationary and the mobile phase, that with the pass through a column, improve the differences of physical and chemical proprieties of the compounds in a sample and their gradual separation.

The correct operation of the instrument together with an analytical development and a validation of the analytical method, are essential conditions to guarantee the quality of results generated by the system.

The large numbers of factors that influence the success of a chromatographic analysis, make of the development and validation of these methods a complex work; so validation should provide information about reproducibility and repeatability of the method. In the same way, it should consider the external factors causing variations of results.

The development and validation of the analytical method to the characterization of natural vanilla extracts has been developed by the Good Laboratory Practice and the specifications of the IUPAC (ICH, 2005). An accurate, repeatable and precise method was obtained, in the same way, calibration curves were created to analyze real samples obtained by different extraction methods.

4.2. High Performance Liquid Chromatography (HPLC) for vanilla extract analysis

HPLC is a separation technique that involves different factors as flow, temperature, and pressure and injection volume.

The main objective of a chromatographic is to separate the compounds in a solution through a column (called stationary phase) and a mobile phase that takes every compound according to an affinity. The sample is carried in a narrow band from the top of the column when the compounds in the sample mixture will have different preferences for the stationary or the mobile phase. The time of retention in the column depends on that difference. The components are carried by the mobile phase down the column one by one. A detector is then used to respond to a physicochemical property of the analyte; this response is digitally amplified and sent to a data system where it is recorded as the chromatogram (AA, 1999).

4.2.1. The effect of experimental conditions in vanilla compounds separation

Every HPLC analysis should have an adequate resolution (Rs) between adjacent peaks of interest. There are three fundamental parameters that describe Rs efficiency. A basic resolution equation (**Eq. 1**) for isocratic separation can be useful to explain the importance of chromatographic conditions in compounds separation:

$$R_s = \underset{\boldsymbol{i}}{0.25N^{0.5}} \; \underset{\boldsymbol{ii}}{[k_1/(1+k_1)]} \; \underset{\boldsymbol{iii}}{(\propto -1)} \qquad \textbf{(Eq.1)}$$

Where:

i, ii, iii are the three fundamental parameters
N is the column plate number
K_1 is the retention factor, *K*, for first peak
α is the separation factor (selectivity)

Hernandez Perez (2005), has recommended the optimization of the three fundamental chromatographic parameters to improve the resolution.

(i) Dolan & Snyder (2013) recommended using a C_8 or C_{18} column with a column plate number range of 5000 ≤ N ≥ 20000. N determines the separation efficiency and can be calculated as follows (**Eq. 2**):

$$N = 16\left(\frac{t_R}{W}\right)^2 \qquad \textbf{(Eq.2)}$$

W represents the peak width, and t_R is the retention time of the peak. The peak width can be modified by the HPLC program (from 1s to 0.05s), but W is also quite affected by the solvent flow, so smaller W gives greater N. When N is higher, the resolution is better because a change in N corresponds to a twofold change in resolution.

(ii) Dolan & Snyder recommended a range of 2 <k> 10, to obtain a good resolution. The modification of the mobile phase strength can modify the retention factor (*k*), the temperature can be useful to improve the elution of compounds. Nevertheless, the retention time and W can slightly change (**Eq. 3**).

$$k = \frac{(t_{R2} - t_{R1})}{t_0} \qquad \textbf{(Eq.3)}$$

(iii) Selectivity (**Eq. 4**) defines the spacing of two peaks; Improve this factor is possible with a modification of the mobile phase (polarity, temperature. Selectivity can be modified at the same time that *k*, because of the dependence of one to the other (see **Eq. 3**).

$$\propto = {}^{k_2}/_{k_1} \qquad \textbf{(Eq.4)}$$

The solvent conditions, defined by Dolan, *et.al,* which modify selectivity, are summarized in ***Table 4***. There are expressed in terms of *Orthogonal power* (OP). If OP > 0.1; a significant change in selectivity will occur.

Table 4: Orthogonal Power for selectivity improvement

VARIABLE	Change	Example	Orthogonal Power
%A (organic solvent)	10%	50% to 60%	0.08
Temperature	20°C	35°C to 45°C	0.07
ACN (Methanol (MeOH)	To MeOH (ACN)	Replace ACN by MeOH or vice versa	0.2
pH	5 units	pH 2.5 to pH 7.5	>>0.7

4.3. Method development

Preliminary runs to analyse vanillin and the other phenolic compounds in vanilla extracts using the HPLC method of Huesgen (2011) revealed that even if is possible to obtain a good resolution, the separation and in effect, the quantification of the main constituents in vanilla extract is not possible in the laboratory conditions of Sevarome.

Waliszewski, *et al*. (2007) and Barry Lavine *et al*. (2012) reported a simple method using water rich mobile phase to quantify vanillin in vanilla extracts. Some parameters of these methods have been taken to obtain the best working conditions to characterize a natural vanilla extract and to get the separation of all compounds.

The study of the different parameters in chromatography was made in function of the goals of this study. The available reported methods are summarized in **Annex D.** A number of methods have used a mobile phase in gradient elution; nevertheless, Waliszewski *et al.* (2007) uses an isocratic method, chosen as the best way of separation for this study.

Flow rate commonly used at the mobile phase by other authors was between 0.6ml/min (ARUN KUMAR SINHA ET AL., 2007) to 2ml/min (MARUENDA ET AL., 2013). In fact, previous essays have shown that the elution time, for vanillin, for example, varied from 2 min (WALISZEWSKI, PARDIO, & OVANDO, 2007) to 14.29 min (MARUENDA ET AL., 2013). Temperature is an important parameter too, and some authors were considered at 30°C (A.G. HUESGEN (AGILENT TECHNOLOGIES), 2011; CICCHETTI & CHAINTREAU, 2009b). The sample volume injection was varied from a method to another from 3µl to 10µl; however, it can change in function of column and flow.

Following the logic of the results of preliminary runs, modifications to the initial method were made. The influence of gradient elution, temperature, injection volume and flow were analyzed. Also, the optimum wavelength was analyzed.

Goals: To understand and to success in an analytical method development, it is necessary to begin by the definition of objectives. The goals will help to understand in a systematic way the most important parameters, which will be modified, during the different phases of the project.

The definition of the goals consists in respond different questions as is showing in the **Annex E.** In that context characterized and quantified the main constituents of a vanilla extract were the essential goal to develop this method. Alike, the method should demonstrate a **good resolution** and **separation** of the chromatogram peaks; **linearity**, **accuracy**, and **precision**.

This method will be used as a **quality control of vanilla extracts**, as well for the creation of a most complete product data sheets.

The most important aim of this method development is separate and quantifies the main constituents of a natural vanilla flavour produced by Sevarome.

4.3.1. Reagents and materials

a. *Reagents:* Methanol use was gradient grade (99.9% based on GC) and was purchased from Merck; ultrapure water and other solvents HPLC grade were purchased from Merck. Trifluoroacetic acid (TFA) for spectroscopy (Merck) was used for acidified water in the mobile solution. Solvents were filtered through 0.45µm glace filter (Fisher Scientific) and were degassed prior to use.

   The standards:

   - Vanillin 97% of purity (Rovanil® Natural)
   - Vanillic acid 95% of purity (Prodasynth)
   - 4-Hydroxybenzaldehyde 98% of purity (Prodasynth)
   - 4-Hydroxybenzoic acid 99% of purity (Sigma-Aldrich)

   Certified Reference Material (For validation step):

   - Vanillin 99.7% of purity (Sigma-Aldrich)

   Vanilla beans, extraction grade which precedence is Madagascar.

b. *Materials:* An HPLC Agilent 1260 Infinity Binary LC System consisting of the following modules was used for the characterization and quantification of the main constituents in vanilla extract:

   - Agilent 1260 infinity Binary Pump
   - Agilent 1260 Infinity Vacuum degasser
   - Agilent 1260 Infinity auto sampler and thermostat
   - Agilent 1260 Infinity Diode Array Detector (DAD) with 10 nm max-light flow cell

   The column used was an Agilent ZORBAX Eclipse Plus C18 (4.6 X 150mm, 5µm) (Agilent technologies).

   Analytical balance:

   Precisa
   Model: XB 1220M
   Precision: 0.001g

   Potentiometer:
   Mettler Toledo

4.3.2. Experimental

Preparation of standards

Stock solutions of individual compounds were prepared separately to perform the development and validation test. The desired concentration range was obtained for a dilution series (A.G. HUESGEN (AGILENT TECHNOLOGIES), 2011) *(see **Table 5**)* from stock solutions.

The serial dilution was used for standard curve preparation. For the stock solution and all others dilutions, a mixture of methanol – distilled water (1:1) was used as solvent for all sample preparation.

Table 5 : Standard solutions

Compound	Stock µg/ml
Vanillin	4620
4-hydroxybenzaldehyde	4510
Vanillic acid	4590
4-hydroxybenzoic acid	4570

Table 6: Flow conditions during HPLC analysis

TIME	FLOW (ml/min)
0 min	1
3 min	1.5
6 min	1.5
7 min	1

Sample preparation

The external calibration was run with solutions of the four target analytes in water: methanol solution. They were mixed at different concentrations to obtain an artificial preparation equivalent to the natural preparation *(See **Table 1** in **Chapter 2**).*

Real Life extract

The vanilla extracts were prepared using the recently developed UAE (**Chapter 3**). Vanilla Extract was prepared in the same solvent that Stocks solutions. 50µl of natural vanilla extract (AVN 3286, Extrait pure de Vanille Gousses, Sevarome) was diluted in 4950µl of water/methanol (1:1) to obtain a solution vanilla/solvent (1:100).

Sample and standards were filtered through a 0.45 µm syringe filter (Titanic RC, Fisher Scientific) before analysis.

Chromatographic conditions

A mixture of methanol (99.9% of purity) and acidified water (0.1% TFA, pH 2.0 – 2.3) were used as mobile phase, as recommended by different previous works (MARUENDA ET AL., 2013). Even if the composition of the mobile phase has not changed in the analysis time, the flow has changed during elution time (***Table 6***) in order to improve resolution (R_s) and column plate number (N).

The mobile phase was circulated through the column at the starting conditions for 10 minutes before analyze with the aim of equilibrating the column. 4µl of the sample was injected with an isocratic mobile phase composed of methanol (solvent A), water (Solvent B, 0.1%TFA, pH 2.3) during 7 minutes. The temperature for the analysis was 35°C with a variation in pressure (influenced by the flow). The detection wavelengths were: 230 nm for vanillin, 260 nm for 4-hidroxybenzoic acid and vanillic acid, and 280 nm for 4-hidroxybenzaldehyde (A.G. HUESGEN (AGILENT TECHNOLOGIES), 2011; MARUENDA ET AL., 2013; ARUN K SINHA ET AL., 2008).

Optimization

The global overview of each equation (**Chapter 3 4.2.1**) that describes the resolution of chromatographic separation has allowed for the optimization of the chromatographic method. Factors as temperature, elution time, flow and composition of the mobile phase were analyzed.

The experimentations with the first analytical method of Agilent shows that the mean compounds of vanilla are not separated completely. Each factor, summarized in ***Table 4*** was studied to obtain a good resolution and separation.

ACN has been changed to methanol, this one recommended for analysis of rich water samples, different solvent proportions were tried. The final mobile phase was methanol and ultrapure water (0.1% TFA). TFA improves separation and resolution of compounds; that is why it is imperative to acidify water of mobile phase.

Determination of three different wavelengths was made to obtain the optimal absorbance for each compound. The method has been validated for the parameter of identification and quantification as recommended by the IUPAC; validation is described in the next chapter.

CHAPTER 5: METHOD VALIDATION

The IUPAC and the ICH have developed an appropriate methodology to validate of methods. They present a discussion of the characteristics that should be considered during the validation of analytical procedures. So it is possible to design the experimental work with this guideline and provide a sound, overall understanding of the capabilities of the analytical method. For instance: Specificity, linearity, range, accuracy, and precision (ICH, 2005) see **Table 7**.

Table 7: Required Specified Parameters for ICH assay categories and specification codes

	Required specified parameters for ICH categories			
Specification code	1	2	3	4 and 5
ICH Category	**I**	**II**	**III**	**IV**
	Identification test	Quantification of impurities	Qualitative limit test for impurities	Quantification of active ingredients
Accuracy	NO	YES	NO	YES
Repeatability Precision	NO	YES	NO	YES
Intermediate Precision	NO	YES	NO	YES
Specificity/Selectivity	YES	YES	YES	YES
Linearity	NO	YES	NO	YES
Assay Range	NO	YES	NO	YES
Limit of Detection	NO	NO	YES	NO
Limit of Quantification	NO	YES	NO	NO

5.1. Selectivity

Selectivity is the ability to assess the analyte unequivocally in the presence of components which may be expected to be present. Selectivity is necessary to ensure the identity of the analyte.

To determinate the selectivity of the system we proceed to prepare the stock solutions and the sample by the preparation process described in the **Chapter 4** paragraph **4.3.2**.

The selectivity is determined by injection of the standards and observing the separation of the different compounds on the chromatogram. In the same way, in terms of quality control and for all quantitative analysis peak purity is an important task. In fact an essential requisite of a separation analysis is the ability to verify the purity of separated species which allow to ensure that not co-eluting or co migrating impurity contributes to the peak's response (STAHL, 2003).

5.2. Linearity

The linearity is the range of concentrations where exist a linear relationship between these and the analytical signals. In this range, the values should submit an acceptable level of precision and accuracy, thus, can not be below the limit of quantification.

Linearity is evaluated with the correlation coefficient (r), which should be higher than 0.999.

The linearity has been determined for the four standards solutions at different concentrations (see **Table 8**). To create the calibration curves, prepare the standards in accordance to the **Chapter 4** paragraph **4.3.2**.

Table 8: Dilution series

Concentration / Compound	1 (µg/ml)	2 (µg/ml)	3 (µg/ml)	4 (µg/ml)	5 (µg/ml)	6 (µg/ml)	7 (µg/ml)	8 (µg/ml)	Stock (µg/ml)
Vanillin	5.77	11.5	23.1	46.2	92.4	138.15	184.3	231	4620
Vanillic Acid	2.05	5.63	11.27	22.55	33.82	45.1	90.2	-	4510
4-Hydroxybenzaldehyde	2.29	4.59	9.18	18.36	22.95	45.9	91.5	183.6	4590
4-Hydroxybenzoic Acid	2.28	5.71	11.42	22.85	34.27	45.7	114.25	-	4570

Each dilution was made with a water: methanol solution (1:1) and at different dilution factors.

5.3. Precision of areas

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions (ICH, 2005; UNDOC, 2010).

- Repeatability: 9 determinations covering the specified range of each compound was made. Three concentrations of the linearity range were chosen and injected triplicate. Standard deviation (SD) and relative standard deviation (RSD) were measured in the confidence interval as recommended by the ICH guidelines for validation.

- Intermediate precision was determined as a part of accuracy. Standard deviation (SD) and relative standard deviation (RSD) were measured in the confidence interval.

5.4. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy may be inferred once precision, linearity and specificity have been established.

Bias can be determined quite reliably, because the reference values of CRM-s are generally quite reliable. The accuracy of the method was determined with a Certified Reference Material (CRM) of vanillin purchased from Sigma-Aldrich (99.7% of purity). 3 determinations were made in the working conditions recommended by supplier.

For the other compounds (**2, 3, 4**) accuracy was done by the recovery method (ICH, 2005; SABLIER & FEINBERG, 2012) where extracts of raw vanilla were spiked by adding each compound at 3 different known concentrations, and compared with vanilla extracts without the added compounds. For the each level of concentration three analysis replications were performed resulting in 54 analyses and the control extract of raw vanilla.

The residuals for each concentration were calculated with the eq. 5.

$$\%R = \frac{F-I}{A} * 100 \qquad \textbf{(Eq.5)}$$

Where:

F is the concentration of the spiked analyte
I is the concentration of the unspiked analyte
A is the concentration of the analyte added to the spiked portion

The spiked solutions were prepared as the stock solutions and were added to an ordinary test portion that was analyzed alongside an unspiked test portion. This kind of recovery is called "surrogate recovery". The difference between that two results is the recovered part of the added analyte, which can be compared with the known amount added (MENDITTO, PATRIARCA, & MAGNUSSON, 2007).

5.5. Limit of detection (LOD) and limit of quantification (LOQ)

These parameters are related to the sensitivity of the method. The limit of detection (LOD) is usually defined as the lowest quantity or concentration of a component that can be reliably detected with a given analytical method. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

The quantification limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

The noise of baseline is previously determined as 0.1, so purity factor and other calculations are made with this value.

5.6. Statistical Analysis

All the analysis was carried out in triplicates in order to verify the reproducibility of the experimental data obtained. The data were analysed using Excel (Office pack 2007). Descriptive statistic were use to analyse the results. Linear regression analysis was used to obtain coefficient of determination (R^2) and to develop the prediction models for each compound, in order to determinate the concentration.

Analysis of variance (ANOVA) was also performed to verify the hypotheses of linearity.

Precision and accuracy are expressed in terms of %RSD and recovery percentage, respectably.

Statistical t-student was calculated to prove the hypotheses of linearity and also to determinate accuracy.

CHAPTER 6: Result and discussion

6.1. Vanilla Flavour Extraction

The results of the two different approaches studied show that UAE is an efficient alternative to improve vanillin extraction, compared with a conventional extraction method (maceration).

The results of the experimental design show a remarkable dependence of amplitude and time of sonication during UAE in vanillin percentage on final extract (see ***fig. 8***). The other factors, which also were studied, pulsations, the percentage of organic solvent and ratio between raw material/solvent had not a significant influence in vanillin percentage.

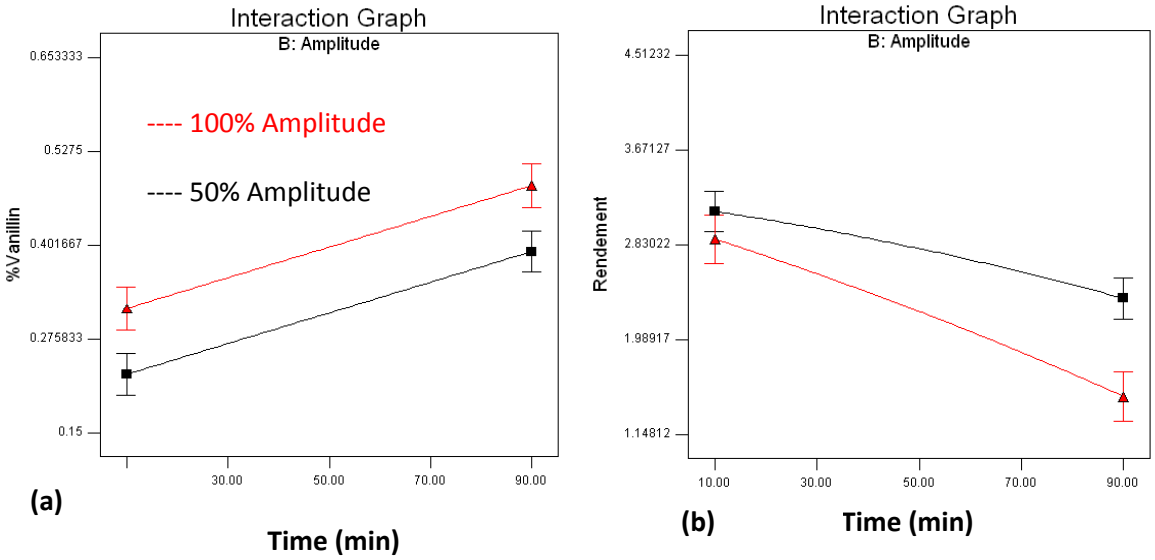

Figure 8: (a) Interaction graph of amplitude and time on % Vanillin (Time in minutes, amplitude in percentage). **(b)** Effects of amplitude and time of sonication on yield extraction (Rendement)

About the other responses such as 4-Hydroxybenzaldehyde percentage, extraction rate, speed extraction and extraction yield were analyzed, but only extraction rate and extraction yield were statistically significant.

Extraction rate is significantly affected only by the raw material/solvent ratio, which is logic because of the quantity of extractable compound (vanillin) present in raw material (vanilla). The best-determined ratio was 12.5g of fresh vanilla pods in 50ml of solvent (water: alcohol).

Extraction yield is highly affected by amplitude and time of sonication (see ***fig.* 9**). In fact, this effect is caused by solvent evaporation that couldn't be controlled during the extraction process. A close extraction system should be used to avoid solvent evaporation during the extraction process.

The optimal conditions that had been determined are a sonication period of 50 min with 85% of amplitude, 60% of sonication (5s on followed by 5s off), 65% of alcohol and 12.5g /50ml (raw material/solvent). The resulting extract containing 0.35% of vanillin, 3.52 g extract/g vanilla pods and 1.32% of extraction yield.

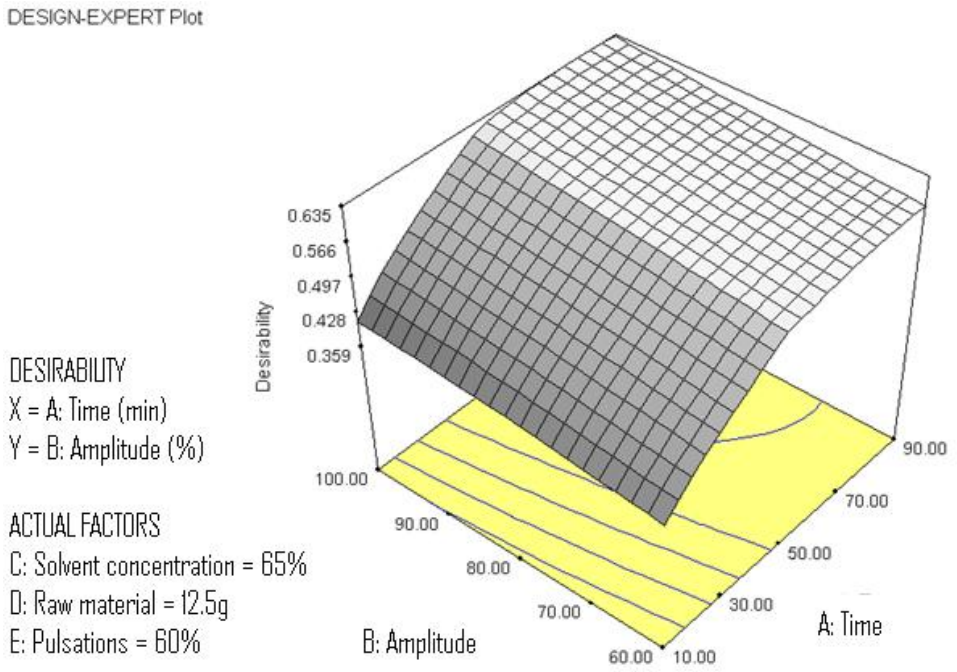

Figure 9: Surface response to obtain the best results in terms of desirability (% vanillin, extraction yield and extraction rate).

Using ultrasonic for extraction purposes in high-cost raw materials, as vanilla pods, can be considered as an economical alternative to conventional extraction processes. Nowadays it is an industry demand for sustainable development.

The second approach, a comparison between traditional extraction and UAE demonstrate that UAE is more efficient than maceration extraction. The mass transfer is better and the extraction time is reducing (see ***Table* 9**) when using UAE.

Table 9: Comparison between traditional extraction method and UAE

For 100g of vanilla pods	Maceration extraction with agitation	UAE without agitation
Alcohol	262 g (327 ml) 96.2%v/v	
Water	209.92 g (209.92 ml)	
Raw material Vanilla /Solvent (g/g)	0.20	
Extraction rate (vanillin)	1.61%	1.30%
Extraction Yield (g extract / g vanilla)	3.94	3.52
Extraction time	91.59 hours	4 hours
speed extraction (g vanillin/hour)	0.002	0.872
% vanillin	0.41%	0.37%

With UAE is possible to extract 0.16g of vanillin during 4 hours at 95% of amplitude and 50% of pulsations, which compared with maceration is equivalent to 73 hours of extraction. It represents a reduction of extraction time of 94%. A study made by Dnyaneshwar Jadhav *et. al* (2009) shows a positive effect on vanilla extraction using UAE. UAE compared with Soxhlet extraction provides high extraction of vanillin in much shorter time periods (*see* ***fig.* 10**)

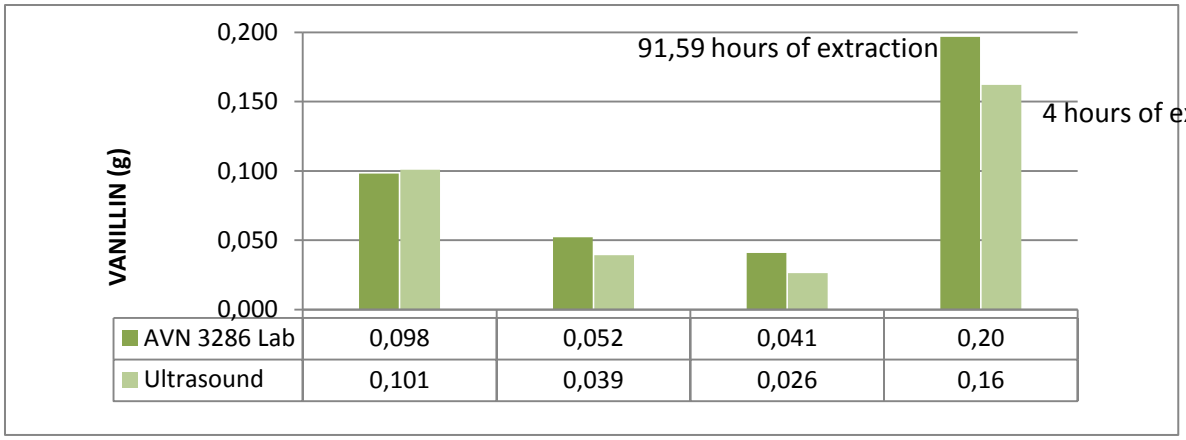

Figure 10: Comparison between UAE and maceration extraction.

Noemi Rasoamandrary *et. al* (2013), has demonstrated that UAE is an effective technology to improve vanillin amount yield, under optimal conditions.

6.2. Method development for HPLC separation of vanilla flavor compounds

For the development of the HPLC separation method, initially ACN (0.09% TFA) was used as the organic solvent in the mobile phase, using the method developed by Agilent Technologies (2013). Nevertheless, separation of the main compounds of vanilla extract is tedious due to a similarity in the tr (retention times) of them. Functional groups as –OH, -CHO and -COOH have pKa values between 3.51-9.82 (ARUN KUMAR SINHA ET AL., 2007), which difficulties separation.

In an attempt to develop an efficient mobile phase ACN was changed by Methanol, which has weaker elution strength but improves separation, in that case, and ultrapure water (0.1% TFA). The addition of acid was based on the fact that it improves the peak tailing of solute by lowering the pH.

The choice of organic solvent and column becomes crucial to achieving an efficiency separation. That is the reason the column (ZORBAX Eclipse Plus C18) was used during the development phase. That column has been used by other authors HUESGUEN et.al (2013) and has a large operation range of pH (2 -9) and temperature (until 60°C). It has showed an excellent separation in operation conditions and has not changed during the method development.

Table 10: Differences between Agilent method and developed method

	AGILENT	DEVELOPED METHOD
Organic solvent	ACN 0.01%TFA	MeOH
Ultrapure Water	0.9%TFA	0.9% TFA
Flow	1ml/min	1 – 1.5ml/min
Temperature	30°C	35°C
Injection volume	3µl	4µl
Gradient/Isocratic	Gradient	Isocratic

The detection was made with a DAD at 230nm, 260nm and 280nm as recommended by (WALISZEWSKI ET AL., 2007). The spectres of compounds are showed in **Annex F.**

To better understand the factors that contribute to better separation of compounds, it was necessary to examine equations showed in **Chapter 4** (**4.2.1**). The variation of different factors was made in function of attended the maximum value in each equation.

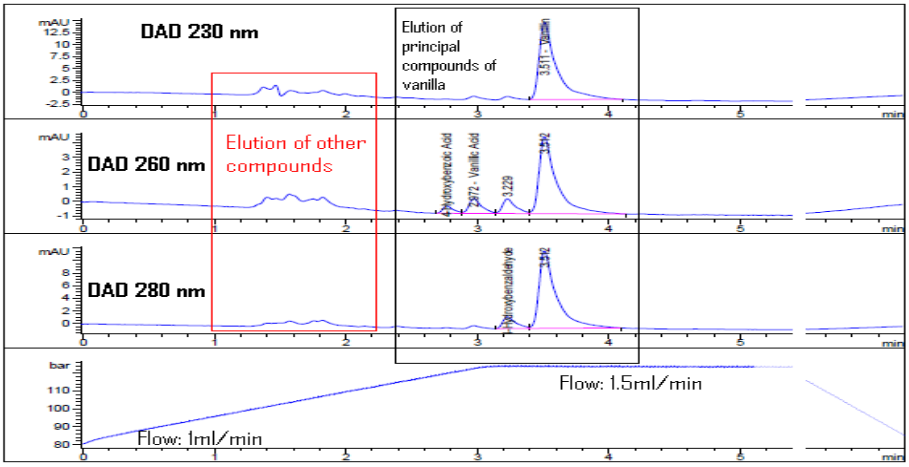

Figure 11: Influence of pressure in compounds elution

To obtain the best separation of the interest compounds in the lowest time the flow was varied between 1-1.5ml/min (see: ***fig. 11***). At the first minutes of the analysis there is an elution of other compounds presents in vanilla. A reduction of 3 minutes of the required time to carry out the analysis has been evidenced because of variation of the pressure. Also, the temperature was evaluated between a range (25-35°C) the best resolution and lower t_R was 35°C. **Table 10** shows some differences between Agilent method and the developed method.

6.3. Method validation

6.3.1. Selectivity

For this analyse the standard solutions, at known concentrations, and the sample were analyzed. The operation system was considered selective because there are not interferences with the compounds of interest on the chromatogram, the baseline has not a significant noise, and the purity of peaks were >95%. Therefore, the peaks have not interferences and could be considered as pure.

For this analysis, a diode array detector was used at three different wavelengths (230nm, 260nm and 280nm) in, accordance to (KUMAR & BALAMOHAN, 2013). Individual chromatograms of each compound show the retention time on the column in addition to an excellent resolution and purity (*see* **Annex G.**)

There is a separation of the four compounds presents in natural vanilla extracts; peaks are detected between the second to the fourth minute (*see* ***fig. 12***).

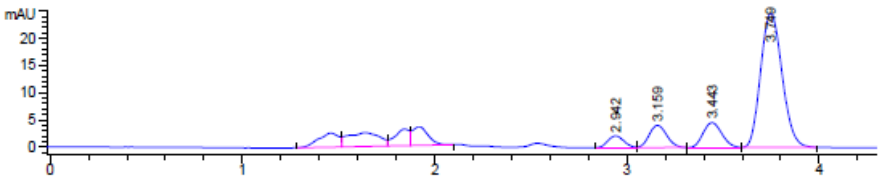

Figure 12: Chromatogram of a natural vanilla extracts, separation, quantification and identification of vanilla compounds.

Before 2 minutes, there are peaks corresponding to solvent and other compounds without interest in this analysis. These peaks do not have interference in the interest compounds response. An individual injection of the blank sample was made to verify the non-interference with the signal.

6.3.2. Linearity

Linearity was evaluated by linear regression across the range (*see* **Table 8** of the **Chapter 5**) of the analytical procedure and by the detector response factor method. A visual inspection of a plot of signals as a function of analyte concentration was realized and, the linear regression showed a linear relation between the area (x) and concentration (y).

To obtain linearity between assays and sample concentrations the test data may need to be subjected to adjustment of range, according with the Beer Law. The test results were evaluated by appropriate statistical methods, as follows.

The equation (**Eq.6**) that describes the model relation is:

$$y = bx + a \qquad \textbf{(Eq.6)}$$

Where:

y: analyte concentration
x: peak area
b: slope
a: intercept on axe *y*

To find *b*: $b = \frac{\sum x_i y_i - \frac{\sum x_i \sum y_i}{n}}{\sum x_i^2 - \frac{(\sum x_i)^2}{n}}$ **(Eq.7)**

To find *a*: $a = y - bx = \frac{\sum y_i - b \sum x_i}{n}$ **(Eq.8)**

To analyse the linear regression, is important to interpret statistically the correlation coefficient (r) which indicates the grade of relation between the factors "x" area and "y" concentration.

The determination coefficient (r^2) is the square of the correlation coefficient. R^2 indicates the total variability of *y*, for validation methods it should be higher than **0.997**.

Hypothesis test for the correlation coefficient:

Ho: there is not a relation between *y* and *x*, r = 0
Hi: "r" is not significantly different to 1 and there is a linear relation between the factors.

Acceptation criteria: If the "t" experimental value is higher than theory "t" of the table, calculated to (n-2) freedom degrees and a significance level of 95% (probability of 0.05), is possible to consider a correlation between "x" and "y". Ho is rejected, and Hi is accepted.

To calculate theory "t", equation 9 is used:

$$t = \frac{|r|\sqrt{(n-2)}}{\sqrt{(1-r^2)}} \qquad \textbf{(Eq.9)}$$

To calculate r:

$$r = \frac{\sum x_i y_i - \frac{\sum x_i \sum y_i}{n}}{\sqrt{\left[\sum x_i^2 - \frac{(\sum x_i)^2}{n}\right]\left[\sum y_i - \frac{(\sum y_i)^2}{n}\right]}} \qquad \textbf{(Eq.10)}$$

The results of the linear regression are resumed in **Table 11** and **Table 12**. The mean correlation coefficient is 0.9999 for all compounds that demonstrate a valid relation between the factors.

The F test helps to prove curve linearity and validate Ho. Of ANOVA (***Table 12***), all F values are higher than F critical, and for all the experiments the probability that the t-stat is greater than t-experimental is under 0,05. For all of compounds texperimental>tstat, so we can reject Ho and accept Hi, that means that there is a linear relation between factors and determination can be made using the calculated model.

Table 11 : Principal results and coefficients of the linear regression

	Vanillin	Vanillic acid	4-Hidroxybenzaldehyde	4-Hidroxybenzoic acid
Correlation coefficient r	0,9998	0,9999	0,9999	0,9999
Determination coefficient r2	0,9996	0,9999	0,9999	0,9999
Standard deviation f (y/x)	0,0029	0,0009	0,0011	0,0005
Variation coefficient f (y/x)	9.0^{E}-06	1.0^{E}-06	1.0^{E}-0.6	$2,8^{E}$-07

Table 12: ANOVA resume

ANOVA							
	Coefficient		**T stat**	**p-value**	**Standard Error**	**F obtained**	**F critical**
Vanillin	**Intercept**	2,4442	3,39664692	0,009407	0,7195921	25589,793	2,6089E-15
	Slope	0,077458	159,9681	2,6089E-15	0,0004842		
Vanillic acid	**Intercept**	0,036040	3,39184525	0,0194223	0,0106256	4709949,04	3,9424E-16
	Slope	0,112425	2170,24170	3,9424E-16	5,1803E-05		
4-Hidroxybenzaldehyde	**Intercept**	-0,40133	-2,97529879	0,0155679	0,1348891	315044,886	9,2076E-22
	Slope	0,058133	561,288594	9,2076E-22	0,0001035		
4-Hidroxybenzoic acid	**Intercept**	0,136698	3,11911593	0,0108903	0,0438259	397698,292	2,4733E-24
	Slope	0,071439	630,633246	2,4733E-24	0,0001132		

With the ANOVA analysis, is possible to conclude:

- The origin of the intercept *a* is significantly different to 0.
- The constant *b* of the regression line is significantly different to 0
- There is a significant relation between area and concentration, and the equation can be used to determinate the concentration of the each compound in a sample.

Graphic of linearity:

The linearity range for the different compounds was different and calculated in function of the analyte concentration in real life samples, so the linearity range cover concentration above and below these concentrations. Besides, linearity range was established by confirming that the analytical procedure provides an acceptable degree of linearity (**Table 13**).

Table 13: Selected linearity range for each compound

Compound	Selected linearity range (µg/ml)
Vanillin	11.5 – 184.8
Vanillic acid	2.05 – 45.1
4-Hidroxybenzaldehyde	4.59 – 183.6
4-Hidroxybenzoic acid	2.28 – 45.7

Vanillin, vanillic acid, 4-Hidroxybenzaldehyde and 4-Hidroxybenzoic acid showed an excellent linearity in the analysis range and with a good correlation coefficient. Therefore, the equations can be used to determinate concentration of these compounds in natural vanilla extracts.

The detector response factor (amount/area) (*see **fig. 13**)*, is another test to determinate linearity, this is typically more accurate and meaningful than regression curves (A.G. HUESGEN (AGILENT TECHNOLOGIES), 2011). All response factors correspond a ±2% range, which is accepted to provide linearity.

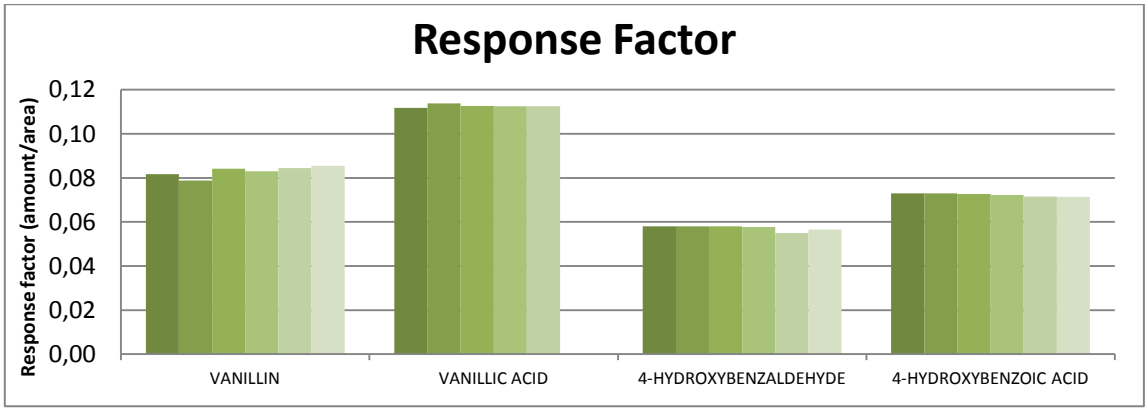

Figure 13: Response factor for each compound (Vanillin, Vanillic acid, 4-Hydroxybenzaldehyde and 4-Hydroxybenzoic acid)

The linearity experiments were used to create a multilevel calibration for each compound (*see **fig.14***). The multilevel calibration was based on dilutions of the linearity range.

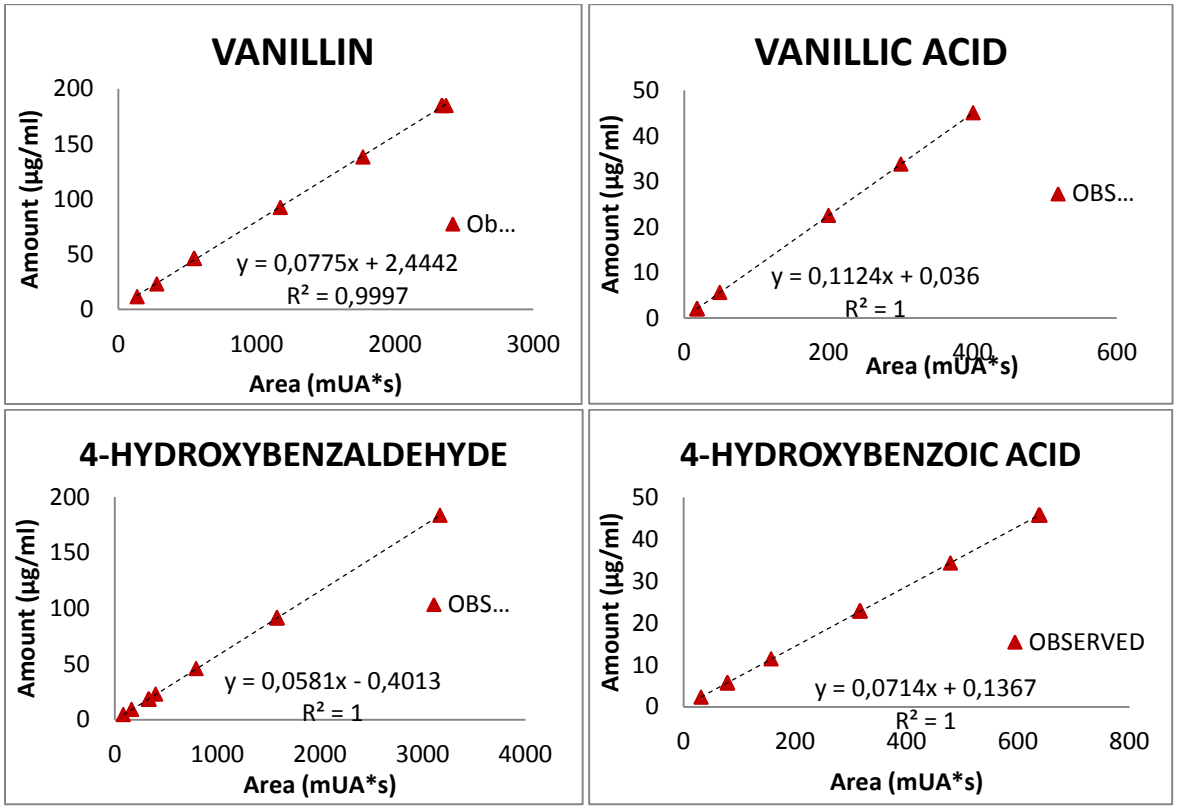

Figure 14: Calibration curves for vanillin, Vanillic acid, 4-Hydroxybenzaldehyde and 4-Hydroxybenzoic acid

6.3.3. Precision

Repeatability: the acceptation criteria is the percentage of relative standard deviation (RSD), it should be less than 3% for intraday essays and less than 5% for interday essays.

The results of the %RSD (*See:* **Table 14***)* are within the acceptable range for all of the compounds; hence the method has a good precision for identification and quantification of the mean compounds (**1**-**4**).

The %error of residuals is within the acceptable range and proves the precision of the developed method. **Annex H (a).** shows all data calculations.

Table 14: Precision values in %RSD

	Level	%RSD	%Error
Vanillin	1	1%	0.30%
	2	0.2%	2.80%
	3	1%	0.1%
Vanillic Acid	1	1%	0.3%
	2	0.2%	0.1%
	3	0.3%	0.4%
4-Hydroxybenzaldehyde	1	0.8%	4.5%
	2	1.3%	2.03%
	3	1.1%	0.1%
4-Hydroxybenzoic acid	1	0.4%	0.73%
	2	0.1%	0.46%
	3	0.2%	0.07%

Intermediate precision was performed as part of accuracy. The samples were analyzed during two days (See: **Annex H (b).**), for the same operator in the same HPLC, to verify interday precision. Intermediate precision is expressed in terms of percentage of relative standard deviation. **Table 15** shows the calculations of intermediate precision for each compound.

The developed method shows a good precision for quantification of all the mean compounds of vanilla extract. %RSD is on the accepted range previously defined.

Table 15: Intermediate precision of the mean compounds of vanilla extract

	CRM-Vanillin			Vanillic Acid			4-Hydroxybenzaldehyde			4-Hydroxybenzoic Acid		
Level	1	2	3	1	2	3	1	2	3	1	2	3
Reference Value (µg/ml)	12,5	25	125	11,25	5,62	2,81	20,6	10,3	5,1	0.55	1	1.8
Number of series	2	2	2	2	2	2	2	2	2	2	2	2
Number of essays	6	6	6	6	6	6	6	6	6	6	6	6
Number of repetitions	3	3	3	3	3	3	3	3	3	3	3	3
RMSr	1,99E-01	2,31E-02	3,82E-01	5,59E-01	9,13E-03	3,80E-03	4,67E-02	8,47E-03	1,31E-02	2.93E-03	2.67E-03	5.67E-03
RMSt	0,199083	0,036133	0,808200	0,739733	0,011533	0,004400	0,064883	0,008733	0,013133	0.004283	0.006933	0.008483
RMS(inter-series) S^2B	1,50E-04	1,31E-02	4,27E-01	1,80E-01	2,40E-03	6,00E-04	1,81E-02	2,67E-04	6,67E-05	1.35E-03	4.27E-03	2.82E-03
Variance (inter-series) S^2	-1,999950	-1,99564	-1,857778	-1,93991	-1,99920	-1,99980	-1,99395	-1,99991	-1,99998	-1.99955	-1.99858	-1.99906
S^2r	0,049733	0,005767	0,095383	0,139867	0,002283	0,000950	0,011683	0,002117	0,003267	0.000733	0.000667	0.001417
Variance Intermediate precision S^2P	0,049733	0,005767	0,095383	0,139867	0,002283	0,000950	0,011683	0,002117	0,003267	0.000733	0.000667	0.001417
PRESICION												
Average	13,6	27,0	118,4	12,1	5,9	2,9	21,1	10,6	5,2	0.5	0.8	1.3
SDr	0,446019	0,151877	0,617684	0,747975	0,095568	0,061644	0,216179	0,092014	0,114310	0.05416	0.05164	0.07527
SDB	0,012247	0,114310	0,653197	0,424578	0,048990	0,024495	0,134722	0,016330	0,008165	0.03674	0.06532	0.05307
SD P	0,223010	0,075939	0,308842	0,373988	0,047784	0,030822	0,108089	0,046007	0,057155	0.027080	0.02582	0.03764
RSD	0.02	0.003	0.003	0.03	0.01	0.01	0.005	0.004	0.01	0.05	0.03	0.03
Mean %RSD		1%			2%			1%			4%	

6.3.4. Accuracy

Certified Reference Material was used to calculate the accuracy of the method for the quantification of Vanillin. The CRM was prepared by the technical specifications (diluting minimal 50mg of CRM in 1ml of ethanol) and was measured at three different concentrations: 12.5µg/ml, 25µg/ml and 125µg/ml.

The recovery percentage was on the range reported by Kumar et.al (2007), and the mean value of bias was 0.04, so it's possible to consider that the method is accurate for quantification of vanillin. **Table 16** shows the obtained values for calculating accuracy. ***Fig. 15*** shows a chromatogram of CRM-Vanillin, there are not interferences of the other compounds while analyzing.

Table 16: Recovery percentage found for CRM - Vanillin

CRM VANILLIN

Day	Reference	% Recovery 1	% Recovery 2	% Recovery 3	Average	Bias	CV
1	125	94	95	95	94,83	0.09	0,17
2		95	95	95			
1	25	110	108	107	108,17	0.08	0,97
2		108	108	108			
1	12,5	108	108	108	108,00	-0.05	2,80
2		111	107	106			

SD	7,65		
Mean	103,67	0.04	1.31
n	18		
tcal	2,033		
ttab	1,734		

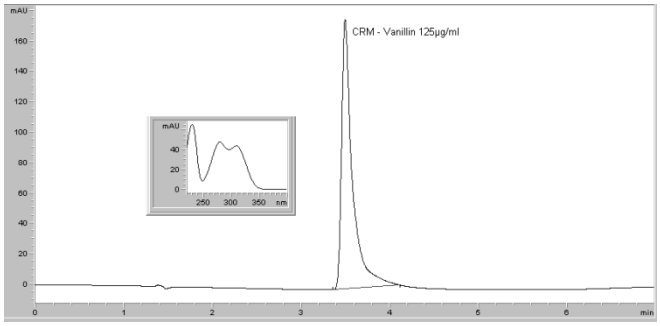

Figure 15 : Chromatogram of Vanillin (CRM), determination of concentration with the developed method.

Corrections should be necessary for calculation of vanillin concentration. Bias, the difference between the true value and the average of the estimate, was calculated with the **Eq. 11**. Moreover, the results should be corrected with this value.

$$bias = Cref - \overline{Clab} \qquad \textbf{(Eq.11)}$$

Uncertainly of bias was also calculated with the **Eq. 12**.

$$u(bias) = \sqrt{RMS_{bias}{}^2 + u(Cref)^2} \qquad \textbf{(Eq.12)}$$

Where:
RMS: Root mean square
Cref: Compound of reference (CRM)
u(Cref): uncertainly of the reference compound

The RMS is found as follows:

$$RMS_{bias} = \sqrt{\frac{\sum(bias_i)^2}{n}} \quad \textbf{(Eq.13)}$$

Where *n* is the number of bias determinations carried out and each $bias_i$ is a result of an individual bias determination and is found as follows:

$$bias_i = Clab_i - Cref_i \quad \textbf{(Eq.14)}$$

Where $Clab_i$ is a mean of the results of analyte determination in the reference sample obtained by the laboratory and $Cref_i$ is the reference value of the reference sample.
The bias obtained and u(bias) obtained for vanillin were 4% and 2.46% respectably which is considered good for Industry.

The correct equation (**Eq.15**) that describes the concentration of vanillin is as follows:

Vanillin $$C_{Vanillin} = (0.0774582 * A + 2.44420039) \pm 0.04 \quad \textbf{(Eq.15)}$$

Determination of accuracy for the other compounds:

Availability of CRM is limited. Therefore, the accuracy of vanillic acid, 4-hydroxybenzaldehyde and 4-hydroxybenzoic were expressed in terms of recovery percentage of the surrogate.

Recoveries of the experiment were performed to studying the accuracy of the method. The developed HPLC method was applied for the determination of percentage recovery of compounds 2-4 in vanilla extracts.

Table 17 : Recovery percentage of vanillic acid, during HPLC analysis

VANILLIC ACID

Day	Ref (µg/ml)	% Rec1	% Rec 2	% Rec 3	Aver %	CV
1	11,25	115	107	107	108	11,7
2		107	106	105		
1	5,62	103	105	104	104	0,73
2		104	105	104		
1	2,81	105	104	103	104	1,11
2		105	105	103		

Mean	105	4.51
SD	2.03	
t_{exp}	11.27	
T_{table}	1.734	
Bias %	0.04	

Table 18: Recovery percentage of 4-Hydroxybenzaldehyde, during HPLC analysis

4-HYDROXYBENZALDEHYDE

Day	Ref (µg/ml)	% Rec1	% Rec 2	% Rec 3	Aver %	CV
1	20,6	102	102	102	102	0,17
2		102	102	103		
1	10,3	103	102	103	103	0,17
2		103	103	103		
1	5,1	103	101	100	101	1,07
2		101	102	101		

Mean	102	0.47
SD	0.751	
t_{exp}	11.31	
T_{table}	1.734	
Bias %	0.02	

Table 19: Recovery percentage of 4-Hydroxybenzoic Acid, during HPLC analysis

4-HYDROXYBENZOIC ACID

Day	Ref (µg/ml)	% Rec1	% Rec 2	% Rec 3	Aver %	CV
1	0.55	85	80	89	88	28.3
2		96	87	87		
1	1	92	88	84	85	17.1
2		81	83	82		
1	1.8	92	91	86	88	5.24
2		87	87	87		
				Mean	87	11.8
				SD	1.61	
				t_{exp}	34.32	
				T_{table}	1.734	
				Bias %	-0.13	

Tables 17, **18** and **19** show the recovery percentages for vanillic acid (**2**), 4-hydroxybenzaldehyde (**3**) and 4-Hydroxybenzoic acid (**4**) respectively. The mean recovery percentage for compounds **2** and **3** were within the range reported in precedent works by Kumar *et.al* (2007); it is 94.331 - 103.96%. Nevertheless, for the compound **4** the recovery factor was less than the reported result.

To determine if corrections are necessary, a t-student was realized. The test allows concluding that the mean recovery percentage is significantly different to 100% for all compounds because texp>ttable, this with α=0.05. So, corrections are necessary for calculations (SABLIER & FEINBERG, 2012). In the case of the compound 4 the application of a correction factor is doubtful because it may be possible that some proportion of the analyte is not recoverable. In fact, the added analyte may not come to effective equilibrium with the native analyte. Nevertheless, the correction will be applied to all of the compounds.

Applying the corresponding correction with the calculated bias the concentration of each compound is expressed as:

Vanillic acid $C_{Vanillic\ A} = (0.112425 * A + 0.03604073) \pm 0.04$ (**Eq.16**)

4-Hydroxybenzaldehyde $C_{4HyB} = (0.0581337 * A - 0.4013356) \pm 0.02$ (**Eq.17**)

4-Hydroxybenzoic acid $C_{4HB\ A} = (0.0714396 * A + 0.13669809) + 0.13$ (**Eq.18**)

6.3.5. Limit of detection and limit of quantification

The limit of detection was calculated based on the calibration curve. LOD can be expressed as:

$$LOD = \frac{3.3\sigma}{S} \quad \text{(Eq.19)}$$

Where: σ is the standard deviation produce by the noise
S is the slope of the calibration curve

The LOD for each compound is show in **Table 20**.

The limit of quantification was calculated based on the calibration curve. LOQ can be expressed as:

$$LOQ = \frac{10\sigma}{S} \quad \textbf{(Eq.20)}$$

Where: σ is the standard deviation produce by the noise
S is the slope of the calibration curve

The LOD for each compound is show in **Table 20**.
The Standard deviation produce by the noise is 0.1.

Table 20: Limit of detection and limit of quantification

Compound	LOD (µg/ml)	LOQ (µg/ml)
Vanillin	0.12	0.38
Vanillic acid	0.03	0.08
4-Hidroxybenzaldehyde	0.06	0.18
4-Hidroxybenzoic acid	0.02	0.07

It was analysed both sample and standards, and no interference was found in the chromatographic peaks of interest. For natural vanilla extract sample, there were additional chromatographic peaks, but with different retention time, so without interference in vanillin, vanillic acid, 4-Hidroxybenzaldehyde and 4-Hidroxybenzoic acid.

Three wavelengths were chosen for analysis of natural vanilla extracts, 230 nm for identification of the aldehyde vanillin; 260 nm for acids, and 280 nm for aldehyde 4-Hidroxybenzaldehyde.

The identification of the different compounds in each wavelength proves the selectivity of the method and the possibility to use it as a quality control for identification and quantification of the main constituents of natural vanilla extracts.

Linear equation between the concentration (amount) and the peak area (in mUA*s) was expressed as *y=(bx+a)±bias*, and a good linearity were observed. The determination and correlation coefficient were on the acceptable range for each compound (**1-4**).

Precision were prove with repeatability, all results were below of the maximal %RSD of 5% for intraday essays and 3% for intermediate precision for interday essays, showing an acceptable precision and accuracy.

A data sheet of validation protocol resumes the conditions and results of this validation method (**Annex I.**).

6.3.6. Robustness

Two critical parameters were changed to verify robustness of the validated method; they are summarized in **Table 21**.

The developed method is robust even by change in the less 16% of temperature. (**Table 21** and ***fig. 16***)

Table 21: Robustness test

Change in temperature	35°C	Low: 30°C	Good elution and separation of the mean compounds.
		High: none	
Change in flow rate	1ml/min followed of 1.5ml/min followed of 1ml/min	Low: 1.0 ml/min	Constant flow, RT highly affected but good separation and identification of the mean compounds.
		High: 1.5 ml/min	Constant flow, faster elution of the mean compounds. RT quite affected.

When flow was changed to a constant flow of 1.0ml/min to 1.5ml/min the changes on chromatogram were non-significant for the high level, but the low level were highly affected (***fig. 17***). Hence, the method is robust to changes in flow. The method is not robust by flow changes if the objective is quantifying the mean compounds, but for identification it can be considered as robust.

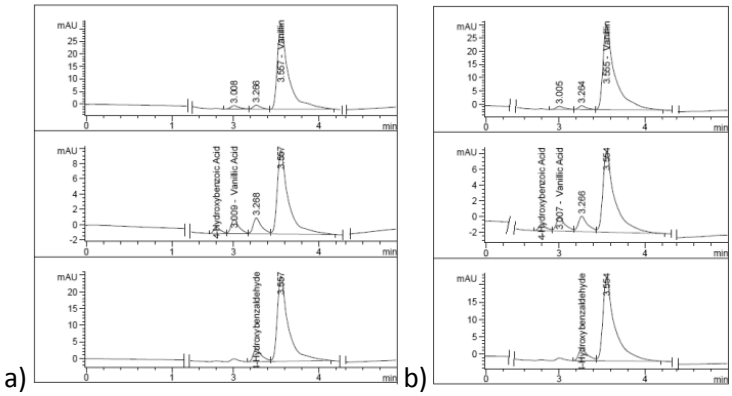

Figure 16 : Chromatograms for robustness with variable temperature a. 30°C and b. 35°C

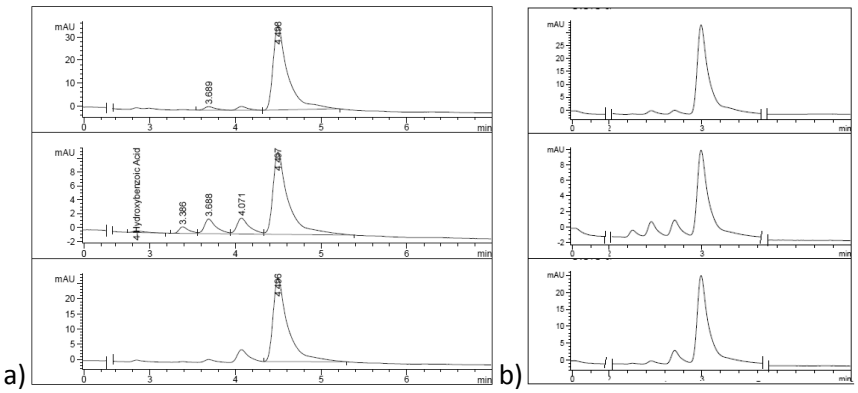

Figure 17: Chromatograms for changes on flow a. 1.0ml/min and b. 1.5ml/min

6.4. Analysis of Real Life Sample

Real samples were analysed to the developed and validated method. Vanilla extract produced by Sevarome and vanilla extract extracted by UAE were compared. Conformity with regulations was verified by calculation of the specifics ratios.

Both extracts, produced by Sevarome and vanilla extract (UAE) were within the values recommended by the SNIAA and regulations.

Samples as oleoresins and absolutes can also be analysed by the current method, but specifics dilutions should be made. They are usually soluble in alcohol, and the current method is correctly suitable for the analysis of alcoholic vanilla extracts.

GENERAL CONCLUSIONS

UAE has demonstrated to be an efficient economical and innovative technology for the extraction of vanillin from vanilla beans. The combination of cavitation with conventional extraction methods or simply the utilisation of this extraction method alone could be a possibility to improve vanillin extraction in vanilla extracts. However, it is necessary to obtain an extraction method that allows combining the advantages of ultrasound with an excellent quality of the final extract. It is a high vanillin content and excellent sensorial propriety.

Related compounds (1-4) presents in natural vanilla extracts responsible for vanilla aroma, can be separated and quantified by an isocratic RP-HPLC using an Agilent 1260 Infinity Binary LC System with an Agilent ZORBAX Eclipse Plus C18 (150 X 4,5mm, 5µm) column, in the conditions which have been previously describes. The result is a more precise, specific, sensitive and reproducible RP-HPLC method for quantitative determination of vanillin, vanillic acid, 4-Hydroxybenzaldehyde and 4-Hydroxybenzoic acid.

The method allows separation of compounds (1-4) and it has been validated in terms of linearity, precision, accuracy, LOD and LOQ as recommended by the ICH guideless. The results are in the range of acceptability, except 4-Hydroxybenzoic Acid that is less accurate than the other compounds. Further, the validation method is in accordance with the established parameters for validation methods of IUPAC.

Expectations:

The development of an RP-HPLC method to vanilla extracts characterisation was just the beginning of a very long process. Characterisation of vanillin is a complex and laborious work because of a vast number of compounds presents in vanilla. Also because of the naturalness of the vanilla bean, which can change from one cultivation country to the other.

The currently developed method will be useful for vanillin determination in vanilla extracts. It will be specifically used to build an extraction curve and to determine the effectiveness of the current extraction method, also to determine performance parameters.

The optimized RP-HPLC conditions will be applied to determination of percentage recovery of compounds (**1-4**) in natural vanilla extract produce by Sevarome. In the same way, this method will be useful to improve the extraction performance of natural vanilla extracts.

Recommendations:

It is necessary verifying the recovery factor of the mean compound 4-hydroxybenzoic acid, to prove the complete effectiveness of the developed method. The verification could be made using other standards with high purity. Further, the quality control of a method should be applied, a method also has a cycle and it will always be at the forefront of news exigencies.

Moreover, there is important to develop a GC/MS- spectrometry method to determine the isotopic deviation ratio, it is a more accurate method for determination of naturalness of vanilla extracts (LAMPRECHT, PICHLMAYER, & SCHMID, 1994).

SYNTHESE :

OPTIMISATION D'UNE APPROCHE ANALYTIQUE BASEE DANS LA CHROMATOGRAPHIE LIQUIDE D'HAUTE PERFORMANCE POUR LA CARACTERISATION DES EXTRAITS NATURELS DE VANILLE

Catalina VELEZ SUAZA – Alimentation et Santé Spécialité : Formulation, Ingrédients et Qualité des Aliments

INTRODUCTION

La vanille est la deuxième épice la plus chère dans le monde. Elle est issue d'une orchidée originaire du Mexique qui a été découverte par les Espagnols pendant l'époque de la conquête. La découverte du mode de pollinisation en 1841 a permis d'intensifier la culture de cette fleur dans d'autres régions du monde et ainsi l'exploitation de sa richesse aromatique (HAVKIN-FRENKEL & BELANGER, 2011).

La vanille doit son nom et son arôme caractéristique à la molécule de la vanilline, elle est produite de différentes façons comme la voie chimique et la voie biochimique. A l'heure actuelle, **75% de la quantité de vanilline produit dans le monde est d'origine synthétique** (BOUTHIN, HIRTZLIN, & SCHMITT, 2006).

L'arôme vanille reste l'arôme le plus populaire au monde, son usage est très diversifié. La valeur commerciale de l'arôme vanille naturelle est environ 20 fous supérieure à celle de l'arôme de synthèse ce qui explique en partie l'augmentation des **fraudes** quant au type d'arôme. Cette fraude est principalement faite lors de l'étiquetage, les fabricants d'arôme vanille ajoutent la molécule de synthèse vanilline aux extraits et inscrivent l'appellation de « arôme naturelle de… ».

En effet, la réglementation est de plus en plus stricte, et la demande de fiches techniques sur le produit plus complète et précise, met en évidence un travail de recherche qui doit être fait par tous les intéressés, dont les industries aromatiques.

Sevarome est une entreprise basée à Yssingeaux, elle fait partie du groupe Nactis dont sa principale activité est la fabrication et la conception d'arômes pour l'industrie agroalimentaire. Sevarome produit, depuis des années, des extraits naturels de vanille. Aujourd'hui, avec l'augmentation de la demande, il est obligatoire d'effectuer une caractérisation fine de l'arôme afin de pouvoir baser son prix sur une définition complète de ses qualités et de sa composition.

L'objet principal de ce travail a été de développer une méthode d'analyse pour caractériser l'arôme vanille et notamment pour quantifier ses quatre molécules principales : la vanilline, l'acide vanillique, le 4-Hydrox benzaldéhyde et l'acide 4- Hydrox benzoïque. Lors du développement de la méthode d'analyse plusieurs phases ont été réalisées, un état de l'art, une optimisation de l'extraction des extraits de vanille, le développement d'une méthode analytique par Chromatographie Liquide d'Haute Performance (HPLC) et finalement une validation de la méthode analytique.

1. PRESENTATION DE L'ENTREPRISE

Sevarome est une entreprise qui fait partie du groupe Nactis, dont le siège est basé à Bondoufle (91). Le site de production est basé à Yssingeaux (43).

La production de Sevarome est assurée par des contrôles de qualité spécifiques pour chaque produit. Le principal objectif de Sevarome est d'assurer la qualité de ses produits et la satisfaction de ses clients. Pour cela, Sevarome utilise la transparence et l'étique professionnelle. En effet, une certification ISO 9001 montre l'engagement de cette entreprise en matière de qualité.

Sevarome dispose d'un laboratoire de recherche et développement où ses aromaticiens développent des produits sur mesure et à la demande des consommateurs. Les activités principales sont la recherche, la création et l'innovation. L'entreprise Sevarome est très reconnue pour son expertise dans le monde des arômes, la société offre des solutions fonctionnelles visant à améliorer la satisfaction du client, et s'engage à garder un équilibre dans son environnement technique, financier et réglementaire.

2. ETAT DE L'ART

Un arôme peut être défini comme le mélange de différentes molécules volatiles suffisantes pour donner une réponse olfactive, ce que l'on appelle « a flavour ». Les arômes sont des produits qui donnent du goût aux aliments, un indicateur qui détermine l'acceptation de ce dernier par le consommateur (INTERNATIONAUX, 2011).

La vanille est une épice qui est convoitée depuis longtemps, elle est encore l'arôme le plus consommé dans le monde. La vanille est présente dans une gousse issue d'une plante orchidée tropicale, principalement de l'espèce *Vanilla pompona,* originaire du Mexique. Aujourd'hui, elle est cultivée dans plusieurs pays dans le monde, notamment Madagascar, l'Indonésie, Tahiti, les Comores, etc. Madagascar reste encore l'acteur principal dans cette filière, sa politique et les aléas climatiques influencent le prix de cette matière première très demandée dans le monde (GRETZINGER & DEAN, 2011).

A l'heure actuelle l'utilisation des arômes naturels, notamment de la vanille, s'accroit de manière continue dans pratiquement tous les secteurs de l'agroalimentaire. Tout d'abord, les modes de vie ont évolué ainsi que les habitudes des consommateurs. Ces dernières sont de plus en plus exigeantes et recherchent des produits naturels à un coût raisonnable.

Afin d'éviter toute confusion entre la vanille naturelle est synthétique la réglementation exige des étiquetages spécifiques pour informer le client. En effet, la loi est stricte concernant toutes les dénominations utilisées pour les produits provenant de la vanille, pour cela toutes les tentatives de fraude sont sévèrement réprimées (PACKER, 2008).

En ce qui concerne le prix de l'arôme vanille, il existe une différence significative entre celle d'origine naturelle et synthétique, le premier ayant un prix 8 fois plus élevé que le deuxième. Pourtant la fraude dans cette industrie est devenue un problème. L'intérêt réside dans une diminution du coût de revient du produit principal la vanille et notamment la vanilline. Le principe est de substituer totalement ou partiellement un ingrédient par un autre (vanilline naturelle par synthétique), sans modification des qualités organoleptiques du produit.

Actuellement plusieurs techniques ont été mises en place afin d'identifier les fraudes. Par ailleurs, les entreprises cherchent aussi à fidéliser ses clients en leur offrant une garantie sur la qualité et la provenance de ses produits. Pour cela, Sevarome cherche à faire des fiches techniques plus complètes permettant justifier la qualité de ses produits vis-à-vis les clients.

3. EXTRACTION DE L'ARÔME VANILLE

L'arôme de vanille est extrait en faisant macérer les gousses de vanille, préalablement broyées, dans un mélange d'eau et d'alcool. Ce mélange pénètre la structure cellulaire des tissus de la gousse pour aller chercher et décrocher la vanilline et d'autres composants aromatiques au cœur du végétal (RANADIVE, 2010).

Les différentes technologies dans les procédés d'extraction, de distillation et d'assemblage sont de plus en plus maîtrisées pour les industrielles. L'arôme vanille a une place prépondérante dans l'industrie, l'amélioration des méthodes d'extraction est primordial pour chercher un meilleur rapport qualité/prix.

Parmi les méthodes d'extraction de la vanille, la macération, la percolation et l'hydro distillation sont les plus utilisées. Cependant, ces méthodes sont longues et consommatrices d'énergie. Par conséquent, la recherche de nouvelles méthodes d'extraction est au cœur de l'actualité.

Les méthodes conventionnelles d'extraction prennent environ de 48 à 72 heures (en laboratoire) d'extraction et utilisent des températures allant de 45°C jusqu'à 60°C, ce qui peut détruire certains composants aromatiques. L'extraction assistée par ultrasons est une technologie récemment utilisée pour l'extraction des composants naturels, les résultats sont très prometteurs pour ce type d'application.

Cette technique d'extraction est basée sur les effets de cavitation. La cavitation est une action physique qui est le résultat de plusieurs cycles de sonication (*cf.* ***fig. 7***), qui désintègrent les structures cellulaires. Les effets mécaniques des ultrasons offrent une pénétration plus rapide et plus complète du solvant dans la matière première (matière végétal). En général, le transfert de masse est effectué plus rapidement en diminuant le temps d'extraction (*cf.* **CHAPTER 3: VANILLA EXTRACTION**)(AZUOLA & VARGAS, 2007; VILKHU ET AL., 2008).

Expériences :

La première approche a été l'exploration des conditions qui affectent le plus l'extraction de la vanille par les ultrasons. Pour ce fait, un plan d'expériences composite par surface de réponse a été élaboré. Les facteurs qui ont été analysés sont : l'amplitude, les pulsations, le temps de sonication, le pourcentage d'alcool et le ratio matière première/solvant. Les réponses mesurées ont été : le pourcentage de vanilline, le pourcentage de 4-hydrox benzaldéhyde, le taux d'extraction (vanilline), la vitesse d'extraction et le rendement (*cf.* **CHAPTER 3: VANILLA EXTRACTION***).*

La deuxième approche était la comparaison de deux méthodes d'extraction : l'extraction par macération en bain marie et l'extraction par ultrasons. Les résultats optimaux d'extraction à l'ultrason obtenu dans la première approche ont été utilisés comme conditions d'extraction pour la deuxième. Pour la macération, les conditions utilisées ont été définies par rapport à des méthodes référencées dans la littérature scientifique. Le Table 9: Comparison between traditional extraction method and UAE résume ces conditions d'extraction.

Une troisième approche est l'optimisation de l'extraction par les ultrasons avec l'objectif d'améliorer ou au moins conserver la qualité de l'extrait d'un point de vue aromatique. En effet, l'arôme naturel de vanille est un mélange complexe de plusieurs composants aromatiques que ne peut pas se réduire

qu'à la vanilline. D'où l'importance de chercher un équilibre entre le temps d'extraction, la quantité de vanilline et le profil aromatique.

4. DEVELOPPEMENT DE LA METHODE D'ANALYSE PAR HPLC

L'identification des composants principaux de la vanille est devenue de plus en plus importante. Plus de 200 molécules ont été identifiées préalablement (CICCHETTI & CHAINTREAU, 2009b). Ils existent plusieurs méthodes analytiques pour déterminer si la composition de l'arôme vanille est complètement naturelle (CICCHETTI & CHAINTREAU, 2009a). Notamment, l'HPLC permet de vérifier cette naturalité par la quantification de ses quatre composants majoritaires dont, la vanilline, l'acide vanillique, le 4-hydrox benzaldéhyde et l'acide 4-hydrox benzoïque, qui représentent un tiers de l'arôme (ELKE ANKLAM, 1993).

La chromatographie permet de faire un tri entre les différentes espèces moléculaires présentes dans un mélange. L'objet principal est de les séparer, les identifier puis éventuellement les quantifier. La Chromatographie Liquide d'Haute Performance (HPLC par ses sigles en anglais), consiste à faire passer par une colonne à haute pression le mélange des composants à analyser. Les molécules sont véhiculées dans un fluide appelé phase mobile. Certaines espèces auront plus de difficultés à sortir, il y aura donc un échelonnement à l'arrivée. La colonne est composée d'une phase stationnaire, dont l'objectif est de retarder la sortie des molécules qui ne sont pas affines à la phase mobile. La séparation s'effectue de cette façon (FEREZ MELGAJERO, 2007).

Conditions expérimentales

Des conditions chromatographiques, telles que la température de la colonne, le débit du solvant, la composition de la phase mobile, et la longueur d'onde à laquelle les molécules doivent être analysées, font du développement de cette technique d'analyse un travail très complexe.

La résolution est l'un des facteurs les plus importants lors de l'optimisation d'une méthode par HPLC. La résolution est donnée par l'équation 1 du texte principal. La résolution est dépendante de plusieurs facteurs comme le nombre de plateaux d'une colonne (N), le facteur de séparation (α), et la résolution (k) (*cf.* **CHAPTER 4: Method development for vanilla flavor analysis**). La combinaison optimale de ces facteurs aide au développement optimal de cette technique d'analyse.

Développement de la méthode

Des essais préliminaires ont été réalisés afin d'analyser les composants principaux de la vanille. Pour ce fait, la méthode développée par Huesguen (Agilent technologies) a été utilisée. La méthode a été standardisée pour les besoins spécifiques et conditions de travail de Sevarome.

Waliszewski, *et al.* (2007) a reporté une méthode qui utilise une phase mobile aqueuse pour la caractérisation de la vanille, ce qui est de grand intérêt pour l'analyse des extraits de vanille (riches en alcool). Plusieurs méthodes par HPLC, dont l'objectif est l'analyse de la vanille ont été publiées dans le passé. Ces techniques se caractérisent principalement par une élution en gradient, utilisation de l'acétonitrile comme phase mobile, un débit entre 0.6 et 2ml/min, et une température moyenne d'analyse de 30°C. L'**annexe D** montre les différents travails qui ont été réalisés.

Réactifs et matériaux

a. *Réactifs :* Du méthanol degré gradient (99.9% basée en GC), l'eau ultra pure et d'autres solvants ont été achetés chez Merk. L'acide trifluoracétique (TFA) (Merk) a été utilisé pour acidifier l'eau dans la solution de la phase mobile. Tous les solvants ont été filtrés à travers un filtre de vitre de 0.45µm de diamètre (Fisher Scientific) et ont été dégazés avant l'utilisation dans l'appareil.

Les standards : Vanilline 97% de pureté (Rovanil ® Natural)
Acide vanillique 95% de pureté (Prodasynth)
4-Hydroxybenzaldehyde 98% de pureté (Prodasynth)
4-Hydroxybenzoic acid 99% de pureté (Sigma-Aldrich)

Matériel de Référence Certifié (CRM): Vanilline 99.7% de pureté (Sigma-Aldrich)

Les gousses de vanille qualité Bourbon pour extraction dont la qualité de la vanille est garantie provient de Madagascar.

b. Matériaux : Pour les analyses un système chromatographique a été utilisé : HPLC Agilent 1260 Infinity Binary LC equipé avec :

- Une pompe binaire Agilent 1260 infinity
- Un dégazeur sous vide Agilent 1260 Infinity
- Un injecteur automatique avec thermostat Agilent 1260 Infinity
- Détecteur à barrettes de diode (DAD) Agilent 1260 Infinity

Une colonne Agilent ZORBAX Eclipse Plus C18 (4.6 X 150mm, 5µm) de Agilent Technologies.

Balance analytique **Precisa**
Modèle: XB 1220M
Précision: 0.001g

Potentiomètre **Mettler Toledo**

Méthodes

Préparation des standards : Des solutions étalons de chaque molécule ont été préparées pour développer et valider la méthode d'analyse. La gamme de concentration a été obtenue par une série de dilutions de chaque solution étalon (*cf.* **Table 5**), d'après la recommandation d'auteurs références.

La série de dilutions a été utilisée pour construire une courbe de calibration. La solution étalon et toutes les autres solutions ont été préparées dans une solution de méthanol : eau distillée (1 :1).

Préparation de l'échantillon : Une solution avec les quatre molécules a été préparée dans la solution méthanol : eau distillée. Les molécules ont été mélangées afin d'obtenir une concentration équivalente à celle d'un échantillon réel (*cf.* **Table 8**).

Préparation d'un échantillon de la vie réel : Des extraits de vanille ont été préparés d'après la méthode précédemment décrite par ultrason. Des extraits naturels de vanille produits par Sevarome ont été aussi analysés. Pour ce faire, 50µl d'extrait ont été dilués dans 4950µl de la solution de travail méthanol : eau.

Toutes les solutions ont été filtrées avant l'analyse, à travers un filtre de 4.5µm (Titanic RC, Fisher Scientific).

Conditions chromatographiques : Un mélange de méthanol et d'eau ultra pure acidifiée (0.1%TFA) a été utilisé comme phase mobile. L'analyse Isocratique été composée de 35% de méthanol et 65% d'eau. Le débit de la phase mobile a varié entre 1 et 1.5ml/min, pour un temps total d'analyse de 7min. La température moyenne de la colonne a été définie à 35°C. Les longueurs d'onde pour l'identification des différentes molécules ont été établies à 230nm pour la vanilline, 260nm pour l'acide vanillique et l'acide 4-Hydrox benzoïque et 280nm pour le 4-Hydrox benzaldéhyde.

5. VALIDATION DE LA METHODE D'ANALYSE

La validation est l'une des étapes les plus importantes avant de mettre en place une méthode d'analyse. La Conférence Internationale d'harmonisation (ICH) et l'Union International de Chimie Pure et Appliquée (IUPAC) sont des organismes qui ont crée des guides dans le but de standardiser les différents types de validation. Dans ces cas spécifiques, on validera une méthode de la catégorie IV (*cf.* **Table 7**). Conformément au document, il est indispensable de valider la méthode pour les paramètres énoncés ci-dessous :

- Sélectivité
- Linéarité
- Range d'analyse
- Précision dont la répétabilité et la précision intermédiaire
- Exactitude

La limite de détection et de quantification sont optionnelles.

Sélectivité : La sélectivité est la capacité d'un système pour déterminer sans erreur la présence d'un « analyte » dans un mélange. Le système peut donc différencier entre les différents composants d'un échantillon.

Linéarité : C'est l'intervalle de concentrations où il existe une relation entre une mesure analytique et une réponse instrumentale. Elle est représentée par l'équation **Eq.6** du texte principal.

Intervalle d'analyse : L'intervalle d'analyse est une section de la courbe linéaire qui suit la loi de Beer. Il doit alors y avoir une certaine précision et exactitude pour tous les niveaux de concentration mesurés.

Précision : La précision d'une mesure analytique est déterminée par la capacité du système d'exprimer des résultats proches entre une série de mesures. On peut donc mesurer la précision intermédiaire (entre les jours d'analyse) mais aussi la répétabilité (dans une même journée avec des conditions identiques).

Exactitude : L'exactitude d'une méthode analytique exprime le degré de concordance entre la valeur mesurée et la valeur réelle ou acceptée. Ces valeurs acceptées sont limitées à l'existence de Matériel de Référence Certifié (CRM), qui n'est pas toujours valable.

Limite de détection et limite de quantification : La limite de détection est la concentration minimale qui peut être détectée ou identifiée sans avoir recours à une quantification. Par contre, la limite de quantification est la concentration minimale qui peut être quantifiée avec précision et exactitude.

La détermination de ces paramètres a été faite d'après la guide élaboré par l'ICH, et sous conditions d'avoir un laboratoire contrôlé. Les analyses statistiques ont été réalisées dans Excel (Pack Office 2007). (*cf* **CHAPTER 5: METHOD VALIDATION**)

6. ANALYSES ET DISCUSSION

Extraction de l'arôme vanille

Les résultats obtenus dans les deux approches montrent l'efficience des ultrasons pour l'extraction des composants de la vanille, notamment de la vanilline et des autres composés aromatiques. En effet, l'extraction par ultrasons en comparaison à la macération en bain marie est beaucoup plus efficace en termes de temps et de vitesse d'extraction. Cependant, une analyse du profil aromatique doit être réalisée afin d'optimiser la méthode d'un point de vue sensoriel.

La surface de réponse obtenue par le plan d'expériences montre une très forte relation entre la quantité de vanilline extraite, l'amplitude de l'appareil et le temps de sonication. Plus l'amplitude est grande et plus il y a de sonication, plus il y aura de vanilline dans le produit fini (*cf.* ***fig. 11***).

Cependant, le rendement d'extraction est fortement affecté par le temps de sonication. Plus il y a de sonication, moins il y aura d'extrait. Pour cela, on a déterminé les conditions optimales d'extraction, dont l'amplitude est 85%, le temps de sonication est 50min, les pulsations 50%, 12.5g de vanilline par 50g de solvant et 60% d'alcool dans le solvant. Avec ces paramètres on obtient un extrait de vanille d'environ 0.35% de vanilline et 1.32% comme taux d'extraction (*cf.* ***fig. 12***).

La deuxième approche a permis de comparer les deux méthodes d'extraction. Pour les deux extractions les mêmes ratios : quantité de gousses par quantité de solvant ont été utilisées et le même pourcentage d'alcool. L'extraction a été réalisée en trois étapes, trois jus ont été crées puis mélangés. Le temps d'extraction et le taux de vanilline ont été mesurés et comparés (*cf.* ***fig. 13***).

Pour finir, on peut affirmer que la quantité de vanilline extraite pendant 4 heures de sonication avec l'ultrason est équivalente à 73 heures d'extraction avec la méthode de macération en bain marie. Rasoamandrary *at. al* (2013), a démontré l'efficacité de la technologie par ultrasons pour améliorer l'extraction de la vanilline, uniquement sous des conditions optimales.

Développement de la méthode d'analyse par HPLC

La méthode analytique développée a été conduite en fonction de l'objectif principal qui est d'identifier et quantifier les principaux composants présents dans l'extrait de vanille. Différentes conditions ont été testées en utilisant la méthode « expérimentale» OFAT (One factor at time). Finalement, une séparation des composants avec une bonne résolution a été obtenue. Les conditions opératoires sont décrites dans **Table 10** du texte original.

Validation

La méthode a été validée pour les paramètres de sélectivité, linéarité, intervalle d'analyse, précision, exactitude, limite de détection et de quantification.

a. **Sélectivité :** Le système a montré qu'il était sélectif pour les 4 composants principaux de la vanille. La ligne de base n'a pas d'interférences lors de l'analyse. Le facteur de pureté a été

mesuré pour chaque composant. Ils ont une pureté >95% avec une résolution <2, donc le système est sélectif pour les conditions souhaitées.

b. **Linéarité :** La linéarité a été évaluée pour différents niveaux de concentration. Une courbe de calibration a été obtenue pour chaque composant, ainsi qu'une régression linéaire.
Une équation de la courbe a été obtenue par chaque composant. On a calculé le coefficient de corrélation (r>0.999) et le coefficient de détermination (r^2>0.9999).
Des tests de student et de fisher ont été faits afin de vérifier la linéarité mais aussi l'hypothèse alternative : r n'est pas significativement différent à 1 donc il y a une relation linéaire entre les facteurs. Une ANOVA montre les principaux résultats et permet l'analyse de la linéarité (*cf.* **Table 12**).
Le système est linéaire et les équations peuvent être utilisées pour la prédiction de la concentration des composants de la vanille.
    a. Intervalle d'analyse : L'intervalle d'analyse a été déterminé en conformité avec la loi de Beer. Les prédictions avec la courbe de linéarité sont donc précis et exacts.

c. **Précision :** La précision a été déterminée en fonction de la répétabilité des mesures intra-jour et inter-jour.
    a. Répétabilité intra-jour : Un critère d'acceptation de 3% de la déviation standard relative a été choisi. Le pourcentage d'erreur des résidus a été aussi mesuré et pris en compte pour les analyses. Les résultats obtenus pour les quatre molécules montrent que la méthode est précise pour les mêmes conditions d'analyse.
    b. Précision intermédiaire (inter-jour) : Le critère d'acceptation de 5% de la déviation standard relative a été défini pour cette précision. Ils sont montrés dans l'**Annex H (b).**

La méthode a montré une précision qui ne dépasse pas les limites d'acceptation. Les quatre molécules peuvent être quantifiées avec précision en utilisant la méthode développée.

d. **Exactitude :** L'exactitude a été mesurée pour 3 niveaux de concentration différents de l'acide vanillique, le 4-hydrox benzaldéhyde et l'acide 4-hydrox benzoïque. La méthode de taux de récupération a été utilisée pour cette mesure. Un CRM a été utilisé pour la détermination de l'exactitude de la quantification de la vanilline.
Les calculs ont été analysés statistiquement. Pour le taux de récupération, le pourcentage de récupération a été utilisé, un test de différence significative « t-student » a été fait aussi afin d'appliquer ou non des corrections aux résultats.
Pour le CRM le pourcentage de récupération et de proximité avec la valeur réel a été déterminé.
Les résultats sont exprimés en %RSD (Pourcentage de déviation standard relative). Les bias ont été aussi calculés et utilisés pour les corrections correspondantes. Les résultats sont résumés dans **Table 17, 18** and **19** du texte original.
La vanilline, l'acide vanillique et le 4-hydrox benzaldéhyde ont montré une exactitude pour les taux de récupération qui sont comparables avec les résultats obtenus par Kumar *et.al* (2007) entre 94% et 104%. Cependant, l'acide 4-hydrox benzoïque n'est pas dans l'intervalle reporté par l'auteur, le taux de récupération pour ce composant est de 87%.
Plusieurs explications sont possibles, différents auteurs ont énoncé que le risque pris lors de la détermination du taux de récupération est dû à la différence de nature de la molécule

analysée. C'est-à-dire que le composant ajouté peut ne pas être en complet équilibre avec la matrice naturelle (le composant naturel) et pour cela empêcher des pertes.

e. **Limite de détection et de quantification :** Ces limites ont été évaluées en utilisant l'échantillon le plus dilué. La quantité d'analyte que peut fournir un signal avec un bruit (S/N) of >3 (LOD) a été considéré entre 0.02 et 0.12µg/ml, cela >10 (LOQ) a été considéré entre 0.07 et 0.38µg/ml.

La méthode a été validée pour les paramètres exigés par l'ICH, dont les valeurs correspondent aux critères définies. Ils sont aussi comparables avec les résultats obtenus par des autres auteurs. Pour terminer, la méthode peut être utilisée pour l'objectif défini (*cf.* **Annex I.**)

CONCLUSSIONS

Une vision globale de l'industrie aromatique a été présentée. Un bilan de la filière vanille a permit la compréhension de la problématique actuelle. La fraude dans cette industrie est évidente, on remarque surtout une différence de prix entre la vanille d'origine naturelle et d'origine synthétique, ce qui a explique la quantité de fraudes dans la filière.

Le consommateur actuel veut de plus en plus des produits naturels, et les falsificateurs en profitent. Pour toutes ces raisons, la réglementation est de plus en plus exigeante et a mis en place des méthodes précises qui servent à contrôler les fraudes. Cependant, les entreprises ont aussi un intérêt prope quant au développement de telles mesures.

Sevarome a voulu mettre en place une méthode d'analyse de la vanille, ce qui lui permet de créer des fiches techniques plus complètes, et ainsi de continuer à assurer et garantir la qualité et l'origine naturelle de ses extraits de vanille.

Une méthode d'analyse par HPLC a été développée et validée. La méthode ayant pour objectif la caractérisation des extraits de vanille a eu comme phase préliminaire une optimisation de la méthode d'extraction issue de la comparaison entre deux méthodes d'extraction.

On a pu démontrer l'efficience de l'extraction par ultrasons, comparée avec une méthode traditionnelle, la macération en bain marie. L'extraction assistée par ultrason est donc un procédé alternatif très prometteur. C'est une technologie émergeante très avantageuse et économique pour l'extraction des composants naturels, surtout si le coût de la matière première est vraiment significatif.

En général, il a été montré que la méthode permet l'identification et la quantification des quatre composants principaux de la vanille. La méthode a été validée et adaptée aux conditions de travail du laboratoire de Sevarome. On peut avoir des résultats précis et exacts sur un intervalle de concentrations qui est déterminée pour chaque composant.

La méthode est efficace et se positionne comme une base pour le développement de projets futurs. Elle sera utilisée pour l'optimisation des extractions de vanille, notamment pour la recherche des extraits plus concentrés en vanilline. Elle sera aussi utile lors du développement des fiches techniques plus complètes et pour des contrôles de qualité (basés sur les taux de vanilline) des extraits de vanille.

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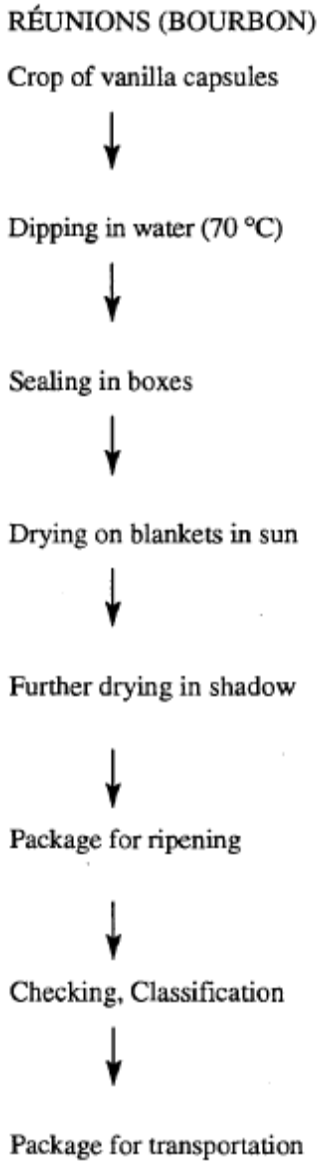
ANNEXES

Annex A. Brief description of Bourbon preparation of vanilla beans:

The Warming of vanilla beans/ Dipping in water:

It is the operation of mortification of the tissues of the "green" beans. A size selection is the first step. Vanilla pods are classified with the aim of improving process performance. Vanilla pods should have a certain maturity imposed by some regulations; in fact, green beans are harder to treat.



After classification, vanilla pods are ready to being scalded "warming" at 70°C for 3 minutes, this operation will stop maturation and starts the hydrolyze that converts the precursors to the rich flavouring elements. Time and temperature of thermal treatment can change in function of the global quality of vanilla beans.

Fermentation/ Sealing:

After warming, vanilla beans are quickly drained. The cooked beans are transferred in boxes padded with blankets during 24 to 72 hours. The temperature is constant; this allows the enzymatic reactions, needed for the development of the rich flavoring.

Drying in the sun:

The drying in the sun helps to stabilize the product. It also avoids the development of molds and them olfactive degradation.

For ten days, the beans dry naturally in the sun during the day and sweet in rolled blankets at night. This process improves the transformation of the beans aroma done this way help to keep a minimum of moisture. After the sun drying steep the beans continue to dry slowing. The entire curing process takes about three months.

Drying in the shade:

During this step, all of the enzymatic reactions that have started during the first stages continues to developing of the natural vanilla flavour. The drying step can last one to two months. Furthermore, the slowing drying on racks in the well-ventilated storage shed allows stabilizing vanilla beans.

A separation of split beans from that ones that have not been split, classification of colour and moisture contain imperative to ensure quality. The beans are packed to isolate the outside humidity that could ruin the quality. Finally, when the moisture content of the bean is reached, the cured process is considered ended.

Vanilla beans can be refined as wine or rum. Refining improves an evolution of the quality of the aroma. The refining period can last up to six months. The "dried" beans are stored in wooden boxes stuffed with sulfurised paper to limiting the desiccation of the beans. After a few weeks, the aroma of the vanilla greatly improves.

Annex B. Names for "non-natural" vanilla flavour and other chemicals names for vanillin, here a compilation of different references found in regulation text and research.

Names for "non natural" vanilla flavour	Other chemicals names for vanillin
Vanilla Flavour (not more than one once of synthetic vanillin) Vanilla – vanillin (not more than one once of synthetic vanillin) Artificially flavoured Artificial vanilla	Bensaaldehyde, 3-Ethoxy-4-Hydroxy Bourbonal Ethavan Ethovan 3-Ethoxy-4-Hydroxybenzaldehyde Ethyprotal Ethyl Vanillin 4-Hydroxy-3-Ethoxybenzaldehyde Protocatechuic Aldehyde Ethyl Ether Quantrovanil Vanillal Vanillin, Ethyl Vanirom

Table 22: Names for "non natural" vanilla flavour and other chemicals names for vanillin

Important remark:

"Natural vanillin is enriched in deuterium and carbon- 13 compared to synthetic vanillin and the adulteration of vanilla extracts by addition of synthetic vanillin can be determined using stable isotope ratio measurements made by high resolution mass spectrometry or nuclear magnetic resonance spectroscopy" (BELAY & POOLE, 1993).

Due to limitations of R&D laboratory in Sevarome, specifically for the absence of a High-Resolution Mass Spectrometry, the determination of carbon-13 was not carry-out on this research work.

Annex C. Summary and interpretation of the most significant regulations for food flavours and aromas and vanilla flavour.

REGULATION		
Decree n°66-319 (French Law)	"Fraud Control in the sale of goods and adulteration of foodstuffs concerning vanilla"	Precision concerning the use of term "vanilla": denomination reserved only for *Vanilla planifolia* fruit and species of Orchidasea family. (*see: article 2)*
Decree n°91-366	"Regulation for flavours"	Function of an flavour Description of the different categories of flavours Composition of a flavour Security and quality of flavours
Regulation (EC) n°178/2002	"Food law"	Security of products Conformity of products Control of conformity of products Traceability of products
Regulation (EC) n°1333/2008	"Food additives"	
Regulation (EC) n°1334/2008	"Flavours and ingredients"	Conditions of use of food ingredients with flavouring proprieties in and on foods. List of flavourings and source materials approved for use in and on foods. Referent to vanilla (*See: Article 3.2 and Annexe II in the text*)
Regulation (EC) 2232/96 **Regulation (EU) 872/2012**	"Flavouring substances"	This Regulation applies to flavouring substances that are used or intended for use in or in foodstuffs to impart **odour or taste**.
Regulation (EU) 873/2012	Concerning the Union list of flavourings and source materials set out in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and the Council.	
Directive 2010/59/EU	"Referent to the extraction solvents used in the production of foodstuffs and food ingredients"	Applies to extraction solvents used or intended for use in the production of foodstuffs or food ingredients. (*See: Annexe I of the text*)
NF T75-006 (French rule) or ISO 9235:2013	"Aromatic raw materials"	Definitions and vocabulary used in food flavouring industry.
NF ISO 5565-1:1999 Part 1	"Quality criteria for vanilla beans"	Importance of the vanillin content in vanilla beans for commercialization. **(Not obligatory)**
Order of June 11/1987	"HPLC Official method for quantification of the main constituents of vanilla"	The content of Vanillin, Aldehyde p-hydroxybenzoic, vanillic acid and p-hydroxybenzoic acid can be used as an element in quality control of vanilla beans and extracts.
Code of federal Regulations Title 21	Chapter 1: FDA "Food dressing and flavourings"	Requirements for Specific Standardized Food Dressings and Flavorings. Specifications of vanilla extracts.

Table 23: Summary of regulations for vanilla (aroma/flavour), interpretation

Annex D. Some of the developed HPLC methods for the analysis of vanillin and/or phenol compounds in vanilla pods.

Name of column	Mobile phase		Injection volume (µl)	Temperature (°C)	Elution type	Flow rate (ml/min)	Reference
	Modifier (A)	**Organic Phase (B)**					
Cromolith RP – 18e (100 X 4.6mm, 5µm)	$1X10^{-3}$ M K-PO_4, pH 3.1	Methanol	10	30	Gradient	1 - 2	(Maruenda et al., 2013)
ZORBAX Eclipse Plus C18 (4.6 X 150mm, 5µm)	Water 0.1%TFA	ACN 0.09% TFA	3	30	Gradient	1	(A.G. Huesgen (Agilent Technologies), 2011)
BDS – Hypersil C18	Water 0.2 pH 4.03	Butanol	5	-	Isocratic	1.2	(Lavine, Corona, & Perera, 2012)
Hypersil C18 RP (250 X 4.6mm, 5µm)	Water, H_3PO_4 (10^{-2}M)	ACN	5	30	Gradient	1.5	(Cicchetti & Chaintreau, 2009b)
Cromolith RP – 18e (100 X 4.6mm, 5µm)	Water 0.05%TFA	ACN	5	35	Isocratic	4	(U. K. Sharma, Sharma, Sinha, Kumar, & Gupta, 2009)
RP Purospher*Star RP 18e (250 X 4.6mm, 5µm)	Water/acetic acid, pH 2.88	ACN/Methanol (1:1)	20	25	Gradient	0.6 -1.5	(Arun Kumar Sinha et al., 2007)
Nucleosil C18	None	Water/Methanol (40:60)	20	-	Gradient	1	(Waliszewski et al., 2007)

Table 24: Comparison between other HPLC developed methods for vanillin analysis

Annex E. Goals determination for the development of the analytical HPLC method

How will the method be used?	The method will be useful for analytical research, for standardize natural vanilla extracts and for routine analysis of vanilla extracts for their quality control.	
Who will use the method?	The method will be utilized by a qualified operator previously training. The training will be conducted by the person who developed the method. This person will be responsible also for preparing the instructions of utilization of HPLC and the protocol of the developed method.	
What are the chromatographic goals?	Resolution Separation Quantification Characterization of the main constituents of a natural vanilla extract.	
What level of validation is required?	The method will be an R&D method but also will be following French regulations for quantification of constituents in vanilla extracts.	
Are sufficient resources available for adequate method development?	Time	Yes
	Personnel	Yes
	Materials	Yes
	Budget	Yes

Table 25: Determination of Goals for the HPLC method development.

Annex F. Spectres of the main compounds of vanilla extract.

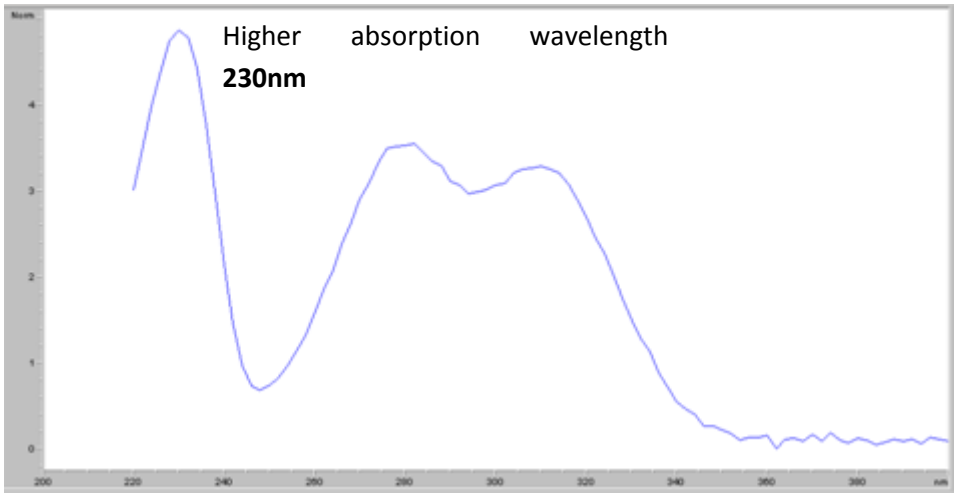


Figure 18: Vanillin spectrum

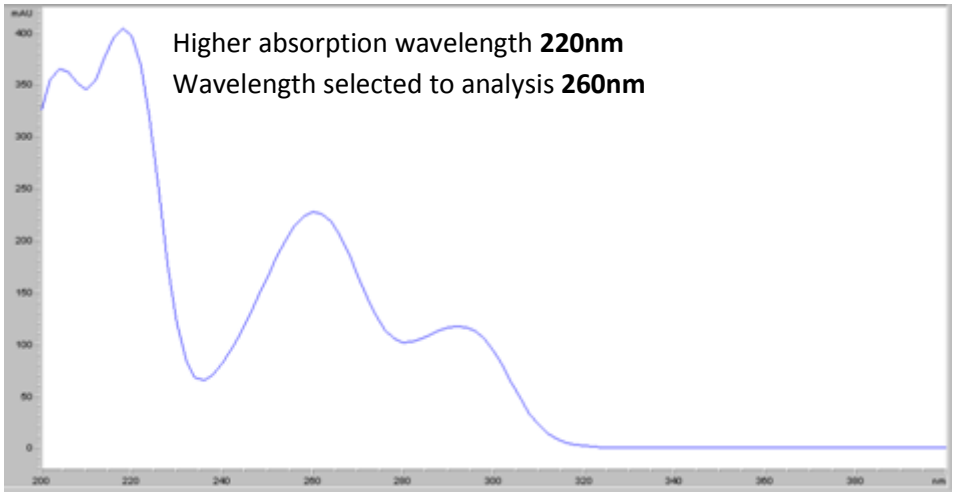


Figure 19: Vanillic Acid spectrum

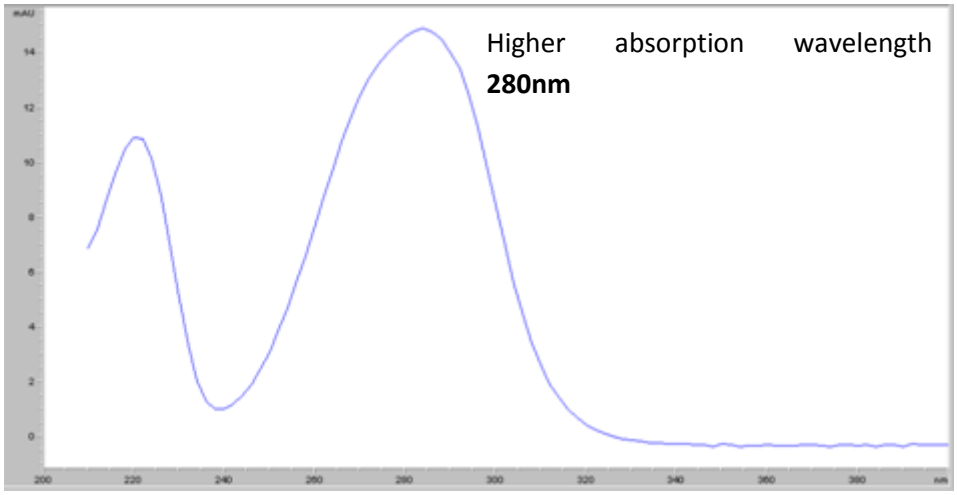


Figure 20: 4-Hydroxybenzaldehyde spectrum

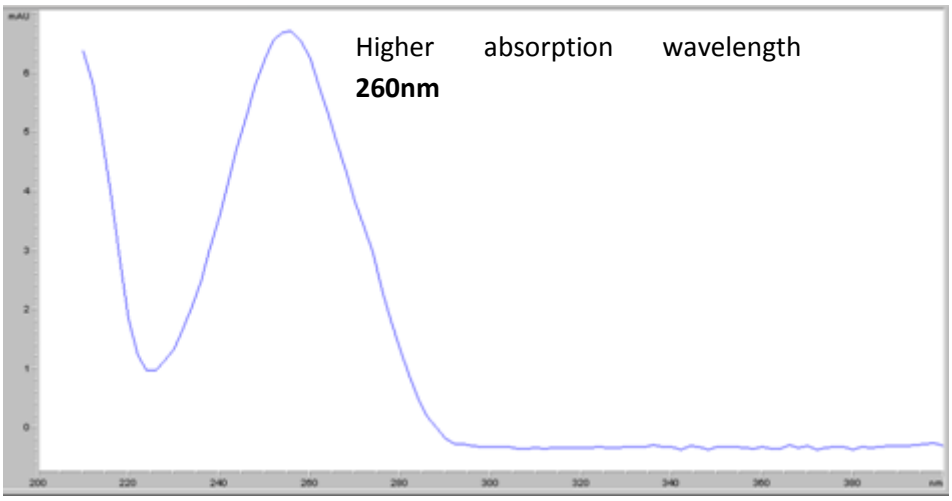


Figure 21: 4-hydroxybenzoic acid spectrum

The wavelength of analysis for each compound was selected in function of it spectrum between 230nm and 400nm.

Annex G. Chromatogram of separation, the main compounds of vanilla extract.

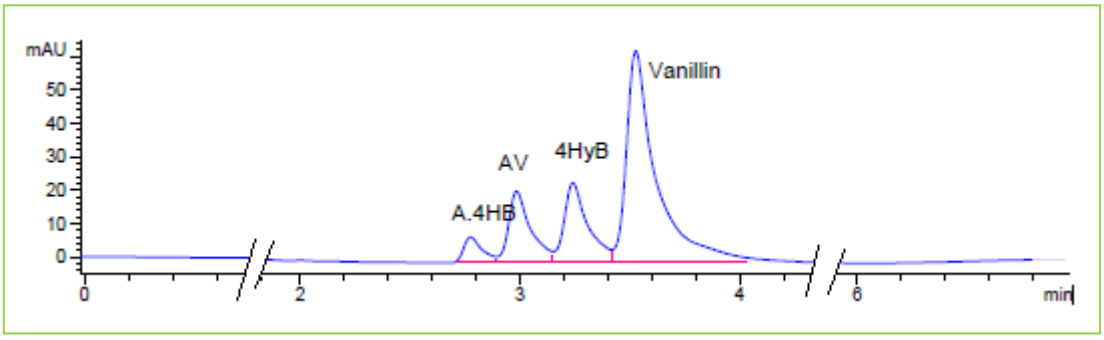


Figure 22: Baseline separation of the mean compounds of vanilla (Imitation of a real solution).

Figure 22 shows a separation chromatogram at 230nm. The first peak corresponds to 4-Hydroxybenzoic acid (A.4HB), the second peak corresponds to vanillic acid (AV), the third peak corresponds to 4-Hydroxybenzaldehyde (4HyB), and the fourth peak corresponds to vanillin. The separation succeeds with all standardized conditions.

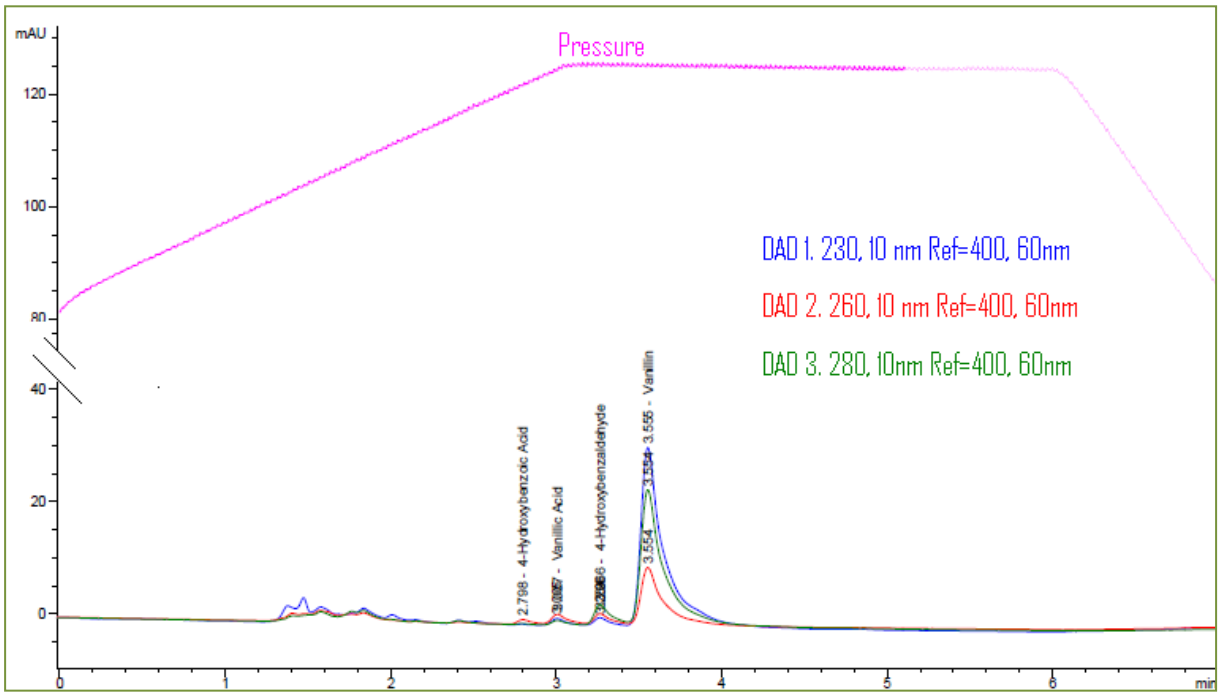


Figure 23: Chromatogram of Natural Vanilla Extract. Identification at t three different wavelengths: 230, 260 and 280nm for identification of each compound.

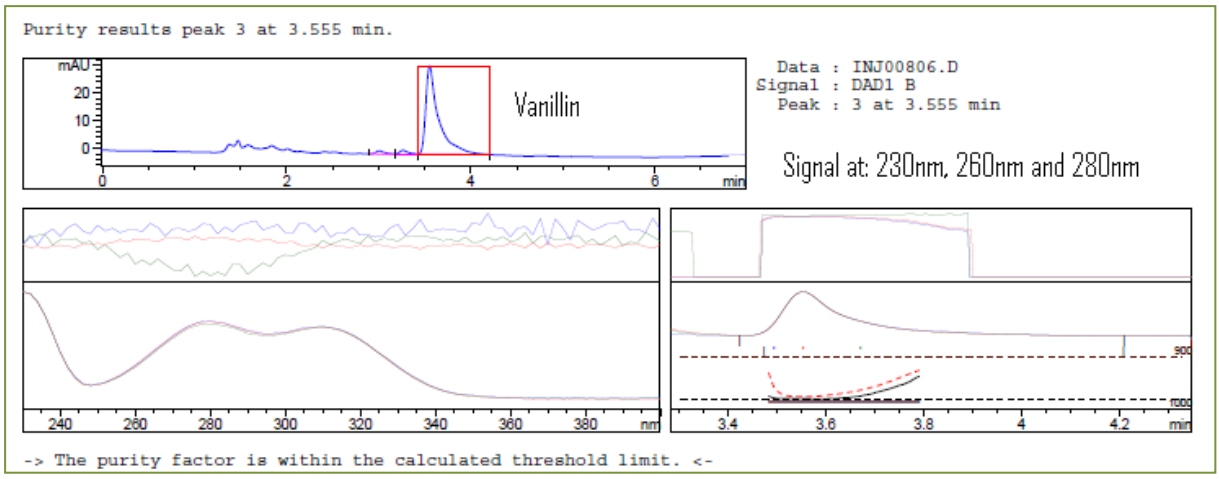


Figure 24: Purity calculations for Vanillin peak

Purity Factor with the calculated threshold limit:

Purity factor: 998.781

Threshold: 996.333

Reference: Peak starts and end spectra (3.474/4.207)

Spectra: 3 selected spectra (230nm, 260nm and 280nm)

Noise Threshold: 0.1

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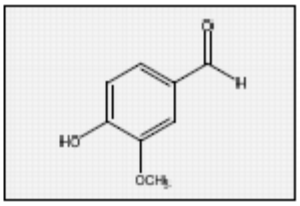
Product name:	**Vanillin**
Product no.:	**30304**
Lot no.:	BCBM7169V
Formula:	$C_8H_8O_3$
Molecular mass:	152.15
Traceability [2]:	NIST SRM 84l (KHP) and NIST SRM 350b (Benzoic acid)
Certificate issue date:	August 13, 2014
Expiry:	AUG 2016



Certified value and uncertainty according to ISO Guide 35 [3] and Eurachem/CITAC Guide [4]		
Substance	Certified value as mass fraction (g/g)	Expanded uncertainty, $U = k \cdot u_c$ ($k = 2$) as mass fraction (g/g)
Vanillin	**99.7 %**	**0.3 %**

Minimum sample: The sample is solid at room-temperature. 10 mg is recommended as the minimal sample amount. If less material is used, it is recommended to increase the certified uncertainty by a factor of two for half of sample and a factor of four for a quarter of sample.

Drying instruction: This material does not require drying before use.

Intended use: Use this certified reference material (CRM) as calibrant for chromatography or any other analytical technique.

Storage and handling: The CRM should be stored in the original bottle at room-temperature (20-25°C). After use the bottle should be tightly closed and protected from excessive moisture and light.

CRM operations: A. Rück
A. Rück, Ph.D.

Certification body:
K.-D. Schmidt, Ph.D.

SWISS CRM-PRODUCTION SRMS 001 **ISO Guide 34**

SWISS TESTING STS 490 **ISO/IEC 17025**

16368 **ISO 9001**

Sigma-Aldrich Production GmbH, Industriestrasse 25, 9471 Buchs/ Switzerland

SIGMA-ALDRICH

Annex H (a). Calculation of precision of areas

Table 26: Results of repeatability

	Concentration (µg/ml)	Area (mUA*s)	%RSD	Predicted (µg/ml)	Residuals (µg/ml)	% Error
Vanillin	34.65	417,91	1.0%	34,814	-0,165	0.30%
		413,51		34,473	0,176	
		416.31		34,691	-0,041	
	46.2	547,27	0.2%	44,835	1,365	2.80%
		549,41		45,000	1,199	
		548,70		44,945	1,254	
	184.8	2370,72	1.0%	186,076	-1,276	0.10%
		2338,40		183,572	1,228	
		2344,03		184,008	0,791	
Vanillic acid	2.05	18,10	1.0%	2,071	-0,021	0.30%
		18,06		2,066	-0,016	
		17,84		2,042	0,008	
	11.27	100,19	0.2%	11,300	-0,030	0.10%
		100,00		11,279	-0,009	
		99,83		11,260	0,010	
	33.82	302,61	0.3%	34,057	-0,237	0.40%
		301,80		33,966	-0,146	
		300,57		33,828	-0,008	
4-Hydroxybenzaldehyde	4.59	82,84	0.8%	4,414	0,176	4.50%
		82,56		4,398	0,192	
		81,52		4,338	0,252	
	18.36	333,90	1.3%	19,009	-0,649	2.03%
		327,61		18,644	-0,284	
		325,86		18,542	-0,182	
	91.5	1577,41	0.1%	91,299	0,201	0.10%
		1579,77		91,436	0,063	
		1580,53		91,481	0,019	
4-Hydroxybenzoic acid	5.7125	78,29	0.4%	5,730	-0,017	0.73%
		78,90		5,773	-0,061	
		78,70		5,759	-0,046	
	22.85	316,27	0.1%	22,731	0,119	0.46%
		316,12		22,720	0,130	
		316,96		22,780	0,070	
	45.7	639,66	0.2%	45,834	-0,134	0.07%
		637,75		45,697	0,003	
		637,38		45,670	0,029	

Annex H (b). Calculation of intermediate precision of areas

Table 27: Essays of intermediate precision

	Sequence	Level	Reference (µg/ml)	1 Result (µg/ml)	2 Result (µg/ml)	3 Result (µg/ml)
CRM-Vanillin	Day 1	1	12,5	13,6	13,5	13,6
		2	25	27,06	27,00	26,91
		3	125	117,70	118,40	118,24
	Day 2	1	12,5	13,93	13,45	13,35
		2	25	27,10	27,15	27,00
		3	125	118,43	118,61	118,90
Vanillic Acid	Day 1	1	11,25	12,9	12	11,99
		2	5,62	5,77	5,89	5,85
		3	2,81	2,94	2,93	2,89
	Day 2	1	11,25	12,02	11,97	11,86
		2	5,62	5,86	5,91	5,86
		3	2,81	2,96	2,96	2,90
4-Hydroxybenzaldehyde	Day 1	1	20,6	21,01	21,02	21,01
		2	10,3	10,64	10,51	10,58
		3	5,1	5,28	5,19	5,13
	Day 2	1	20,6	21,09	20,99	21,29
		2	10,3	10,59	10,59	10,59
		3	5,1	5,19	5,24	5,19
4-Hydroxibenzoic Acid	Day 1	1	1.8	1.65	1.63	1.55
		2	1	0.83	0.79	0.73
		3	0.55	0.47	0.44	0.49
	Day 2	1	1.8	1.57	1.56	1.57
		2	1	0.73	0.75	0.74
		3	0.55	0.53	0.48	0.48

Annex I. Data sheet of validation protocol.

Table 28 : Data Sheet of Validation Protocol

Product: Natural Vanilla extract		VALIDATION TYPE	
Compounds:	Vanillin	Analytic	
	Vanillic Acid		
	4-Hidroxybenzaldehyde		
	4-Hidroxybenzoic Acid		
ESSAYS		**SPECIFICATIONS**	**RESULTS**
1. *Selectivity*			
Solvent without sample (placebo)		Without interference	According
Peak purity		Whit the calculated threshold limit	According
Other compounds		Without interference	According
2. *Linearity*			
Correlation coefficient (r)		<0.999	0.999
Determination coefficient (r^2)		<0.997	0.998-0.999
Linearity between *x* and *y*		F observed > F critical	According
t-Student value for 0.05 of confidence		t-student > 2.306	According
3. *Precision*			
%RSD		>3.0%	0.02 - 1.3%
4. Accuracy			
Residuals (Bias %)		>5%	2 – 4%
CRM (only for vanillin)			4% *13%[2]
5. LOD LOQ		Enough to quantify an average sample	According

[2] Only for the compound 4-Hydroxybenzoic Acid