UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE FARMÁCIA

MICHELY CAPOBIANGO

# ASSESSMENT OF VOLATILE ORGANIC COMPOUNDS IN DIFFERENT ALCOHOLIC FERMENTATION PROCESS OF BANANA *TERRA* PULPS AND IDENTIFICATION OF AROMA-IMPACT VOLATILES IN BANANA SPIRIT

Belo Horizonte, MG
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**MICHELY CAPOBIANGO**

***Assessment of volatile organic compounds in different alcoholic fermentation process of banana terra pulps and identification of aroma-impact volatiles in banana spirit***

**TESE APROVADA EM 09 DE OUTUBRO DE 2014**

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*I dedicate this thesis to my dear mom!*

*Eu dedico este trabalho à minha querida mãe!*

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## ABSTRACT

Banana (Musa *spp*.) is an important food crop, which grows extensively in tropical and subtropical regions. Losses in fruit production represent a significant cost in the market. In this context, developing alternative products for banana is imperative. Considering its nutritional value and aroma quality, manufacturing of banana-based, fermented and spirit beverages is of great interest to the industry. It could also influence the region's economy by adding value on the product. Firstly, in this study, the effects of enzymatic treatment, centrifugation and commercial strain of yeast were investigated during the production of alcoholic fermented banana *Terra*. The impacts of these factors were evaluated according to the following kinetic parameters: ethanol yield, efficiency and productivity, and the profile of the volatile organic compounds (VOCs). The volatile composition of different fermented must was determined by headspace solid phase microextraction (SPME) using gas chromatography coupled with mass spectrometry detection (GC/MS). All the kinetic parameters, ethanol yield, efficiency and productivity were significantly different ($p<0.05$). The optimum results, achieving a maximum ethanol yield (85.97 % and 86.39 %, respectively) were shown employing enzymatic treatment, wet commercial yeast and with or without centrifugation of the must. Two selected strains from production of cachaça of *Saccharomyces cerevisiae* UFMGA-1007 and UFMGA-1031 were also tested to improve yeast performance alcoholic fermentation, using the select previous conditions but the results showed a lower ethanol yield for both yeast (39.83 and 42.24%, respectively). Twenty-two compounds of distinct chemical classes were analyzed by SPME GC/MS, including alcohols, esters, organic acids, aldehydes, ketones, among others. The concentrations of the volatile compounds differed depending on the fermentation condition. The values of higher alcohols per 100 mL of anhydrous alcohol in the fermented banana ranged from 353 to 1017 mg. The fermented banana *Terra* studied showed a volatile composition similar to other fermented fruit. Secondly, this work aimed to characterize the banana *Terra* spirit aromatic compounds obtained by alcohol fermentation and distillation using enzymatic treatment, wet commercial yeast and without centrifugation of banana must. A multidimensional gas chromatography (MDGC) in a multi-hyphenated system – i.e. coupled to flame ionization detection (FID), MS, and olfactometry (O) were employed to analyze the sample. SPME was used to isolate the headspace of

the banana spirit aromatic compounds. The detection frequency (DF) technique was applied, and aroma regions detected in $^{1}$D GC separation with over 60 % Nasal Impact Frequency (NIF) value were screened as the target potent odor regions in the samples. GC/O analysis enabled identification of 18 aroma-impact regions, such as those comprising spicy, whisky, fruity, and others. Using a polar/non-polar phase column set, the potent odor regions were further subjected to MDGC separation with simultaneous MS and O detector for identification of individual resolved aroma-impact compounds. Compounds were tentatively identified through mass spectral data matching and retention indices in both first and secondary dimensions. The principal volatile compounds identified in this work were 3-methylbutan-1-ol, 3-methylbutan-1-ol acetate, 2-phenylethyl acetate and phenylethyl alcohol and they were responsible for the characteristic aroma of banana spirit This is the first such study that reveals important information about the major aroma compounds that contribute to the banana spirit aroma.

**Keywords*:*** Alcoholic fermentation. Banana *Terra* spirit. Olfactometry. Volatile Organic Compounds. MDGC. GC×GC/FID

## RESUMO

A Banana (Musa *spp*.) destaca-se no cenário mundial de produção de frutas. O elevado índice de perdas pós-colheita representa um custo significante à toda cadeia produtiva. A fruta, mesmo no seu último estágio de maturação, continua apresentando características de boa qualidade, e desenvolvimento de produtos à base de banana torna-se uma alternativa de industrialização. Considerando o seu valor nutricional e aroma, a produção de bebidas a base de banana, como o vinho e a aguardente, são opções para a indústria. Primeiramente, o objetivo do presente estudo foi investigar os efeitos da hidrólise enzimática com enzimas pectinolíticas e da centrifugação do mosto de banana e do emprego da levedura comercial (fermento úmido e seco) na fermentação alcoólica para produção do mosto fermentado de banana, variedade *Terr*a. O impacto destes fatores foram avaliados pelos parâmetros cinéticos de fermentação (rendimento em etanol, eficiência e produtividade) e pelo perfil dos compostos orgânicos voláteis (COVs). Os COVs foram extraídos via headspace por microextração em fase sólida (SPME) e analisados por cromatografia gasosa com detector de espectrometria de massas (GC/MS). Todos os parâmetros cinéticos de fermentação apresentaram diferença estatística significativa ($p<0,05$). O emprego da hidrólise enzimática, do fermento comercial úmido com o mosto centrifugado ou não centrifugado apresentaram o máximo de rendimento em etanol (85,97 % e 86,39 %, respectivamente). Duas linhagens selecionadas para produção de cachaça de *Saccharomyces cerevisiae,* UFMGA-1007 e UFMGA-1031, também foram empregadas na fermentação alcóolica de banana *Terra* hidrolisada e centrifugada, porém apresentaram baixos valores de rendimento em etanol (39,83 e 42,24%, respectivamente). Foram identificados 22 COVs de diferentes classes incluindo álcoois, ésteres, ácidos orgânicos, aldeídos, cetonas dentre outros. As concentrações dos COVs foram distintas para cada condição testada. O valor de álcoois superiores por 100 mL de álcool anidro nos vinhos de banana variou de 353 a 1017 mg. Os fermentados alcóolicos de banana *Terra* apresentaram o perfil dos compostos voláteis similar à outros fermentados de frutas. Sequencialmente, o segundo objetivo deste trabalho foi caracterizar os compostos odoríferos da aguardente de Banana *Terra*, obtida pelas condições de fermentação alcóolica previamente estabelecidas seguida de destilação. Para tal foi empregada a cromatografia gasosa multidimensional (MDGC), utilizando um sistema

acoplado aos detectores de ionização por chama (FID), MS e olfatometria (O). Os COVs foram extraídos por via headspace por SPME. As regiões de importância aromática foram identificadas pela técnica de detecção por frequência (DF) por $^{1}$D GC/FID/O empregando 60 % de Frequência de Impacto Nasal (NIF) e pela análise por GC×GC/FID. Pela análise olfatométrica foi possível identificar 18 regiões de aroma de impacto, dentre algumas das descrições dos compostos destacam-se aroma floral, uísque e frutado. Um conjunto de colunas polar/apolar foi empregado na separação subsequente por MDGC com detecção simultânea no MS e O. Os principais compostos responsáveis pelo aroma característico da aguardente de banana *Terra* neste trabalho foram 3-metilbutan-1-ol, etanoato de 3-metilbutan-1-ol, etanoato de 2-feniletila e 2-feniletanol. Este estudo destaca-se por apresentar, pela primeira vez, os principais compostos odoríferos responsáveis pelo aroma da aguardente de Banana *Terra* e apresenta a necessidade de dar continuidade ao estudo dos demais compostos característicos das aguardentes de frutas.

**Palavras-chave**: Fermentação alcóolica. Aguardente de Banana *Terra*. Olfatometria. Compostos Orgânicos Voláteis. MDGC. GC×GC/FID.

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# 1. INTRODUCTION

Tropical fruits have been used as substrates for the production of fermented beverages and spirit drinks. Banana is one of the most consumed fruits in the world. It has a rich aromatic flavour, nutritional value and contains a high concentration of sugar (SILVA, 2004; AURORE; PARFAIT; FAHRASMANE, 2009). These characteristics could provide yeast growth and the action of yeast to convert the sugars into alcohol, volatile organic compounds (VOCs) and non-volatile compounds. Although bananas are widely produced and consumed, the amount of fruit waste generated is in constant increase and might result in production losses as much as half the crop (PEREIRA *et al.*, 2008; AURORE; PARFAIT; FAHRASMANE, 2009; FAO, 2013a). The large amount of these wastes presents the problem of disposal without causing environmental pollution. Thus, the valorization and processing industry of this by-product have come up with more useful applications of these agro-waste foods (EMAGA *et al.*, 2007). The banana on the last stage of maturation doesn't have commercial value but still having the nutritional value, which can be used in the production of fermented fruit and spirit, and also as an ingredient in other products, including pies, cookies, juice, flour.

Recently, new spirits obtained by distillation of fermented from fruits have been attracting new markets. Especially the distillates industry, that have demonstrated large interest in producing novel alcoholic beverage from residue or unusual raw materials (SAMPAIO *et al.*, 2013). In this way, banana spirit has been produced using the banana residue, which is the banana in the last stage of maturation without commercial value.

Alcoholic beverages are highly complex mixtures of compounds, especially by VOCs. These compounds define its aroma, appearance and taste properties. So, these compounds are the most important parameters responsible for the beverages character, quality and hence for consumer acceptance. Different factors influence the composition and concentration levels of VOCs. In addiction, to the parameters involved when processing wine and spirits, such as metabolic pathways and their genesis fermentation, distillation and ageing, others are oak derived influence the presence of VOCs (OLIVEIRA; ROSA*; et al.*, 2005; PLUTOWSKA; WARDENCKI, 2008; CARDEAL; MARRIOTT, 2009; SAMPAIO *et al.*, 2013).

Besides, the legislation steps for the production process to obtain the fermented and the spirit fruits is absent and fermentation is generally carried out empirically. As a result, there may be variations in ethanol yield, efficiency, productivity and organoleptic characteristics of the beverage, including the aroma compounds (OLIVEIRA; CARDELLO*; et al.*, 2005; IVANOVA *et al.*, 2012).

Hence, these factors must be studied with more details, especially their effects on quality of alcoholic fermented of banana. For these reasons, the first aim of the present study was to evaluate the influence of enzymatic treatment, filtration and yeast strains by the kinetic parameters and the VOCs profile of the banana's must fermented. The kinetic parameters evaluated were ethanol yield, efficiency and productivity. Furthermore, the profile volatile composition of different must fermented was analyzed by SPME-GC/MS.

The volatile odour compounds provide important information about the quality assessment and organoleptic characteristics present in alcoholic beverages and it's attributes for consumer's preference. For this reason it is essential to investigate their quantitative and qualitative composition, by both chemical and sensory analysis (PLUTOWSKA; WARDENCKI, 2008).

Gas chromatography with olfactometry detection (GC/O) has shown to be a valuable method for identifying aroma active compounds from a complex mixture as spirits. The aroma perception is detected and evaluate by the human assessors sniffing the eluate from a GC separation via a specifically designed odour port (RUTH, 2001; DELAHUNTY; EYRES; DUFOUR, 2006). Experience shows that the application of GC/O in complex products such spirits, peak overlap can occur in odour-active regions, leading to ambiguous or problematic peak identification (VILLIÈRE *et al.*, 2012). A powerful solution is to use a heart-cut (H/C) multidimensional GC/olfactometry (MDGC/O) to resolve this problem. These co-eluting peaks were heart-cut from the first column ($^1$D) and cryotrapped before subsequent release and separation in the second column ($^2$D) in the MDGC/MS/O system. Another possibility is use comprehensive GC (GC×GC), which offers a fast and simple approach for analysis of an entire sample's profile via two different separation mechanisms on $^1$D and $^2$D capillary columns. However, the combination of GC×GC and olfactometry is very complicated for judges. This is because the human breathing cycle is about 3-4 s, which is too slow to reliably assess the narrow, rapid eluting peaks produced by GC×GC (DELAHUNTY; EYRES; DUFOUR, 2006; EYRES; MARRIOTT; DUFOUR, 2007;

MARRIOTT; EYRES; DUFOUR, 2009; VILLIÈRE *et al.*, 2012; MAIKHUNTHOD; MARRIOTT, 2013).

Additionally, the second part of this study was carried out to identify, for the first time, the key aroma compounds in banana *Terra* spirit by means of GC/FID/O, GC×GC/FID and H/C MDGC/MS/O. The aroma regions obtained from GC/O were aligned with parallel GC×GC/FID contour plots, and then correlated with targeted for better identification in the H/C MDGC mode with simultaneous olfactory and MS detection.

# 2. CHAPTER 1: LITERATURE REVIEW

## 2.1. Banana production and properties

Banana (*Musa* spp.) is an important fruit crop, which is grown extensively in tropical and subtropical regions and is an economically essential food product. Considering nutrition aspect and an attractive aroma, it is widely consumed throughout the world. Its aroma is one of the most significant factors, which determines the character and the quality of this fruit (AURORE; PARFAIT; FAHRASMANE, 2009; SELLI *et al.*, 2012).

The world production of bananas in 2012 was at 101,992,743 tonnes and the main producers were India, China, Philippines, Ecuador and Brazil, respectively (FAO, 2013b). Banana is an important fruit crop in Brazil with an annual production of 6,902,184 tonnes in 2012 (IBGE, 2014). This production was mainly concentrated in the northeast and southeast of the region. The most widespread varieties in Brazil are “Prata” (*Musa* AAB, Prata subgroup), “Cavendish” or “Nanica” (*Musa* AAA, Cavendish subgroup), “Maçã” (*Musa* AAB, Maçã subgroup) and *Terra* (*Musa* AAB, Plantain subgroup) (PEREIRA *et al.*, 2008; AURORE; PARFAIT; FAHRASMANE, 2009). The variety *Terra* is commonly located in local food markets in Africa, the Caribbean and Latin America and is frequently used for desserts and cooking. It is very similar to other varieties, although often larger and their flesh is starchy (EMAGA *et al.*, 2007). Although the literature reports alcoholic fermentation experiments with other varieties of banana, this is the first time that this experiment has been done using banana *Terra*.

Although bananas are widely produced and consumed, losses from poor handling, packaging and transportation mean a significant cost for economy, and might result in the wastage of as much as half the crop, achiving 40 to 60% of the production (PEREIRA *et al.*, 2008; AURORE; PARFAIT; FAHRASMANE, 2009; FAO, 2013a). However, the banana on the last stage of maturation have a rich substrate and can be used for industrial purposes, as an ingredient in other products, including pies, cookies, juice, flour and beverages, such as wine, beer and spirits. (EMAGA *et al.*, 2007; AURORE; PARFAIT; FAHRASMANE, 2009). Production of beverages using banana residue is considered in this study because of their abundance, relatively high

concentration of fermentable sugars and provision of yeast growth. (SILVA, 2004; BOGUSZ JUNIOR *et al.*, 2006).

A possible alternative to provide new resources, innovative products and minimize crop wastage is using the banana on the last stage of maturation for the production of alcoholic fermented and spirit of banana. Agricultural residues are potential raw materials for this purpose due to their low cost and different flavours, attracting new markets. In this sense, the current commercialization of known alcoholic Brazilian beverages obtained from sugarcane, especially *cachaça*, could facilitate market penetration of fruit beverages, such as alcoholic fermented and spirit of banana. In addition, *cachaça* has been recognized for its quality worldwide and manufacturing is limited during sugarcane harvesting seasons. However, between harvesting periods, stills can be used for the distillation of banana spirits, thus optimizing production site (BOGUSZ JUNIOR *et al.*, 2006; AURORE; PARFAIT; FAHRASMANE, 2009; SAMPAIO *et al.*, 2013).

## 2.2. Alcoholic beverages from fruits: fermented must and banana spirit

Brazilian Legislation, through law number 6871 (BRASIL, 2009) classifies two kinds of alcoholic fruit beverages; the first applies to those fermented from a unique type of fresh fruit, its juice or pulp with an alcohol content of 4 to 14 % v/v at 20 ℃. The second refers to fruit spirits obtained directly from the fermented of fresh fruit or must of fruit, with or without stones, by distillation with an alcohol content of 36 to 54 % v/v at 20 ℃.

There are many studies in the literature that have investigated the suitability of fruits to produce alcoholic beverages, include the potential use for wine and spirits. (HERNÁNDEZ-GÓMEZ; ÚBEDA; BRIONES, 2003; REDDY; REDDY, 2005; DUARTE; DIAS; OLIVEIRA; TEIXEIRA*; et al.*, 2010; OLIVEIRA *et al.*, 2011). The production of alcoholic fermented from other fruit than grapes have increased in last years such as kiwi (BORTOLINI; SANT'ANNA; TORRES, 2001), mango (REDDY; REDDY, 2005), raspberry (DUARTE; DIAS; OLIVEIRA; VILANOVA*; et al.*, 2010), cagaita (OLIVEIRA *et al.*, 2011), *masau* (NYANGA *et al.*, 2013).

Spirits are distilled beverages obtained by distillation of fermented must usually produced from grains or fruits (SAMPAIO *et al.*, 2013). They are widely consumed all over the world, and an example of these spirits commercially includes whisky, vodka,

rum, *cachaça*, and tequila (HERNÁNDEZ-GÓMEZ; ÚBEDA; BRIONES, 2003; OLIVEIRA; CARDELLO*; et al.*, 2005; PINO *et al.*, 2012). Other distilled alcoholic beverages are obtained from fermentation and distillation of fruits. Some of these are very popular, especially those produced from grape marc, such as grappa from Italy (LÓPEZ-VÁZQUEZ *et al.*, 2010), bagaceira from Portugal (CORTÉS *et al.*, 2011) and with other fruits as melon (HERNÁNDEZ-GÓMEZ; ÚBEDA; BRIONES, 2003), orange (DA PORTO; CORDARO; MARCASSA, 2006), pear (GARCÍA-LLOBODANIN *et al.*, 2007), jaboticaba (ASQUIERI; SILVA; CÂNDIDO, 2009) and cherry (NIKIĆEVIĆ *et al.*, 2011).The flow chart about the production of banana spirit was presented in Figure 2.1 following Lara (2007) process. The conditions of the process can influence the formation of volatile compounds and the quality of spirit. The second chapter of this research focused in three main variables employed in alcoholic fermentation of banana must: the enzymatic hydrolysis, centrifugation and yeast strain. The steps, hydrolysis and centrifugation can or not be applied to obtained the beverage. The hydrolysis was applied on the banana must. The juice of banana must was called when the centrifugation was used. After the fermentation it was obtained the fermented must. And the favourable alcoholic fermentation conditions were chose to obtained the distillate after the distillations for analyzed their aromatic composition (Chapter 3).

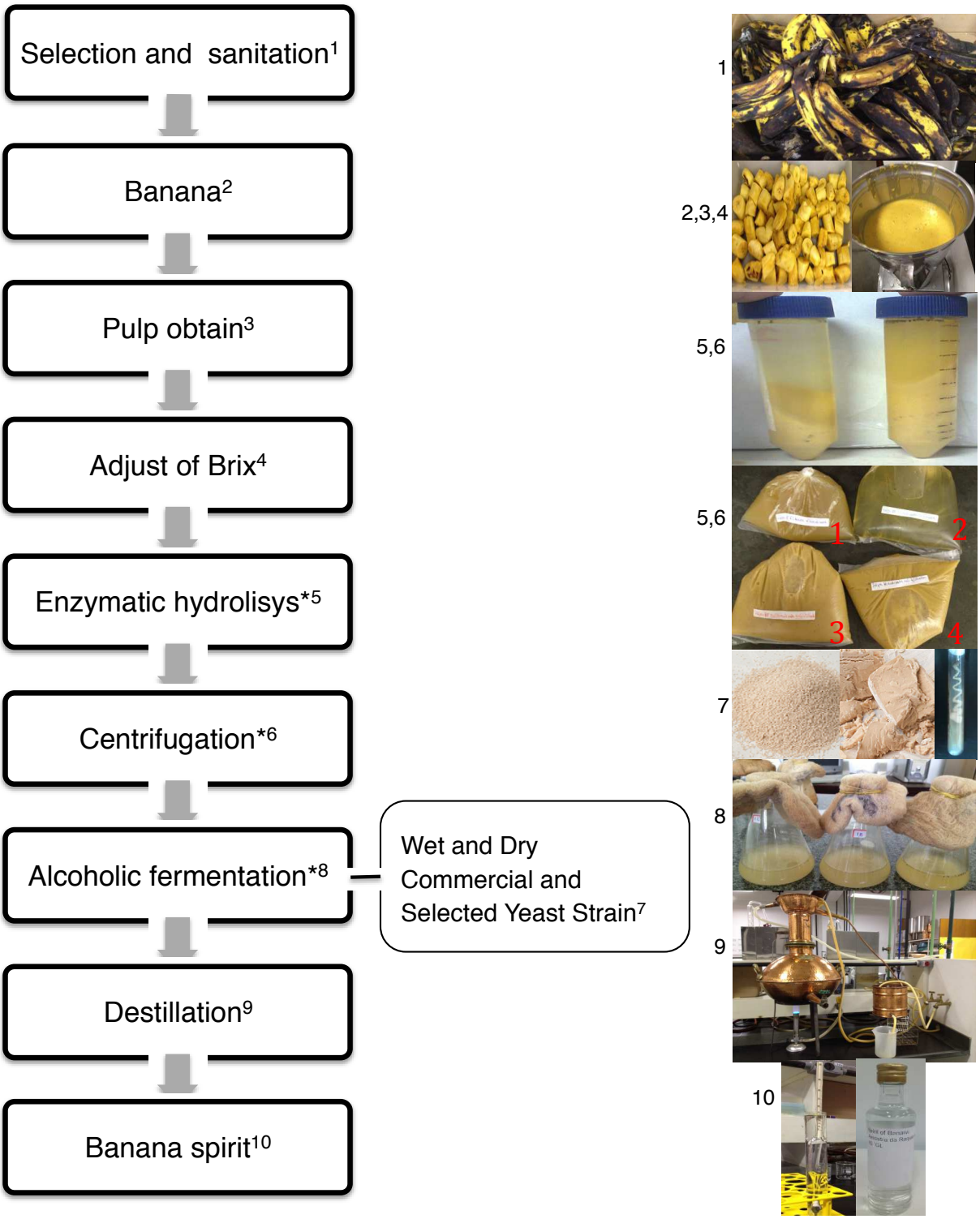


FIGURE 2.1 - Flow chart of banana spirit production
From: own Author, 2014
*Variables that was evaluated in this study
[5-6]Treatment of Banana Pulp: 1-Non-hydrolyzed and Centrifuged; 2-Hydrolyzed and Centrifuged; 3-Hydrolyzed and Non-Centrifuged; 4-Non-hydrolyzed and Non-centrifuged

## 2.3. Influence of parameters in alcoholic fermentation

Fruit wine and spirits are defined by the legislation (BRASIL, 2009), however, legislation concerning the production process is absent. In this sense, alcoholic fermentation and distillation are generally carried out empirically. As a result, there may be variations in ethanol yield, efficiency and productivity. Even more, the quality of the final product, including the aroma is related with the chemical composition, which is directed related with the presence and concentrations of volatile organic compounds (VOCs) of the beverages (OLIVEIRA; CARDELLO*; et al.*, 2005; REDDY; REDDY, 2005; DUARTE; DIAS; OLIVEIRA; VILANOVA*; et al.*, 2010). So the production of these beverages is influenced by different factors involving a complex making process. In this context, coming up with a controlled process with known conditions such as filtration, enzymatic treatment and yeast strain could lead to the production of a commercial valued beverage.

The enzymatic treatment has been used in juice extraction and clarification during the production of beverage with aim to filter the must and wine, which accelerates fermentation and alcohol yield (CARDOSO *et al.*, 1999; LEE *et al.*, 2006). It also increases the physic-chemical stability and strengthens the aromatic profile (JAYANI; SAXENA; GUPTA, 2005). The process of filtration removes spongy substances that could decrease the amount of higher alcohol. These practices have been used in the food industry, biotechnology and particularly in alcoholic beverages to improve the process (HSIEH *et al.*, 2013).

A number of studies have demonstrated the key role of yeast strains on the alcoholic fermentation, producing the greater part of the aromatic compounds present in fermented fruits (DATO; JÚNIOR; MUTTON, 2005; OLIVEIRA; CARDELLO*; et al.*, 2005; ALVARENGA *et al.*, 2011). The yeast strain *Saccharomycces cerevisiae* (*S. cerevisiae*) is extensively used in the production of different alcoholic beverages, due to its positive performance in higher ethanol production (OLIVEIRA; CARDELLO*; et al.*, 2005). The influence of yeast in the formation of VOCs in the fermentative process must be taken into account during the selection of suitable *S. cerevisiae* that impact the quality of alcoholic beverages. The beverage industries are greatly interested in yeast strains that produced a unique flavour and have a positive performance to achieve high ethanol yields and productivity (DATO; JÚNIOR; MUTTON, 2005; OLIVEIRA; ROSA*; et al.*, 2005).

## 2.4. Evaluation of methods used for the analysis of volatile organic compounds of sugarcane (cachaça) and fruit spirits

The chemical assessment of alcoholic drinks should contribute to the attainment of a high level of consumer protection, the prevention of deceptive practices and the attainment of market transparency and fair competition. Assessing the chemical composition of wine and spirit drinks should safeguard the reputation that locally produced alcoholic beverages have achieved in the community and on the world market by continuing to use the traditional practices for the production of wine and spirit drinks while also meeting an increased demand for consumer protection and information. With respect to the export of high-quality wine and spirit drinks, these drinks can be evaluated to maintain and improve the reputation of drinks on the world market. There are many methods that have been developed and tested for the analysis of volatile organic compound content from alcoholic beverages matrices reported in the literature. The scope of this review was intended to present the main analytical methodologies used in the analysis of VOCs because there is not a consensus about the best method for these analysis.

### 2.4.1. Volatile organic compounds from sugarcane and fruit spirits

The quality of spirits depends mainly on the composition of the volatile organic compounds present. Distilled beverages, such as sugarcane and fruit spirits, are organoleptically characterised by the presence of these compounds that fall into the following classes: organic acids, aldehydes (including furfural), esters, higher alcohols, terpenes, lactones, furans, pyrazines and others (NONATO *et al.*, 2001; CARDEAL; MARRIOTT, 2009; NASCIMENTO; CARDOSO; FRANCO, 2009; SILVA *et al.*, 2009)

The nature and quality of these compounds depends on the raw materials, fermentation, distillation, aging and wooden materials used for storage. These factors modify the chemical composition and sensory aspects of the beverage, affecting its aroma and flavour (DATO; JÚNIOR; MUTTON, 2005; SOUZA *et al.*, 2006; SILVA *et al.*, 2009). Several studies suggest that differences in aromatic alcohol components can be principally attributed to different yeast strains (DATO; JÚNIOR; MUTTON, 2005; SILVA *et al.*, 2009).

Some of these compounds are considered favourable, such as ethyl esters of long chain fatty acids and small concentrations of higher alcohols, acetaldehyde and ethyl lactate; however, they are responsible for an unpleasant flavour when present in high concentrations. In contrast, others such as methanol, furfural and ethyl carbamate are considered toxic (GARCÍA-LLOBODANIN *et al.*, 2007; CARUSO; NAGATO ; ALABURDA, 2010).

According to Brazilian legislation, the volatile components of sugarcane spirits (secondary components), except alcohol, are established as the sum of volatile acids, total esters, aldehydes, furfural/hydroxymethylfurfural and higher alcohols. The coefficient of congeners in 100 mL of anhydrous ethanol must be between 200 and 650 mg (BRASIL, 2005a). The maximum limits for each component of this coefficient of congeners in 100 mL of anhydrous ethanol is 150 mg for volatile acidity (expressed as acetic acid), 200 mg of esters (as ethyl acetate), 30 mg of total aldehyde (expressed as acetaldehyde), 5 mg of furfural and hydroxymethylfurfural and 360 mg of higher alcohols (expressed as the sum of n-propyl, isobutyl and isoamyl alcohols) (BRASIL, 2005a). The European Union defines the volatile substance content as the quantity of volatile substances other than ethyl alcohol and methanol contained in a spirit drink obtained exclusively by distillation, as a result of the distillation or redistillation of the raw materials equal to or exceeding 200 mg 100 $mL^{-1}$ of 100 % v/v alcohol (EC, 2008).

Sugarcane spirits contain several harmful organic compounds, such as methyl alcohol, ethyl carbamate, acrolein (2-propenal) and diacetyl (2,3-butadione) that must be monitored according to the maximum quantities allowed by legislation. The total phenolic compounds can also be measured in aged sugarcane spirits and *cachaças* (BRASIL, 2005a).

The volatile acidity, expressed as acetic acid, is an important parameter correlated to the sensory characteristics of distilled alcoholic beverages (BOGUSZ JUNIOR *et al.*, 2006). The volatile acid content of *cachaça* and spirit drinks may be influenced by several factors, such as the time and temperature of fermentation, type of yeast used, and the management of the must and hygiene in the beverage manufacturing process (PARAGGIO; FIORE, 2004; CARDOSO, 2006; VILELA *et al.*, 2007).

Ethyl acetate is the major ester present in distilled beverages, representing 75 % of all esters on average. However, other aliphatic esters may be formed from different sources such as ethyl butanoate, which originates during alcoholic

fermentation via the intracellular secondary metabolism of yeast (NONATO *et al.*, 2001; NASCIMENTO; CARDOSO; FRANCO, 2009), esters generated by the interconversion of phenolic compounds such as ethyl syringate and ethyl vanillate, and esters extracted from wood (methyl homovanillate and methyl syringate) as a consequence of the aging process (VICHI *et al.*, 2007). Nascimento; Cardoso; Franco (2008) Nascimento; Cardoso; Franco (2009) reported the presence of ethyl lactate in spirits, stressing the fact that this is the second most abundant ester in *cachaça*, representing an average of 26.8 % of the total ester content. The presence of ethyl lactate in *cachaça* is related to the contamination of must by bacteria (*Lactobacillus* spp.) responsible for lactic acid fermentation, as well as poor control of alcoholic fermentation.

Dato; Júnior; Mutton (2005) compared the composition of *cachaça* produced by different yeast strains. *Saccharomyces cerevisiae* was employed as a reference along with wild yeast strains (*Pichia silvícola, Pichia anomala and Dekkera bruxelensis*). The levels of volatile organic compounds (VOCs) were determined by gas chromatography. The authors observed that VOCs concentrations were influenced by the pH of the respective must, which depends on the yeast strain used in the fermentation process. *S. cerevisiae* presented slightly higher concentrations of esters, carboxylic acids and methanol, while the wild strains produced more of the higher alcohols. The wild yeast strains used were demonstrated to be adequate for the production of high quality *cachaça*. The findings of this study corroborated those of other researchers who reported a lower production of higher alcohols by *S. cerevisiae* and higher contents of ethyl acetate, suggesting that the strains that produce greater concentrations of higher alcohols also generate lower ester contents (LONGO *et al.*, 2002). Aldehydes, although normal constituents of wines and spirits, are compounds that decrease the quality of spirits and undesirably modify its flavour and aroma. Ingestion of aldehydes may interfere with the central nervous system and, in excess, may induce nausea, vomiting, headache, decreased blood pressure and tachycardia (BOGUSZ JUNIOR *et al.*, 2006; VILELA *et al.*, 2007; SILVA *et al.*, 2009). Aldehyde formation is directly linked to the strain of yeast used, distillation conditions and the aging process (DATO; JÚNIOR; MUTTON, 2005). The formation of furfural and hydroxymethylfurfural may be associated with an inappropriate temperature of distillation and the presence of non-volatile compounds in the spirit. The burning of sugarcane leaves also influences the synthesis of furfural and can adversely affect

product quality. The burning of leaves before harvest generates combustion residues, including solid particles, minerals and others, that are transferred from the burnt sugarcane to the spirit during the production process, reducing the quality of the beverage (MASSON *et al.*, 2007).

Higher alcohols, expressed as the sum of n-propyl, isobutyl and isoamyl alcohol, are often found in distilled beverages. Yokoya (1995) reported that the formation of higher alcohols is greater when the yeast has a low biological activity, which slows the fermentation process. Bogusz Junior *et al.* (2006) associated the increased levels of higher alcohols with the presence of excess must during the fermentation process. Oliveira (2001) reported the increase of these compounds as characteristics of the yeast, noting that the production of higher alcohols is an individual characteristic of the strain. Large amounts of higher alcohols decrease the commercial value and quality of *cachaça* (VILELA *et al.*, 2007). Dato; Júnior; Mutton (2005) verified the increased production of higher alcohols in mashes with elevated pH, especially in *cachaças* produced with wild yeast strains.

The production of methanol in *cachaça* and fruit spirits is mainly derived from the activity of pectic enzymes, which act on pieces of sugarcane bagasse resulting from process flaws in filtering and pressing (BOGUSZ JUNIOR *et al.*, 2006; VILELA *et al.*, 2007) or fruit musts (REDDY; REDDY, 2005; GONZÁLEZ *et al.*, 2011). Additionally, post-fermentation and distillation contamination may occur in beverage containers or by using an inadequate distillation procedure. Methanol is quantified for the purpose of toxicological safety because its presence in the body causes severe acidosis, affects the respiratory system and can lead to coma. Its ingestion, even in small quantities over long periods of consumption, can cause blindness and even death (CLETO; RAVANELI; MUTTON, 2009; GONZÁLEZ *et al.*, 2011).

Ethyl carbamate (EC), or urethane, is a potentially carcinogenic substance found in significant concentrations in fermented–distilled beverages. It is considered an organic contaminant of spirits and is the principal precursor to nitrogen and cyanide compounds (ARESTA; BOSCOLO; FRANCO, 2001; CARUSO; NAGATO ; ALABURDA, 2010). The quantification of EC has been extensively evaluated, and its formation appears to be associated with the alcoholic fermentation process, as well as with the type and process of distillation (BRUNO *et al.*, 2007; NÓBREGA *et al.*, 2009). Nóbrega *et al.* (2009) studied the effect of cooling the distillation column on the formation of ethyl

carbamate in *cachaças*, showing that distilled *cachaças* without cooling presented high levels of this compound, exceeding the limit set by legislation (150 μg $L^{-1}$).

2.4.2. Analytical methods for assessing volatile organic compounds in spirits

Market demands for sugarcane spirits increase concerns regarding its quality, primarily due to the large diversity associated with this product. Some studies have been conducted to evaluate the chemical composition and quality of *cachaça*. Therefore, several analytical techniques have been proposed, and different methods have been employed, for the determination of organic compounds in spirits (TABLE 2.**1**). Among The Official Methods Adopted By The Ministry Of Agriculture, Livestock and Supply (MAPA) in Brazil, volumetric methods have been used to determine the concentrations of acids, total esters and total sugars. The use of colorimetric methods is recommended for the quantification of total aldehydes, methyl alcohol, furfural and total acrolein (BRASIL, 2005b). These volumetric and colorimetric methods do not provide reliable results in coloured samples nor allow for the identification of specific analytes but simply determine the total content of each class of compounds analyzed. Thus, results for aldehydes and acids are expressed in terms of the majority components of the classes, i.e., acetaldehyde and acetic acid (NASCIMENTO *et al.*, 1998; NASCIMENTO; CARDOSO; FRANCO, 2009). The European Community (EC) regulation 2870/2000 and 2091/2002 (EC, 2000; EC, 2002) references pycnometric, electronic densimetric and densimetric methods for the analysis of spirit drinks using hydrostatic balance methods for the determination of alcoholic strength by volume. This regulation suggests that the determination of total dry extract should be performed with a gravimetric method and that determination of the volatile acid content, expressed as acetic acid, should be performed with a volumetric method. A gas chromatographic method was suitable for the determination of acetal, amyl alcohol, isoamyl alcohol, methanol, ethyl acetate, n-butanol, sec-butanol, isobutyl alcohol, n-propanol, acetaldehyde and anethole. The majority of studies described in the literature use chromatographic methods to analyze organic compounds. Liquid chromatography has been used to analyze amino acids, phenolic compounds, glycerol, ethanol, furfural, 5-hydroxymethylfurfural and sugar as parameters for monitoring the fermentation process (AQUINO *et al.*, 2006; GARCÍA-LLOBODANIN *et al.*, 2007; AQUINO *et al.*, 2008).

Liquid chromatography has also been used by EC 2091/2002 to analyze chalcones and glycyrrhizic acid (EC, 2002). Gas chromatography with flame ionisation detection (GC/FID) has been used in most studies for the determination of VOCs, including esters, higher alcohols, aldehydes, methanol, volatile acids, ethanol and furfural (NONATO *et al.*, 2001; BRASIL, 2005b; BOGUSZ JUNIOR *et al.*, 2006; GARCÍA-LLOBODANIN *et al.*, 2007; MASSON *et al.*, 2007; NASCIMENTO; CARDOSO; FRANCO, 2009).

Other studies have used gas chromatography with mass spectrometry (GC/MS) to determine several VOCs in spirits, including dimethylsulphide and the compounds used as markers of beverage aging and contaminants, such as ethyl carbamate (NONATO *et al.*, 2001; CARDOSO *et al.*, 2004; BRUNO *et al.*, 2007) (NASCIMENTO; CARDOSO; FRANCO, 2009; SOUZA; OLIVEIRA*; et al.*, 2009). Nonato *et al.* (2001) developed a headspace solid-phase microextraction (SPME) method with GC/FID to determine ethyl acetate and higher alcohols in Brazilian *cachaça*. A method employing GC/MS was also employed to identify the secondary compounds of artisanal and industrial *cachaça* samples. This method uses an SPME fibre with an 85 µm polyacrylate coating. This novel method was compared with an optimised method: liquid–liquid extraction (LLE). Both methods showed good linearity, but the repeatability of analyzes performed using the SPME technique (% RSD 1.8–3.9) was better than that of samples analyzed by LLE (% RSD 10.3–11.7). Concentrations of the analytes obtained in the analysis of 12 *cachaça* samples with the SPME technique were higher than those obtained with LLE. In the SPME method, the extraction wastes are smaller. The presence of a larger number of compounds with intense responses was observed in the chromatograms obtained by SPME procedures, which should be the technique of choice for assaying ethyl acetate and higher alcohols from Brazilian sugarcane spirits.

Determination of the total ester content, expressed as ethyl acetate, can be performed by two techniques according to the MAPA (BRASIL, 2005b): a volumetric technique that involves the titration of total esters after alkaline hydrolysis and a gas chromatography method (GC/FID), in which only ethyl acetate is monitored (BRASIL, 2005b).

Nascimento; Cardoso; Franco (2009) compared volumetric and chromatographic (GC/FID) methods with a GC-MS method operating in the selective ion mode (SIM) for the determination of nine esters, including ethyl lactate. It was observed that the

results of samples analyzed by the volumetric method and GC/MS showed no significant differences. On the other hand, the results obtained by GC/FID analysis differed significantly at a confidence level of 0.05 % from those obtained by the other two techniques. The authors noted that these results were expected because the GC/FID technique recommended by the MAPA does not take into account the presence of ethyl lactate, although this ester is the second most abundant in *cachaça*. It was concluded that determination of the total ester content in *cachaça* samples using GC/MS is an important methodology for routine analysis of beverages, permitting the specification of the desired analytes, because the volumetric technique is not selective and does not determine which esters are present in the samples. Vilela *et al.* (2007) evaluated the chemical composition of *cachaça* produced in southern Minas Gerais, Brazil using the volumetric method. Results obtained determined that the concentrations of total esters, expressed as ethyl acetate, range from a minimum of 24.75 to a maximum of 114.89 mg 100 $mL^{-1}$ of anhydrous ethanol. These values were lower than those determined by Nascimento; Cardoso; Franco (2009) when employing the volumetric method for analysis of *cachaça* samples from the state of São Paulo, whose concentrations ranged from 54.0 to 159.7 mg 100 $mL^{-1}$ of anhydrous ethanol. Bogusz Junior *et al.* (2006) used GC/FID to quantify aldehydes expressed as acetaldehyde, esters expressed as ethyl acetate, higher alcohols (the sum of n-propanol, isobutanol, isoamyl alcohol) and methanol in *cachaça* samples produced in the northwest region of the Brazilian state of Rio Grande do Sul. Ester concentrations in mg of ethyl acetate per 100 mL of ethanol (average of 34 mg) were lower than the values found by Nascimento; Cardoso; Franco (2009) using the same methodology (75.25 mg), and well below those determined by GC/MS (106.3 mg). These results are confirmed by Parazzi *et al.* (2008) who used GC/FID to analyze acetaldehyde, ethyl acetate, n-propanol, n-butanol, isobutanol and isoamyl alcohol in sugarcane spirits aged in oak barrels.

Masson *et al.* (2007) developed a GC/FID method for the determination of furfural in 24 samples in three different groups. The first one group was composed of 12 artisanal brandies from burnt and unburnt cane, fermented with the same yeast and distilled in the same still. The second was composed of six samples of burnt cane from an industrial brandy producer. The last was composed of six samples of unburnt cane obtained from another artisanal producer. In the first group, furfural

concentrations were significantly different (*p<0.01*) between sugarcane spirits obtained with and without prior burning; however, the values were still below the maximum allowed limit of 5 mg 100 $mL^{-1}$ of anhydrous ethanol. The second group also presented values below the maximum allowed limit. In the third group, spirits from unburnt sugarcane presented higher furfural contents (8.80 mg 100 $mL^{-1}$ ethanol), which is above the allowed limit. This was despite the sugarcane not being burnt, which may be related to the presence of residual sugar, polysaccharides derived from bagasse, poor fermentation and excessive heat during distillation.

Some published reports link aminoacid content in beverages to quality parameters such as smell and appearance (HERNÁNDEZ-GÓMEZ; ÚBEDA; BRIONES, 2003; PERPÈTE *et al.*, 2005; AQUINO *et al.*, 2008). Aquino *et al.* (2008) developed an analytical procedure for the determination of 20 amino acids in *cachaça*, rum and whisky by employing reverse-phase liquid chromatography (HPLC) with fluorescence detection using a C18 column (5 µm particle size, 30 cm×3.9 mm i.d.). The results indicated that the method was linear in the range of 0.10-6.00 mg $L^{-1}$. The precision for repeatability varies from 0.61 % (serine) to 13.4 % (glutamic acid), with an average value of 5.91 and 8.35 %, respectively. The method presented recovery levels ranging from 69.5 (lysine) to 100 % (tyrosine) with an average value of 88 %. Analysis of rum and whisky (median total concentration of 3.20 and 3.18 mg $L^{-1}$, respectively) showed higher levels of amino acids than in *cachaça* (median total concentration of 0.63 mg $L^{-1}$). Cardoso *et al.* (2004) described a GC/MS method for the analysis of dimethylsulphide (DMS), the predominant volatile sulphur component in *cachaça*, which can strongly influence the sensory quality of the drink. Samples of *cachaça*, grappa, whisky, brandy, “tiquira” spirit, rum and vodka were analyzed by GC/MS using an esterified polyethylene glycol column (HP-FFAP, 50 m×0.2 mm×0.3 µm) and a mass-selective detector operating in SIM mode (m/z 62). The detection limit was $8\times10^{-9}$ mol $L^{-1}$ and the repeatability was good (RSD<2 %). Results showed that the variation of DMS content in alcoholic beverages is due to the raw material used in fermentation, as well as the difference in microbiological activity developed in this process.

Bruno *et al.* (2007) employed GC/MS to determine ethyl carbamate (EC) in 28 samples of sugarcane spirits from the state of Rio de Janeiro, Brazil. The results demonstrated that 45 % of the samples showed EC levels above 150 µg $L^{-1}$ (the maximum that is allowed by Brazilian legislation) with average values of 160 µg $L^{-1}$.

Labanca; Glória (2008) used a similar GC/MS method to determine EC in sugarcane spirit samples produced in the state of Minas Gerais. A total of 71 samples were analyzed, presenting ethyl carbamate concentrations from < 30 to 2,609 μg $L^{-1}$, where only 7 % presented levels below the allowed limit (150 μg $L^{-1}$). Caruso et al. (2010) also analyzed EC in *cachaças* obtained from 61 samples of sugarcane acquired from different regions of Brazil using a GC/MS method. The average results were found to be in the range of 20 to 960 μg $L^{-1}$, and 53 % of the samples presented EC values above the maximum tolerated limit.

#### *2.4.2.1. Analytical methods for assessing volatile organic compounds in spirits*

Several studies with grape spirits have been reported; however, only a small number of studies on spirits obtained from other fruits are described in the literature.

Guimarães Filho (2003) studied spirits produced from bananas, using GC/FID to evaluate the volatile organic compounds (acetaldehyde, ethyl acetate, methanol, n-propanol, isobutanol, isoamyl alcohol, amyl alcohol, n-hexanol and ethanol) generated in the fermentation and distillation processes. Among the compounds evaluated, the concentrations of higher alcohols and methanol were above the maximum concentrations allowed by Brazilian legislation. Reddy; Reddy (2005) studied the fermentation viability of six varieties of mango (*Mangifera indica L.*) produced in India and made comparisons with grape fermentation. Volatile organic compounds (ethanol, methanol, acetaldehyde, higher alcohols, ethyl acetate and total glycerol) were analyzed by GC/FID. Ethanol concentrations ranged from 7 to 8.5 %, and the levels of methanol were slightly higher than in fermented grapes. Regarding the other volatile organic compounds, concentrations were equivalent to those of fermented grapes. The effects of distillation processes and the presence of the wine lees on the quality of pear distillates were studied by García-Llobodanin *et al.* (2007). Pear wine obtained by fermentation of pear juice using *Saccharomyces cerevisiae* was distilled with its lees using three different types of equipment: a glass alembic (a glass pot still coupled to a glass column), a copper alembic and a glass alembic with the addition of copper shavings to the pot still. An HPLC with a refractive index detector was used to characterise the pear juice and wine and also to observe the fermentation process. GC/FID was used to quantify methanol and volatile organic compounds (higher alcohols (propan-1-ol, 2-methylpropan-1-ol, 1-

butanol, 2-methylbutan-1-ol, 3-methylbutan-1-ol, and hexan-1-ol), total esters (methyl acetate, ethyl acetate, ethyl decanoate, and ethyl-2-trans-4-cis-decadienoate) aldehydes (acetaldehyde, furfural and acetal) and phenethyl alcohol) in pear wine and to characterise the different samples collected during distillation. The results indicated that methanol, ethyl acetate and furfural either decreased or showed no change in their concentrations when distilled in the presence of lees and in the copper alembic.

Other compounds (ethyl decanoate and ethyl-2-trans-4-cisdecadienoate) showed increased concentrations in the presence of lees in all equipment tested. It was assumed that the distillation of pear wine in the presence of the lees led to better product quality. As for the equipment used, the copper still appeared to be the best, reducing the levels of 3-methyl-1-butanol, 2-methyl-1-propanol and acetaldehyde (GARCÍA-LLOBODANIN *et al.*, 2007).

Alvarenga (2006) studied the effect of enzymatic treatment of mango pulp to increase the extraction efficiency of the juice and to reduce its viscosity in the production of mango spirits. Procedures were compared with and without enzymatic hydrolysis of two mango varieties, Tommy Atkins and Palmer, using two types of commercial pectinolytic enzymes (Allizin PP and Pectinex Ultra SP) at different concentrations, times and temperatures. The alcohol content was determined by spectrophotometric measurements performed in the visible spectrum (690 nm), total titration acidity. The determinations of total titration acidity and total ester content, expressed as ethyl acetate, and acetaldehyde were carried out using a volumetric method. Higher alcohols (n-propanol, isobutyl alcohol and isoamyl alcohol) and methanol were analyzed by GC/FID. Mango spirits produced under optimum hydrolysis and fermentation conditions showed good quality but presented levels of higher alcohols and copper exceeding those allowed by existing legislation.

Lara (2007) evaluated the treatment conditions of banana pulp and the fermentation process necessary to decrease the viscosity of the must and reduce the formation of higher alcohols during the production of banana spirits. The alcohol content was quantified by spectrophotometric methods. Determinations of the volatile acidity and ester content, expressed as ethyl acetate, were performed by volumetric methods while higher alcohols (n-propanol, isobutyl alcohol and isoamyl alcohol) and methanol were analyzed by GC/FID. The results demonstrated that only higher

alcohols and the total acidity were outside of the limits set by Brazilian legislation for the production of banana spirits.

The quality of jabuticaba (*Myrciaria jabuticaba Berg*) spirits from fermented jabuticaba juice was evaluated by Asquieri; Silva; Cândido (2009). GC/FID was used for the determination of furfural, methanol, aldehydes, alcohols (n-propyl, nbutyl, isobutyl, isoamyl) and sec-butyl-ether. The other constituents (alcohol by volume, density, dry extract, reducing sugars and sucrose, total acidity and fixed acidity, pH, ash, protein, calcium, iron, magnesium, phosphate, copper, total and free carbon dioxide, and tannins) were determined by volumetric or spectrophotometric methods. Subsequently, the results were compared to the parameters established by Brazilian legislation regarding the production of fruit spirits. These authors found that jabuticaba spirits presented values compatible with the parameters established by legislation, with the exception of high ester concentrations (357 mg 100 $mL^{-1}$ anhydrous alcohol).

López-Vázquez *et al.* (2010) proposed a GC/FID method using direct injection to determine 33 volatile organic compounds present in distillate obtained from the fermentation of grape residue in the region of Galicia known as the “Orujo de Galicia”. The developed and validated methodology showed good linearity, precision and accuracy, permitting evaluation of distillate quality in a single analysis. Four samples of commercial grape distillates were analyzed. Concentrations of VOCs varied according to the origin of the distillate but remained within the ranges established by the European Commission regulations 2870/2000 (EC, 2000) for fruit-based distilled beverages. The same methodology was used to evaluate the volatile organic compounds of distilled beverages obtained from raspberry and medronho (GONZÁLEZ *et al.*, 2011). In this study, the total VOCs and anhydrous ethanol contents were 41.3 and 200.1 g $L^{-1}$, respectively, in raspberry spirits, and 44.3 and 267.1 g $L^{-1}$ in medronho spirits. The total VOCs for medronho spirits was above the limits set by European Commission regulations (EC, 2000) for volatile compounds in fruit spirits (38.5 to 200 g $L^{-1}$ of anhydrous ethanol). In another study, GC/FID and GC/MS methods were employed for the analysis of volatile organic compounds in cherry distillates. This study compared the influence of five cherry varieties in brandy, evaluating both chemical and sensory characteristics. The analysis of these distillates led to the identification of 32 components, including esters, benzaldehyde,

terpenes and acids. Ethyl esters were the most abundant in all samples (NIKIĆEVIĆ *et al.*, 2011).

#### *2.4.2.2. Aging*

The aging process causes several changes in beverage composition and the concentrations of compounds. Thus, both the aroma and taste of the aged beverage change. Such changes are caused by the extraction of compounds from the wood and the degradation of their macromolecules, such as cellulose, with subsequent reaction and extraction of the reaction products, reactions between the distillate compounds and the wood, and reaction between the distillate components themselves and evaporation of volatile compounds (PIGGOTT; HUNTER; MARGOMENOU, 2000; MIRANDA *et al.*, 2008).

Aging of the beverage is normally characterised by a decrease in pH and concentrations of methyl alcohol and ethyl alcohol, while there is an increase in acidity, colour and the concentrations of ethyl acetate, acetaldehyde, acetone and phenolic compounds (tannins) (CARDELLO; FARIA, 1997; AQUINO *et al.*, 2006; MIRANDA *et al.*, 2008). The international literature, particularly when referring to whisky and wine, describes several compounds that can be used as markers of aging in beverages. Among these are low molecular weight phenolic compounds that are extracted from wood during the aging process (PIGGOTT; HUNTER; MARGOMENOU, 2000; AQUINO *et al.*, 2006).

AQUINO *et al.* (2006) studied the profile of low molecular weight phenolic compounds in aged *cachaças* obtained from small producers in five regions of the Brazilian State of Ceará. The following compounds were quantified by HPLC with UV–vis detection as indicators of aging: gallic acid, 5-HMF, furfural, vanillic acid, syringic acid, vanillin, syringaldehyde, coniferaldehyde, synapaldehyde and coumarin. Although it was not possible to precisely determine the aging time of a beverage based only on the quantification of low molecular weight phenolic compounds, their determination may be used as an indication of beverage authenticity, because these compounds are not found in *cachaça* that has not been aged. This analysis is useful for quality control of the beverage because Brazilian law allows the addition of caramel colouring for the colour correction of *cachaças*. Thus,

the presence or absence of aging markers in the beverage may reveal fraud or a low quality (AQUINO *et al.*, 2006).

In order to monitor the evolution of the chemical composition of spirits aged in 20 L oak barrels, physicochemical analyzes were performed at 0, 76, 147, 228, 314 and 390 days of storage. Analysis of alcohol content was performed using a densimetric method and volatile acidity, dry extract and furfural analyzes were performed using a volumetric method (BRASIL, 1986). Copper, tannin and colour were determined by spectrophotometry. GC-FID was used for the analysis of acetaldehyde, furfural, ethyl acetate, higher alcohols (n-propyl, isobutyl and isoamyl alcohol) and methyl alcohol. The results demonstrated that the concentrations of volatile acids, esters, aldehydes, furfural, higher alcohols, dry extract and tannins in the spirits increased with the aging period, reaching a total increase of approximately 43 %. This caused the beverage to become yellow. The concentrations of ethanol and methanol did not change, and the copper concentration showed a slight decline. Aging the spirit for 390 days in oak barrels altered its chemical composition; however, all parameters were within the quality standards established by Brazilian legislation (MIRANDA *et al.*, 2008).

Souza *et al.* (2007); Souza; Oliveira*; et al.* (2009) developed a direct infusion electrospray ionisation mass spectrometry method for the study of *cachaça* samples. This method was able to differentiate samples of Brazilian artisanal *cachaça* aged in four different types of wood casks, identify adulteration including the addition of essences, sawdust or dyes, and discriminate *cachaça* from rum (SOUZA *et al.*, 2007). Discrimination of *cachaça* distilled in copper stills, which is considered to be artisanal, from *cachaça* distilled in stainless steel columns, referred to as industrial, was also analyzed (SOUZA; OLIVEIRA*; et al.*, 2009).

TABLE 2.1 - GC applications in *cachaça* and fruit spirit analysis

| **Matrix** | **VOCs analyzed** | **Sample treatment** | **Technique of analysis** | **Reference** |
|---|---|---|---|---|
| Brazilian *cachaça* | Ethyl acetate and superior alcohols organic acids, esters, and cyclic and aromatic hydrocarbons totalized 38 compounds. | Headspace solid-phase microextraction (SPME) and Liquid-liquid extraction (LLE) with pentane and dichloromethane | GC/FID GC/MS | Nonato *et al.* (2001) |
| Dwarf banana spirits | Acetaldehyde, ethyl acetate, methanol, n-propyl, isobutyl, isoamyl and amyl alcohol, n-hexanol and ethanol | Direct injection | GC/FID | Guimarães Filho (2003) |
| *Cachaça*, grappa, whiskey, brandy, “tiquira” spirit, rum and vodka | Dimethylsulphide | On column injection direct mode | CG/MS | Cardoso *et al.* (2004) |
| Mango wine | Ethanol, methanol, acetaldehyde, higher alcohols, ethyl acetate and glycerol total | - | GC/FID | Reddy; Reddy (2005) |

| Matrix | VOCs analyzed | Sample treatment | Technique of analysis | Reference |
|---|---|---|---|---|
| Mango spirits | Acetaldehyde, ethyl acetate, superior alcohols, and methanol | - | GC/FID | Alvarenga (2006) |
| *Cachaça* from the northwest region of Rio Grande do Sul, Brazil | Aldehydes, esters, superior alcohols and methanol | - | GC/FID | Bogusz Junior *et al.* (2006) |
| Sugarcane spirits | Ethyl carbamate | Samples with an alcohol content are used directly | CG/MS | Bruno *et al.* (2007) |
| Sugarcane spirits | Furfural | - | GC-FID | Masson *et al.* (2007) |
| Banana spirits | Acetaldehyde, ethyl acetate, superior alcohols, and methanol | - | GC/FID | Lara (2007) |
| Pear distillates | Methanol, esters, aldehydes and furfural | Direct injection | GC-FID | García-Llobodanin *et al.* (2007) |
| Sugarcane spirits aged | Aldehydes, esters, superior alcohols (n-propyl alcohol, isobutyl alcohol and isoamyl alcohol) and methyl alcohol | Direct injection | GC/FID | Miranda *et al.* (2008) |

| Matrix | VOCs analyzed | Sample treatment | Technique of analysis | Reference |
|---|---|---|---|---|
| Sugarcane spirits | Aldehydes, esters, superior alcohols | - | GC/FID | Parazzi *et al.* (2008) |
| Sugarcane spirits | Ethyl carbamate | Direct injection | CG/MS | Labanca; Glória (2008) |
| *Cachaça* samples | Esters | - | GC/FID & CG/MS | Nascimento; Cardoso; Franco (2009) |
| *Cachaça* samples | Ethyl acetate and superior alcohols, organic acids, esters, and cyclic and aromatic hydrocarbons totalized 70 compounds. | SPME | GC×GC/TOFMS | Cardeal *et al.* (2008) |
| *Jabuticaba* spirits | Furfural, methanol, aldehydes, alcohols, n-propyl, n-butyl, isobutyl, isoamyl and sec-butyl-ether | - | GC/FID | Asquieri; Silva; Cândido (2009) |
| Artisanal *cachaça* | Higher alcohols, ethyl, isoamyl and isobutyl esters, acetates, phenyl esters, aldehydes, ketones and alkanes, more than 100 VOCs identified | SMPE | GC×GC/TOFMS | Souza; Cardeal*; et al.* (2009) |

| Matrix | VOCs analyzed | Sample treatment | Technique of analysis | Reference |
|---|---|---|---|---|
| *Cachaças*, rum, vodka, whisky, tequila | Aldehydes, esters, superior alcohols organic acids, and cyclic and aromatic hydrocarbons totalized 168 compounds. | SMPE | GC×GC/FID GC×GC/TOFMS | Cardeal; Marriott (2009) |
| Grape ditillate from "Orujo de Galicia". | 33 Volatile organic compounds | Direct injection | GC/FID | López-Vázquez *et al.* (2010) |
| Raspberry and *medronho* distillates | 33 Volatile organic compounds | Direct injection | GC/FID | González *et al.* (2011) |
| Cherry distillates | 32 Aroma compounds were identified, including esters, acids, benzaldehyde and the monoterpene linalool | Liquid-liquid extraction (LLE) with dichloromethane | GC/FID CG/MS | Nikićević *et al.* (2011) |

**- Not described**

### 2.4.3. Advanced technology for analysis

The determination of volatile organic compounds in spirits is performed largely by conventional chromatographic methods (TABLE 2.**1**). However, some improved separation techniques have been studied because chromatograms may exhibit insufficient resolutions due the presence of dozens of peaks, which should ideally be separated (MARRIOTT; SHELLIE, 2002; GOMES DA SILVA; CARDEAL; MARRIOTT, 2008; CARDEAL; MARRIOTT, 2009; HERRERO *et al.*, 2009; WELDEGERGIS *et al.*, 2011). Peak overlap causes difficulty in detection and identification in one-dimensional gas chromatography. Moreover, the determination of VOCs is complex because components may be present in a very wide range of concentrations. Consequently, trace compounds can sometimes remain undetected if they co-elute with major compounds of the matrix. It is not rare for trace compounds to present appreciable biological activity, or even be the active compounds of the studied matrix (NONATO *et al.*, 2001; GOMES DA SILVA; CARDEAL; MARRIOTT, 2008).

In this context, comprehensive two-dimensional gas chromatography (GC×GC) is a much more powerful technique to overcome resolution deficiencies for complex aroma mixtures. This is valuable for both qualitative and quantitative applications. GC×GC involves the combination of two serially coupled columns with different compositions through a special interface that allows peaks from the primary column to be transferred onto the second column for additional separation. Focusing obtained through this modulation process reduces peak width and thus increases peak capacity, improves resolution and enhances mass sensitivity, as well as allows for faster run times (MARRIOTT; SHELLIE, 2002; ADAHCHOUR *et al.*, 2006; HERRERO *et al.*, 2009).

The optimization and development of a suitable GC×GC method is dependent not only on the separation mechanism and the properties of the solutes, but also on the separation conditions such as column combinations, modulation period, temperature, etc. The modulator presents three main functions: accumulate or trap; refocus; and release of narrow adjacent zones of $^{1}D$ effluent rapidly into $^{2}D$. Even though the modulator is considered the key component of the system for successful operation, the separation will ultimately be the key factor behind the power and versatility of GC×GC applications. The stationary phase types in the $^{1}D$ and $^{2}D$, the column lengths and the internal diameters, film thicknesses and the oven

temperature program of the first and second dimension columns influence the column separation parameters (CORDERO *et al.*, 2008; KOEK *et al.*, 2008; ZHU, 2009; MARRIOTT *et al.*, 2012).

Conventional GC×GC (FIGURE 2.**2**) is performed using a non-polar stationary phase column in the $^{1}$D, typically 15-30 m long coupled with a short polar column in the $^{2}$D suitable to run the separation in a few seconds. This kind of column set is so-called "normal column combination". However, a "reversed-type" column combination, with a polar column used in the $^{1}$D and a non-polar column in the $^{2}$D, was shown to be even more effective for certain separations of highly complex samples containing many types of semi- and highly-polar classes of compounds such as alcohols, aldehydes, ketones, esters, acids, etc. (CORDERO *et al.*, 2008; KOEK *et al.*, 2008; ZHU, 2009; MARRIOTT *et al.*, 2012).

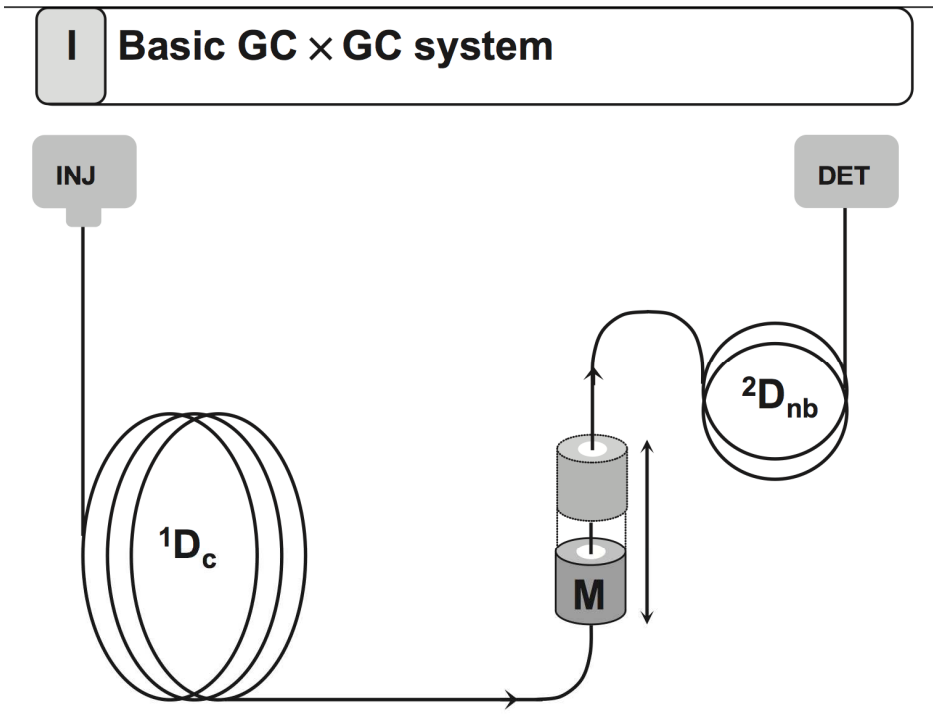


FIGURE 2.2 – GC×GC system arrangement
$^{1}D_{c}$ = conventional first- dimension column, $^{2}D_{c}$ = conventional second-dimension column, 2Dnb = narrow-bore second-dimension column, M = modulator (longitudinally modulated cryogenic system), DET = universal, specific or mass-selective detector.
From: Marriott *et al.* (2012)

In a previous study Nonato *et al.* (2001) developed a headspace solid-phase microextraction (SPME) method for the determination of secondary compounds from Brazilian *cachaça* using GC/FID and GC/MS. Although this study demonstrated good performance capabilities of SPME for the analysis of VOCs in *cachaça*, only 38 compounds were identified, and the chromatogram indicated that there was inadequate resolution. Hence, Cardeal; Marriott (2009) proposed an SPME method for the analysis of VOCs in *cachaça* using the GC×GC with FID and time-of-flight mass spectrometry (TOFMS) detectors. To enable a straightforward comparison of the new GC×GC method with the previous study, which used a conventional one-dimensional GC analysis, the same general experimental parameters were used. More than 168 compounds were identified in *cachaça* by the GC×GC method. As an extension, other spirits, including rum, vodka, whisky, etc. were also analyzed by GC×GC/TOFMS to compare the VOCs present in these spirits with those in the *cachaça* sample. When comparing the inherent structure present in the peak apex plots obtained with GC×GC/TOFMS, it can be observed that *cachaça* plots are very different from those of rum, proving that these two varieties of sugarcane-based spirits are distinct. This includes the differences between the aged beverages. Vodka, whisky and tequila have presentation patterns quite distinct from the *cachaça* plots with respect to the classes of compounds and their general regions of occupancy in $^{2}$D space. Therefore, the results of this study allow for proposing GC×GC/TOFMS as the ideal technique for VOCs analysis in spirits due to its high separation and resolution power, precise analytical measurement and enhanced sensitivity (CARDEAL; MARRIOTT, 2009).

In another application, Cardeal *et al.* (2008) employed the GC×GC/TOFMS method for fingerprint monitoring of the distillation process for *cachaça* production and after aging in different wood materials. The SPME sampling method was employed according to a previous study (NONATO *et al.*, 2001). The SPME-GC×GC/TOFMS method facilitated determination of the turning points of the process by simple visualization and comparison of the contour plots for different fractions of the distillate (head, core and tail). Wood storage materials and the aging period can be also monitored using the fingerprint, making this method suited for product quality analysis.

The SPME CG×CG/TOFMS method was later applied to investigate the effects of bidistillation and the use of filters (resin or charcoal) in the production of artisanal

*cachaça*, as well as the effects of multi-distillation processes on VOCs products in industrial *cachaça*. Volatile organic compounds were sampled onto a polyacrylate solid-phase microextraction fibre and analyzed using GC×GC/TOFMS with a non-polar (5 % phenyl) and polar (20 % phenyl) column set. The GC×GC analysis was efficient for identifying a large number of different functional groups for both polar and non-polar compounds in the samples, structured in $^{2}$D plots. A large difference in the composition of volatile compounds was clearly observed between the bidistillate *cachaça* and the single distillation product. The use of resins in the process for the removal of copper ions results in contamination with phthalate. However, charcoal can successfully remove this contamination product. *Cachaça* samples from the multidistillation process bear close similarity to the fingerprints obtained from the GC×GC analysis of the other *cachaças* produced via a simple column distillation (SOUZA; CARDEAL*; et al.*, 2009).

## 2.5. Evaluation of aroma in spirits by gas chromatography-olfactometry

The content and composition of volatile organic compounds that define the quality of alcoholic beverages include aroma. In spirits these compounds occur at widely differing concentrations (PLUTOWSKA; WARDENCKI, 2008). It has been clearly established that only a small fraction of these odorants actually influence aroma perception. Quite frequently, compounds appearing in trace quantities have a greater influence on the sensory properties than those which appear in high concentrations (VAN RUTH, 2001; DELAHUNTY; EYRES; DUFOUR, 2006). It should be emphasized that sometimes compounds are not at all detectable with conventional detectors (PLUTOWSKA; WARDENCKI, 2008). These compounds under various conditions can provide important information for the improvement and optimization of the process to obtain the beverage. Therefore, the identification of these compounds is considerable to determine the aroma characteristics of the beverage, being also useful to detect illicit spirits, and to identify anomalies that are indicative of inconsistent manufacturing practices (PLUTOWSKA; WARDENCKI, 2008; SAMPAIO *et al.*, 2013).

Gas chromatography olfactometry (GC/O) is based on sensory evaluation of the eluate from the GC separation, using the nose as a detector. A specifically device called sniffing port is connected in parallel to conventional detectors, such as flame-ionisation detector (FID) or mass spectrometer (MS) (FIGURE 2.**4** and FIGURE 2.**4**).

Assessors sniff the effluent via this port in order to study odor active components. GC/O has been applied to investigate flavors and fragrance in complex matrix, including spirits (ACREE, 1997; DELAHUNTY; EYRES; DUFOUR, 2006; PLUTOWSKA; WARDENCKI, 2008; PINO *et al.*, 2012).

The transfer line to the sniffing port is heated to avoid volatile compound condensation and to eliminate residual odor. Addition of humidified air at the sniffing outlet has been generally applied to prevent drying of nose mucous membranes of the evaluating personnel, as this could cause discomfort, especially in longer analyzes. Furthermore, some studies had demonstrated the need for an air makeup to increase odor detection frequency and intensity rating (HANAOKA; SIEFFERMANN; GIAMPAOLI, 2000; DELAHUNTY; EYRES; DUFOUR, 2006).

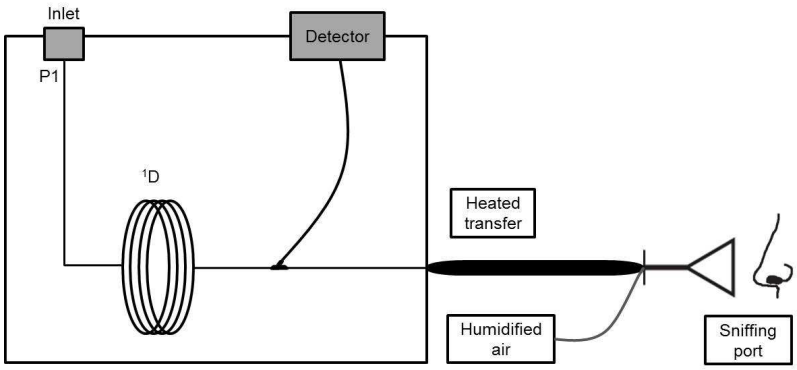


FIGURE 2.3 - Scheme of the gas crhomatography equipped with olfactometry detector
From: own author, 2014

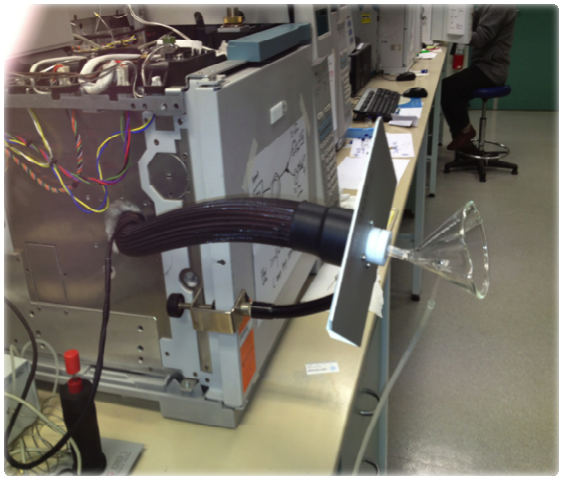

FIGURE 2.4 – Picture of the gas chromatography equipped with olfactometry detector
From: own author, 2014

Different GC/O methods have been developed and that can be categorized into three main techniques: dilution to threshold, direct intensity, and detection frequency (ACREE, 1997; POLLIEN *et al.*, 1997; DELAHUNTY; EYRES; DUFOUR, 2006; PLUTOWSKA; WARDENCKI, 2008). Dilution to threshold methods: CHARM (Combined Hedonic Aroma Response Measurement) and AEDA (Aroma Extract Dilution Analysis). These methods consist on evaluations of a series of extract dilutions until no odor is perceived. Usually, the evaluators describe the type of smell (ACREE; BARNARD; CUNNINGHAM, 1984; ULLRICH; GROSCH, 1987; POLLIEN *et al.*, 1997). Direct intensity method: OSME (mean smell in Greek). The assessors are required to use a scale to measure the perceived intensity of the odor of the eluting compound (DELAHUNTY; EYRES; DUFOUR, 2006). Finally, detection frequency (DF) method based on the proportion of panellists detecting an odor at a particular retention time together with a tape recorded description. The individual responses are combined and normalised to produce an aromagram. Compounds, which are sensed more frequently, reflect the most importance of the odor component. The peak height corresponds to the number of assessors to detect that odor, and have been described as nasal impact frequency (NIF). The NIF is the unit of olfactogram (POLLIEN *et al.*, 1997; DELAHUNTY; EYRES; DUFOUR, 2006). This technique has been applied to alcoholic beverages such as gin (DUSSORT *et al.*, 2012), wine (FALCÃO *et al.*, 2008) and French ciders (VILLIÈRE *et al.*, 2012).

In complex samples, as spirits, the identification of aroma compounds using conventional GC/O with FID or MS can have a limitation due a peak co-elution of compounds in the odor regions. There is also the possibility to make an incorrect identification, if a trace odorant is masked by a large odourless compound (CAMPO; CACHO; FERREIRA, 2006; EYRES; MARRIOTT; DUFOUR, 2007; MARRIOTT *et al.*, 2012).

Multidimensional GC (MDGC) with olfactometry seems to be a solution to overcome this problem. The MDGC design with heart-cut (H/C) and cryo-trapping devices for isolation and transfer of target solute (s) from a first dimension column ($^1$D) to a second dimension column (2D) with a different column and equipped with a sniff port can be used to perform multidimensional analysis. (DELAHUNTY; EYRES; DUFOUR, 2006; EYRES; MARRIOTT; DUFOUR, 2007; PLUTOWSKA; WARDENCKI, 2008). MDGC combined with olfactometry analysis have been applied to the study of beverages, such as beer, wine and spirit. Wanikawa *et al.* (2002) studied green note compounds in malt whisky using multidimensional MDGC/MS/O. The system was

effective in examining small amounts of compounds in malt whisky, which contains many volatiles, since this method enables the volatiles in a complicated matrix to be highly resolved without the influence of large amounts of volatiles. Campo; Cacho; Ferreira (2006) studied a systematic multidimensional chromatographic strategy with different techniques of extraction and fractionation for the identification of the target odourants. It was shown that MDGC strategy must be complemented with specific headspace method for ensure a correct isolation and identification of trace and ultra-trace odourants in wine. Several odourants potentially relevant to the aroma of sherry white wine and Madeira wines were identify by the first time. Sasamoto; Ochiai (2010) developed a selectable $^{1}D$ or $^{2}D$ system using simultaneous mass spectrometry and olfactometry to assure selection of heart-cut region and correct identification of compounds of interest. The proposed system was demonstrated with an identification of trace amounts of aroma components in beverages (beer and coffee).

In H/C MDCG only a proportion of the eluate from the $^{1}D$ is separated in $^{2}D$ while in comprehensive GC×GC the whole first dimension effluent is transferred to the second column (MARRIOTT *et al.*, 2012). A GC×GC/O was proposed by d'Acampora Zellner *et al.* (2008) for odourants analysis in commercial perfume matrices. However, although comprehensive GC×GC has presented enhanced separation and better sensitivity, it is not suitable for olfactory detection using human assessor. This is due the human breathing cycle is about 3–4 s, which is too slow to reliably assess the narrow, rapid eluting peaks produced by GC×GC. The regular modulation during in GC×GC creates multiple slice of each peak with peaks widths between 100-400 ms (DELAHUNTY; EYRES; DUFOUR, 2006; EYRES; MARRIOTT; DUFOUR, 2007; PLUTOWSKA; WARDENCKI, 2008; VILLIÈRE *et al.*, 2012). In addition, this technique is demanding for the assessors to maintain their concentration and unsettled due the chance of missing an odour was high. Thus, H/C MDGC/O have more often been applied to resolve a number of selected co-eluting aroma regions maintain one discrete peak per compound with a broader peak width and identify the compound responsible for the perceived odour (EYRES; MARRIOTT; DUFOUR, 2007; PLUTOWSKA; WARDENCKI, 2008; MARRIOTT; EYRES; DUFOUR, 2009; MAIKHUNTHOD *et al.*, 2010).

# 3. CHAPTER 2: ASSESSMENT OF VOLATILE ORGANIC COMPOUNDS FROM BANANA SUBJECTED TO DIFFERENT ALCOHOLIC FERMENTATION PROCESSES USING SOLID PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHY MASS SPECTROMETRY


## Abstract

In this study, the effects of enzymatic treatment, centrifugation and commercial yeast strain were investigated using the fermented banana. Although the literature reports experiments with other varieties of banana, this is the first time that this experiment has been done using banana *Terra*. After optimizing the fermentation processes, two selected strains of *Saccharomyces cerevisiae* UFMGA-1007 and UFMGA-1031 were also tested to determine if they could improve yeast performance in alcohol fermentation. The commercial yeast still proved to be superior. The impacts of these factors were evaluated according to the following kinetic parameters: ethanol yield, efficiency and productivity, and the profile of the volatile organic compounds (VOCs). The VOCs were determined by headspace solid phase microextraction (SPME) using gas chromatography mass spectrometry (GC/MS). The optimum results for achieving a maximum ethanol yield (85.97 % and 86.39 %, respectively) were achieved shown employing enzymatic treatment, wet commercial yeast and with or without centrifugation. Twenty-two compounds of distinct chemical classes were analysed, including alcohols, esters, organic acids, aldehydes and ketones. The concentrations of the VOCs differed depending on the fermentation condition. The values of higher alcohols ranged from 353 to 1017 mg per 100 mL of anhydrous alcohol. The fermented banana studied showed a composition similar to other fermented fruit pulps.


*Keywords:* Fermented banana . Volatile composition. GC/MS. SPME.

**Resumo**

O presente estudo teve por objetivo avaliar os efeitos da hidrólise enzimática da polpa de banana *Terra* com enzimas pectinolíticas, centrifugação do mosto e emprego de levedura comercial (fermento úmido e seco) para fermentação alcoólica de banana. O impacto destes fatores foram avaliados pelos seguintes parâmetros cinéticos da fermentação: rendimento e produtividade em etanol, e eficiência da levedura e pelo perfil dos compostos orgânicos voláteis (COV) nos mostos fermentados de banana. Embora sejam encontrados trabalhos na literatura com outras variedades de banana, esta é a primeira vez que este experimento é realizado com a variedade *Terra*. Após otimização do processo fermentativo, duas linhagens de leveduras *Saccharomyces cerevisiae* UFMGA-1007 e UFMGA-1031 selecionadas para produção de cachaça foram testadas para fermentar o mosto de banana *Terra*. A levedura comercial continuou apresentando melhor desempenho. Os COV foram extraídos via headspace por microextração em fase sólida (SPME) e analisados por cromatografia gasosa com detector de espectrometria de massas (GC/MS). Todos os fatores avaliados influenciaram significativamente ($p<0,05$) os parâmetros cinéticos de fermentação. Os melhores resultados foram alcançados ao empregar a hidrólise enzimática da polpa, o mosto centrifugado ou não, com o uso do fermento comercial úmido, proporcionando rendimento em etanol de 85,97 % e 86,39 %, respectivamente e eficiência de 97,98 %. Foram identificados 22 compostos de diferentes classes incluindo álcoois, ésteres, ácidos orgânicos, aldeídos, cetonas dentre outros. A concentração dos compostos voláteis diferiram significativamente para todas as condições de fermentação alcóolica testadas. O valor de álcoois superiores por 100 mL de álcool anidro nos fermentados do mosto de banana variou de 353 a 1017 mg. Os fermentados alcóolicos de banana *Terra* apresentaram composição química similar à outros fermentados de frutas.

*Palavras-chave*: fermentação alcoólica. banana. Composição de voláteis. GC/MS. SPME

## 3.1 Introduction

Banana is a tropical fruit widely consumed throughout the world. It has a rich aromatic flavour, nutritional value and a high concentration of sugar. The variety *Terra* (Musa AAB, Plantain subgroup) is one of the most popular banana varieties consumed in Brazil, Africa, the Caribbean and Latin America. Although bananas are widely produced and consumed, post-production loss represents a significant cost to the economy, and could result in waste of as much as half the crop (AURORE; PARFAIT; FAHRASMANE, 2009; FAO, 2013b). In this context, alternative products for banana have been proposed, that would provide new resources, innovative products and minimize crop waste. The banana, in the last stage of maturation, presents high fermentable sugar content and does not have commercial value; although, it still presents nutritional value and aromatic quality. So this raw material can be used in the production of fermented and spirit beverages and is considered in this study (SILVA, 2004; AURORE; PARFAIT; FAHRASMANE, 2009).

Alcohol beverages are highly complex mixtures of compounds, in addition to ethanol, including a series of volatile organic compounds (VOCs). These compounds define the aroma, appearance and taste properties of these beverages (NONATO *et al.*, 2001; CARDEAL; MARRIOTT, 2009; MORAKUL *et al.*, 2012). Different factors influence the composition and concentration levels of VOCs, such as raw materials, fermentation parameters, yeast strain and/or the distillation process, as well as aging (FERREIRA *et al.*, 1996; NONATO *et al.*, 2001). Additionally, standardization of the production process is absent and is generally carried out subjectively. As a result, there may be variations in ethanol yield and productivity, yeast efficiency, and organoleptic characteristics of the beverage. Hence, any one of these factors, for example enzymatic treatment, centrifugation and yeast strain, should be studied in more detail regarding their effects on the quality of fermented banana products.

For these reasons, the aim of this study was to evaluate the influence of enzymatic treatment, centrifugation and yeast strains on the alcohol fermentation of banana *Terra* by kinetic parameters and VOCs profile. The impacts of these factors were evaluated according to the following kinetic parameters: ethanol yield and productivity and yeast efficiency. Furthermore, the volatile composition of different fermented banana was analysed using solid phase microextraction (SPME) with gas chromatography mass spectrometry (GC/MS).

## 3.2 Materials and Methods

### 3.2.1 Sampling, yeast strain and reagents

Banana *Terra* (*Musa sapientum*) in the last stage of maturation was purchased from local markets in Belo Horizonte (Brazil). The pulp was obtained by mashing the bananas in a domestic blender and the total soluble solids were adjusted to 15° Brix (AOAC, 2000a). The must was frozen and stored for future use. The effect of freezing banana must was evaluated and findings revealed that it has no impact on ethanol yield.

Itaiquara brand *Saccharomyces cerevisiae* (Minas Gerais, Brazil) was used as the wet commercial yeast (WCY) and Fleischmann brand (Sorocaba, Brazil) was used as the dry commercial yeast (DCY). All were purchased from a local market in Belo Horizonte (Brazil). The selected yeast strains (*S. cerevisiae*), UFMGA-1007 and UFMGA-1031, were obtained from the Laboratory of Taxonomy, Biodiversity and Fungi Biotechnology at UFMG and originally isolated at a sugarcane spirits distillery in Minas Gerais (Brazil). The selected yeast strains were mantained and after activated in GYMP agar for 48 h at 30 °C (OLIVEIRA; CARDELLO*; et al.*, 2005).

The reagents were purchased from different suppliers: ethanol (J.T. Baker, Mexico) 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol (Vetec, Brazil), ethyl acetate, acetic acid, hexanol, ethyl lactate (Fluka, Germany), hexanoic acid (Aldrich, Germany) and ultrapure water from a Milli-Q purification system (Millipore, Milford, Massachusetts).

### 3.2.2 Effects of different variables on alcoholic fermentation

A $2^3$ two-level full factorial design was used in the investigation of the effects of the independent variables; enzymatic treatment, centrifugation and commercial yeast on the alcoholic fermentation of banana. Then, the banana must was fermented under eight different conditions: HCD – Hydrolyzed, centrifuged and dry yeast; NHCD – Non-hydrolyzed, centrifuged, and dry yeast; HNCD – Hydrolyzed, non-centrifuged and dry yeast; NHNCD – Non-hydrolyzed, non-centrifuged and dry yeast; HCW - Hydrolyzed, centrifuged and wet yeast; NHCW – Non-hydrolyzed, centrifuged and wet yeast; HNCW – Hydrolyzed, non-centrifuged and wet yeast; NHNCW – Non-

hydrolyzed, non-centrifuged and wet yeast. The optimum conditions, of theses eight, involved an enzymatic hydrolysis and centrifugation of the commercial wet yeast. (TABLE 3.**1**)

TABLE 3.1 - Experimental design for different conditions of alcoholic fermentation of banana must

| **Designate experiments** | **Independent Variables** | | |
|---|---|---|---|
| | **Enzymatic treatment (hydrolysis)** | **Centrifugation** | **Commercial yeast strains** |
| HCD | With | With | Dry |
| NHCD | Without | With | Dry |
| HNCD | With | Without | Dry |
| NHNCD | Without | Without | Dry |
| HCW | With | With | Wet |
| NHCW | Without | With | Wet |
| HNCW | With | Without | Wet |
| NHNCW | Without | Without | Wet |

HCD – Hydrolyzed, centrifuged and dry yeast; NHCD - No hydrolyzed, centrifuged, and dry yeast; HNCD – Hydrolyzed, no centrifuged and dry yeast; NHNCD - No hydrolyzed, no centrifuged and dry yeast; HCW - Hydrolyzed, centrifuged and wet yeast; NHCW - No hydrolyzed, centrifuged and wet yeast; HNCW – Hydrolyzed, no centrifuged and wet yeast; NHNCW - No hydrolyzed, no centrifuged and wet yeast.

The enzymatic treatment was performed using pectinolytic enzymes *Pectinex Ultra SP* (Novozymes, São Paulo, Brazil) at 0.025% (v/w) at 30 ℃ for 78 min. Banana must was submitted to centrifugation at *2683 g* (Model T23, Janetzki, Germany) for 10 min in order to obtain the banana juice. The commercial yeast was suspended in sterile water at 30 ℃ at 20 g/L to in itiate fermentation. The yeast viability was previously analysed in a Neubauer chamber (OLIVEIRA; CARDELLO*; et al.*, 2005).

### 3.2.3 Employing selected yeast

The optimized fermentation condition described in the preceding section was applied to test the selected yeast and the banana must was inoculated with the suspension cells of strains, UFMGA-1007 and UFMGA-1031. Cells were suspended in sterile water to provide the same concentration before undertaking the fermentation tests.

### 3.2.4 Fermentation conditions

Inoculation was done by addition of a cell suspension of yeast corresponding a 10 % of the volume of must adjusted previously, yielding a concentration of 8 x $10^9$ cells/mL. Fermentation was conducted using Erlenmyer flasks containing 100.000 g of must, and covered with cotton plugs. The must was incubated under static conditions at a controlled temperature at 30 ± 1 ℃ until the release of $CO_2$ was less than 0.099 g for each flask. (OLIVEIRA; ROSA*; et al.*, 2005)

### 3.2.5 Kinetic parameters of fermentation

The kinetic parameters of fermentation were calculated as a function of the ethanol yield (%) and productivity (g/L/h), and the efficiency of the yeast (%). In order to calculate these parameters, total reducing sugars (TRS), density, pH, and total titrable acidity (TTA) of the banana and fermented musts were determined. The ethanol content in the fermented must also was measured. TRS was determined according Miller (1959). The ˚Brix and TTA were measured following AOAC (2000a); AOAC (2000b) methods, respectively. The pH was measured using a pH meter (QUIMIS, São Paulo, Brazil). The ethanol content was determined using a method described by Zimmermann (1963) after steam-distillation of fermented must in a microstill (Te-012 Tecnal, São Paulo, Brazil).

The ethanol yield (%) was calculated using the following expression: (Ethanol produced/Theoretical quantity) x 100.

The fermentation efficiency (%) was calculated using the following expression: Ethanol produced/[($TRS_{must}$ – $TRS_{fermented\ must}$) x 0.511] x 100.

The productivity (g $L^{-1}$ $h^{-1}$) expresses the mass of ethanol produced (g) per volume (L) by the must fermented over time (h).

### 3.2.6 Analysis of volatile compounds

The SPME method for VOCs extraction was performed using a polyacrylate (PA) 85 □m fibre (Supelco, Bellefonte, PA, USA). The sample (10.0 mL) was added to 22 mL Pyrex vials containing 0.5 g of sodium chloride and the vials were immediately sealed. The fibre was inserted into the headspace for 25 min at 60 ˚C. Subsequently, the fibre was introduced into the GC injector to allow thermal desorption of the analytes at 240 ˚C for 3 min, in splitless mode (NONATO *et al.*, 2001). For analysis of the blank, the same procedure was performed using the matrix with 5% ethanol.

A commercial mixture of *n*-alkanes, $C_9$–$C_{22}$, at 40 mg $L^{-1}$ in hexane (Sigma–Aldrich, St. Louis, MO) was injected to calculated the linear retention indices. The calibration curve was determined using SPME and prepared in six-point concentrations in the range of 10.0 to 340.0 mg $L^{-1}$ of 1-propyl alcohol, 9.6 to 240.6 mg $L^{-1}$ of isobutyl alcohol, 99.9 to 999.9 mg $L^{-1}$ of isoamyl alcohol, 11.0 to 121.0 mg $L^{-1}$ of hexanol, 3.4 to 56.3 mg $L^{-1}$ of ethyl acetate, 1.0 to 103.4 mg $L^{-1}$ of ethyl lactate, 11.6 to 127.9 mg $L^{-1}$ of hexanoic acid.

The VOCs analysis was carried out using an Agilent 7890A gas chromatograph coupled to an Agilent 5975C quadrupole mass spectrometer (Agilent Technologies, Wilmington, DE, USA) with a VOCOL capillary column (60 m × 0.25 mm × 0.15 µm) (Supelco, Bellefonte, PA, USA). The oven temperature was set at 70 ℃ for 2 min, raised to 150 ℃ at 5 ℃ $min^{-1}$, then to 180 ℃ at 10 ℃ $min^{-1}$ and maintained for 20 min. The carrier gas used was helium at a flow rate of 0.66 mL $min^{-1}$. Data were acquired in electron-impact mode (EI) at 70 eV using full scan mode (mass range: 30–550 m/z). The collection of data was performed using ChemStation E.02021431 software (Agilent Technologies, Wilmington, DE, USA).

### 3.2.7 Statistical Analysis

All steps were carried out in triplicate. One-way analysis of variance (ANOVA) and Tukey's test were applied to the experimental data. The Pareto Chart was also

applied to check for interactive effects among the variables using the Statistica 8.0 software (Statsoft Inc., Tulsa, OK, USA). The significance level was 0.05.

The parameters of merit were evaluated according to Eurachem guidelines (CITAC/EURACHEM, 2002). To evaluate the linearity of the curve, the following statistical tests were applied: normality of residues (Ryan-Joiner Test), independence of residues (Durbin-Watson Test), homoscedasticity of residues (Brown-Forsythe Test), significance regression, and deviation from linearity based on ANOVA test (DE SOUZA; JUNQUEIRA, 2005).

## 3.3 Results and Discussion

### 3.3.1 Effect of enzymatic treatment, centrifugation and commercial yeasts strains

The amounts of ethanol produced in fermented banana musts were significantly different and ranged from 1.87% to 6.38% (v/v) (TABLE 3.2). These values were comparable among different varieties of banana and fruit wines (SILVA, 2004; GONZÁLEZ *et al.*, 2010).

The independent variables significantly influenced the kinetic parameters (TABLE 3.2). The results showed that the use of bananas submitted to enzymatic treatment, centrifugation and WCY provided maximum ethanol yield (85.97%) and efficiency (97.98%). There was no significant difference between HCW and HNCW and they presented high ethanol yield. Therefore, in the industrial process, HNCW could be a better option than HCW since it reduces the beverage production process by one step. The productivity evaluates the rate of sugar converted to ethanol. All conditions of the experiments using WCY showed higher values for productivity.

TABLE 3.2 - Kinetic parameters for alcoholic fermentation of banana *Terra* musts

| Experiments[a] | Ethanol amount (g 100 mL$^{-1}$) | Ethanol Yield (%) | Efficiency (%) | Productivity (g L$^{-1}$ h$^{-1}$) |
|---|---|---|---|---|
| HCD | 2.44±0.11 | 32.94±0.22e | 94.38±1.81bc | 1.04±0.007e |
| NHCD | 1.94±0.11 | 25.34±0.05f | 96.00±0.76ab | 0.84±0.003f |
| HNCD | 2.73±0.11 | 36.25±0.12d | 89.16±0.73d | 1.18±0.002d |
| NHNCD | 1.87±0.08 | 23.49±0.06f | 73.92±0.60g | 0.81±0.001f |
| HCW | 6.21±0.20 | 85.97±0.10a | 97.98±0.13a | 2.24±0.003c |
| NHCW | 6.21±0.26 | 83.02±0.41b | 88.98±0.47d | 2.27±0.009c |
| HNCW | 6.38±0.21 | 86.39±2.43a | 92.26±2.77cb | 2.68±0.072a |
| NHNCW | 6.18±0.11 | 77.77±0.35c | 81.39±0.38f | 2.43±0.010b |

[*] Average ± SD (*n*=3). Different letters in same column indicate significant differences (*p<0.05*).
[a] HCD – Hydrolyzed, centrifuged and dry yeast; NHCD – Non-hydrolyzed, centrifuged, and dry yeast; HNCD – Hydrolyzed, non-centrifuged and dry yeast; NHNCD – Non-hydrolyzed, non-centrifuged and dry yeast; HCW - Hydrolyzed, centrifuged and wet yeast; NHCW – Non-hydrolyzed, centrifuged and wet yeast; HNCW – Hydrolyzed, non-centrifuged and wet yeast; NHNCW – Non-hydrolyzed, non-centrifuged and wet yeast. .

The Pareto Chart (FIGURE 3.**1**) shows the effects and interactions of the kinetic parameters. All variables and interaction effects were significant for ethanol yield and productivity. The commercial yeast was negligible for efficiency; whereas the interaction between yeast and enzymatic treatment was significant. These results showed that the kinetic parameters were strongly influenced by the process of fermentation, mainly the enzymatic treatment and yeast strain. The enzymatic treatment promotes pectin degradation, thus reducing the capacity of water retention. This water is then released into the system, thereby decreasing the viscosity (JAYANI; SAXENA; GUPTA, 2005; LEE *et al.*, 2006). Furthermore, centrifugation improves the fermentation process, making the must more fluid and supporting the performance of the yeast. Both variables positively affected alcoholic fermentation, which promoted the growth of yeast and increased the ethanol yield. The WCY presented higher ethanol yield and productivity than DCY. This may possibly be due to cell viability remaining at 96% and 87%, respectively, despite having the same concentrations of

inoculated cells. Also, the DCY requires less time (mean of 19 hours) to complete the alcoholic fermentation compared to WCY (mean of 22 hours). This result could be influenced by cell viability, reducing the capacity of the yeast to ferment the sugar into ethanol. However, no comparisons between wet and dry commercial yeasts have been reported in the literature, to date.

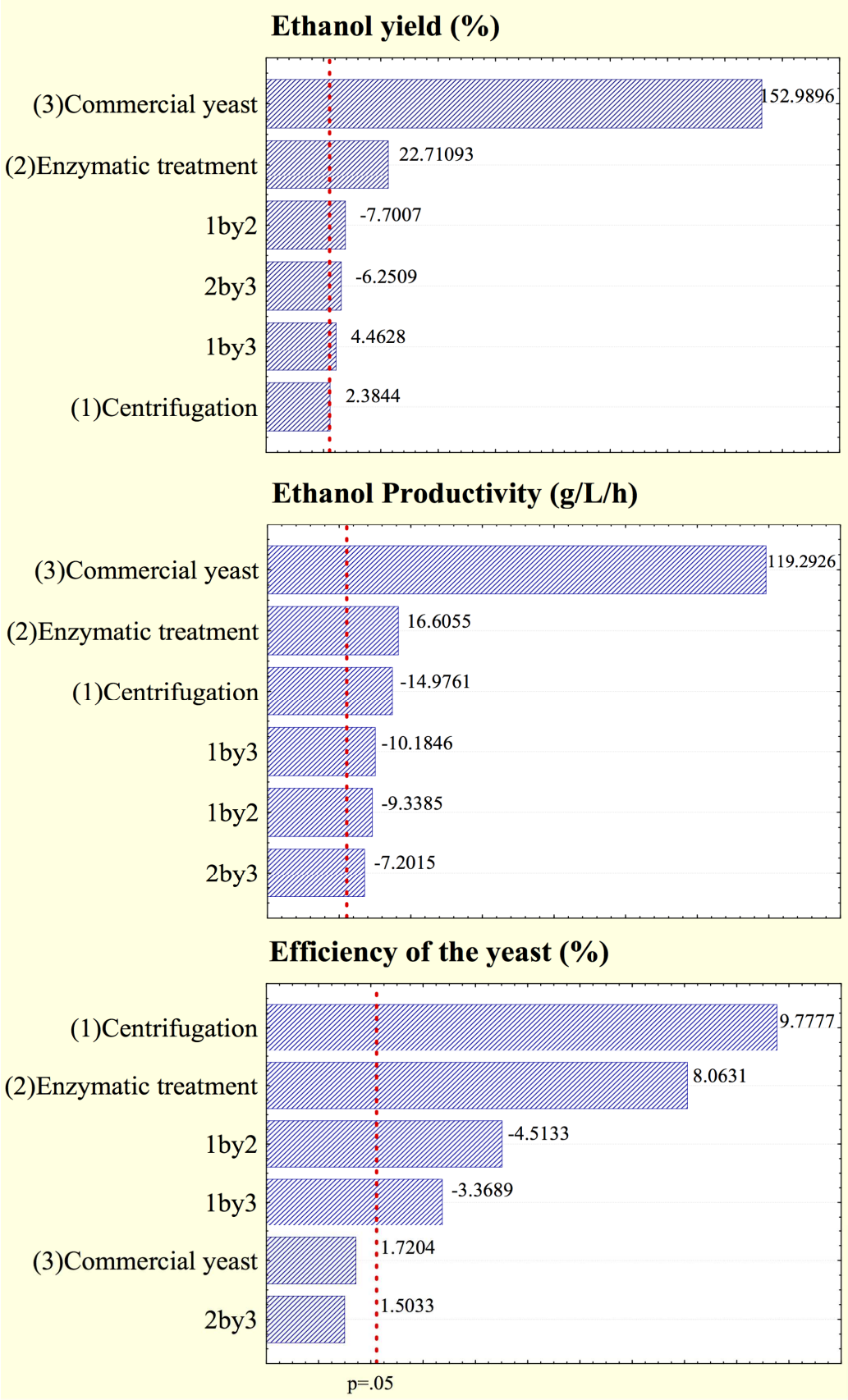


FIGURE 3.1 Pareto Charts showing the effect of the variables enzymatic treatment, centrifugation and commercial yeast strain in kinetic parameters of alcoholic fermentation of banana must

### 3.3.2 Yeast Strain Select

The results of using selected strains UFMGA-1007 and UFMGA-1031 compared to WCY are presented in TABLE 3.**3** T. All kinetic parameters demonstrated significant difference and the commercial yeast presented the highest performance (ethanol yield 85.97%; efficiency 97.98%; productivity 2.24 g $L^{-1}$ $h^{-1}$).

TABLE 3.3 - Effect of selected yeast strain on the alcoholic fermentation of banana must*

| **Conditions** | **Ethanol (g 100 $mL^{-1}$)** | **Ethanol Yield (%)** | **Efficiency (%)** | **Productivity (g $L^{-1}$ $h^{-1}$)** |
|---|---|---|---|---|
| HCW | 6.21±0.20 | 85.97±0.10[a] | 97.98±0.13[a] | 2.24±0.003[c] |
| UFMGA-1007 | 2.93±0.33 | 39.83±0.29[c] | 93.53±1.59[c] | 1.00±0.01[c] |
| UFMGA-1031 | 3.10±0.42 | 42.24±0.26[b] | 94.87±1.39[b] | 1.06±0.79[b] |

*Average ± SD (*n*=3). Different letters in same column indicate significant differences (*p<0.05*). HCW - hydrolyzed, centrifuged and wet commercial yeast; UFMGA-1007 - hydrolyzed, centrifuged and selected yeast UFMGA-1007; UFMGA-1031 - hydrolased, centrifuged and selected yeast UFMGA-1031.

The ethanol yields obtained with *S. cerevisiae* UFMGA-1007 and UFMGA-1031 were 39.83 and 42.24%, respectively. Alvarenga *et al.* (2011) evaluated the effect of different selected yeast strains on the fermentation of the banana juice must, variety “Nanica”. The use of commercial yeast (96.41%) also showed better results for ethanol yield than the selected strains (72.91 to 90.75%).

Productivity also was higher with the WCY than the selected yeast. WCY requires less time (22 hours) than selected yeasts (mean of 25 hours) to complete the alcoholic fermentation and would benefit the industrial process in terms of efficiency of alcoholic fermentation of banana must.

The WCY showed better results than the selected strains. This could be explained by differences in yeast strain and yeast tolerance to the adaptation of must, even though the cell viability of UFMGA-1031 (91%) and UFMGA-1007 (92%) was similar to that of WCY. It might also be due to differences in experimental conditions of the alcoholic fermentation processes, such as an initially harsh

environment and the different varieties of banana. Therefore, the identification or types of cell strains in the commercial yeast need to be clarified.

### 3.3.3 Analysis of volatile organic compounds (VOCs) by CG/MS

A total of 22 non-target VOCs were tentatively identified by library search and retention index. Among the most prevalent were alcohols, esters, acids, and aldehydes (TABLE 3.**4**). The VOCs were more influenced by using different yeasts than by manipulating other parameters. However, analysis of VOCs shows numerous differences in the intensities and concentrations of some compounds.

TABLE 3.4 - Volatile organic compounds analysed in different fermented banana *Terra* musts

| **Compounds** | **I Literature[c]** | **I Calculated** | **HCD** | **NHCD** | **NHCD** | **NHNCD** | **HCW** | **NHCW** | **HNCW** | **NHNCW** | **UFMGA-1007** | **UFMGA-1031** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1-Propanol[b] | 627 | 604 | x | x | X | x | x | x | x | x | x | x |
| Acetic Acid[b] | 576 | 542 | x | x | X | x | x | x | x | x | x | x |
| Acetaldehyde Hydroxyl | 706 | 659 | | | X | | x | | | | x | x |
| Ethyl Acetate[b] | 628 | 672 | x | x | X | | x | x | x | x | x | x |
| 2-Methyl-1-Propanol[b] | 660 | 682 | x | x | X | x | x | x | x | x | x | x |
| Propanoic acid, 2-Methyl | 793 | 821 | x | x | X | x | x | x | x | x | | |
| 3-Methyl-1-Butanol[b] | 844 | 828 | x | x | X | x | x | x | x | x | x | x |
| 2-Methyl-1-Butanol | 852 | 834 | x | x | X | x | x | x | x | x | x | x |
| 2,3-Butanediol | 897 | 924 | x | x | X | x | x | x | x | x | x | x |
| 3-Methylbutanoic Acid | 971 | 953 | x | x | X | x | x | x | x | x | x | x |
| Ethyl (S)-2-hydroxypropanoate[b] | 993 | 970 | x | x | X | x | x | x | x | x | | x |
| 1-Hexanol | 990 | 1044 | x | x | X | x | x | x | x | x | x | x |
| 2-Hydroxypropanoic Acid | 1058 | 1035 | x | x | X | x | | | | x | x | x |
| 3-methylbut-1-yl ethanoate | 1117 | 1054 | | | | | x | x | x | x | x | x |
| 2,3-Dihydroxypropanal | 913 | 1062 | x | | X | x | x | | | | x | x |
| Hexanoic Acid[b] | 1186 | 1194 | x | x | X | x | x | x | x | x | x | x |
| Ethyl Hexanoate | 1229 | 1233 | x | x | X | x | x | x | x | x | x | x |
| Propane-1,2,3-triol | 1196 | 1257 | x | x | X | x | x | x | x | x | x | x |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Octanoic Acid | 1370 | 1379 | x | x | X | x | x | x | x | x | x | x |
| Phenylethyl Alcohol | 1272 | 1459 | x | x | X | x | x | x | x | x | x | x |
| Ethyl Octanoate | 1436 | 1415 | x | x | X | x | x | x | x | x | x | x |
| D-Glucose | 1698 | 1695 | x | x | X | x | x | x | x | x | x | x |

[a] Compounds are listed in sequence of elution.

[b] Retention index calculated using authentic standard

[c] NIST (2009); El-Sayed (2012); Acree; Arn (2013)

HCD – Hydrolyzed, centrifuged and dry yeast; NHCD - No hydrolyzed, centrifuged, and dry yeast; HNCD – Hydrolyzed, no centrifuged and dry yeast; NHNCD - No hydrolyzed, no centrifuged and dry yeast; HCW - Hydrolyzed, centrifuged and wet yeast; NHCW - No hydrolyzed, centrifuged and wet yeast; HNCW – Hydrolyzed, no centrifuged and wet yeast; NHNCW - No hydrolyzed, no centrifuged and wet yeast. UFMGA-1007/UFMGA-1031 = Hydrolyzed, centrifuged and selected yeasts UFMGA-1007 and UFMGA-1031, respectively.

Seven higher alcohols were identified in fermented banana musts and several of them have already been found in banana fruit (VERMEIR *et al.*, 2009; SELLI *et al.*, 2012). In spirits, the sum of 1-propanol, 2-methyl-1-propanol and 3-methyl-1-butanol expresses the total concentration of higher alcohols. If these compounds are higher than 360 mg/100 mL of anhydrous alcohol (aa) it is an indication of poor beverage quality, due to their strong, pungent smell and taste (LÓPEZ-VÁZQUEZ *et al.*, 2010). However, they may contribute to the floral nuance of alcoholic beverages when present in low concentrations (DUARTE; DIAS; OLIVEIRA; TEIXEIRA*; et al.*, 2010). Favorable keynotes to alcoholic beverages have been attributed to 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 1-hexanol (GONZÁLEZ *et al.*, 2010; LÓPEZ-VÁZQUEZ *et al.*, 2010; REDDY; REDDY, 2011). 1-Hexanol is partially a non-alcoholic fermentation product which contributes to a green, grassy and leafy odour affecting both the aroma and taste of the beverage (GONZÁLEZ *et al.*, 2010; SELLI *et al.*, 2012).

Methanol is reportedly associated with harmful effects on health and its presence in distilled fruit spirits is strictly controlled. Methanol was not detected in the fermented banana analysed in this study, which was verified with a standard.

Volatile components belonging to the group of fatty acids such as 2-methylpropanoic, 3-methylbutanoic, octanoic, hexanoic and decanoic acids can contribute significantly to the aroma of wines and spirits. Therefore, their odour may be as strong as that of acetic acid and also could be associated with the odour descriptor “rancid” (ZHANG; WOODAMS; HANG, 2011).

Some of these fatty acids, including hexanoic, octanoic and 3-methylbutanoic acids occur naturally in fruit, they may be formed during the fermentation as secondary products of the yeast metabolism, and they could inhibit the alcoholic fermentation (DUARTE; DIAS; OLIVEIRA; TEIXEIRA*; et al.*, 2010; OLIVEIRA *et al.*, 2011; IVANOVA *et al.*, 2012). All these compounds were detected in the fermented banana studied.

Different studies of the banana aroma profile indicate that the characteristic of banana maturity is determined by the presence of esters (3-methylbut-1-yl ethanoate, acetate esters and butyl esters) (VERMEIR *et al.*, 2009; SELLI *et al.*, 2012).

Some esters, such as ethyl acetate, lactate, hexanoate, octanoate and 3-methylbut-1-yl ethanoate were detected in the fermented banana studied. Esters constitute one of the most interesting classes of aromatic VOCs, mainly ethyl esters,

in alcoholic beverages such as mango wine (REDDY; REDDY, 2011) and grape pomace spirits (LÓPEZ-VÁZQUEZ *et al.*, 2010). Indeed, esters are associated with a pleasant aroma because of their fruity and floral notes (HERNÁNDEZ-GÓMEZ; ÚBEDA; BRIONES, 2003; DUARTE; DIAS; OLIVEIRA; TEIXEIRA*; et al.*, 2010).

### 3.3.4 Analytical validations and quantification of VOCs

TABLE 3.**5** reports the results of parameters of merit that were evaluated according to Eurachem guidelines (CITAC/EURACHEM, 2002). The tests performed showed that the residues followed a normal distribution, were independent and possessed homoscedasticity. ANOVA showed that the regression was significant and that there was no deviation from linearity. The limits of detection (LOD) and quantification (LOQ) were calculated using ten consecutive measurements of the blank. LOD and LOQ ranged from 0.056 to 2.694 mg $L^{-1}$ and 0.057 to 2.904 mg $L^{-1}$ respectively. The intra- and inter-assay precision presents coefficients of variation less than 20 %, which is considered suitable for food analysis (CITAC/EURACHEM, 2002).

TABLE 3.5 - Linearity ($R^2$) limits of detection (LOD) and quantification (LOQ) of the method SPME-GC/MS used for quantification of s in fermented banana musts

| **Compound**[a] | **Quantitation ions** | **Linearity ($R^2$)** | **LOD (mg $L^{-1}$)** | **LOQ (mg $L^{-1}$)** | **Coefficients of Variation** | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | **intra-assay** | | **inter-assay** | |
| | | | | | **X2** | **X5** | **X2** | **X5** |
| 1-propanol | 31 | 0.97216 | 2.694 | 2.721 | 16.28 | 12.11 | 18.13 | 11.55 |
| 1-propanol, 2-methyl | 43 | 0.99759 | 0.475 | 0.511 | 18.79 | 8.30 | 16.10 | 17.78 |
| 1-butanol, 3-methyl | 55 | 0.99131 | 2.688 | 2.904 | 17.76 | 6.96 | 19.11 | 14.31 |
| 1-hexanol | 56 | 0.99719 | 0.056 | 0.057 | 19.94 | 5.47 | 18.51 | 7.05 |
| ethyl acetate | 43 | 0.98094 | 0.435 | 0.452 | 18.70 | 7.62 | 10.34 | 14.36 |
| ethyl (S)-2-hydroxypropanoate | 45 | 0.98222 | 1.224 | 1.228 | 18.87 | 19.21 | 19.64 | 14.93 |
| hexanoic acid | 60 | 0.98644 | 0.372 | 0.373 | 19.86 | 10.73 | 19.69 | 15.13 |

[a] Compounds are listed in sequence of class. Concentrations of VOCs of second and fifth point of curve calibration

The validated method was applied to quantify seven compounds in fermented banana musts. The results, presented in FIGURE 3.**2** as mg of compound per 100 mL of aa, showed that differences in the final amounts of some VOCs were statistically significant when employing the different fermentation conditions especially for the yeast employed: WCY, DCY and selected yeasts. These results could be attributed to differences in genetic material and variability in physiological properties of yeasts (DELAHUNTY; EYRES; DUFOUR, 2006).

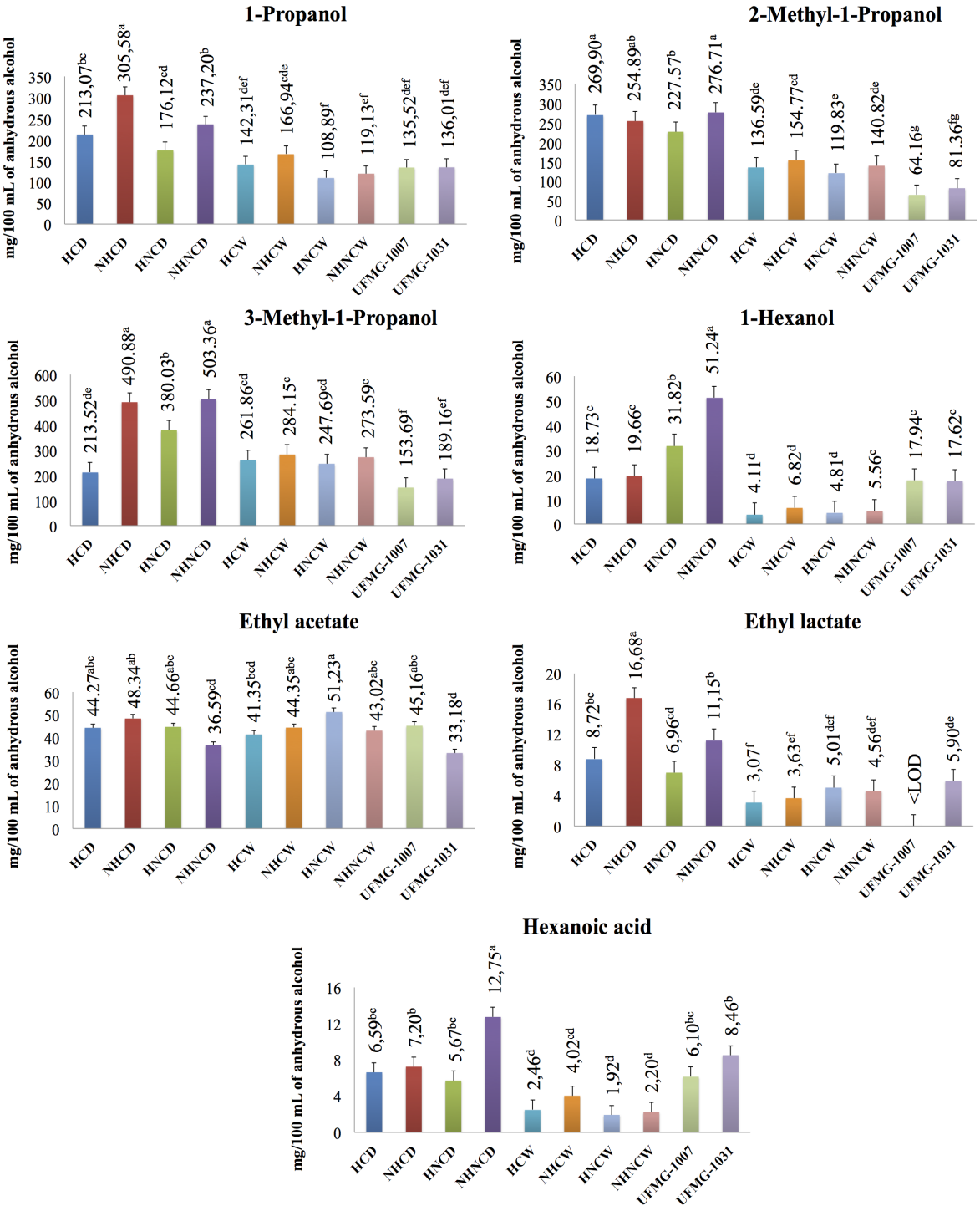


FIGURE 3.2 - Volatile organic compounds concentration (mg 100 $mL^{-1}$ of anhydrous alcohol) in fermented banana musts

In this work almost all fermented musts presented concentrations of higher alcohols above 360 mg per 100 mL aa, except for the UFMGA-1007 (353.37 mg 100 $mL^{-1}$ aa). Globally, the selected yeasts UFMGA-1007 and UFMGA-1031 produced lower content of higher alcohols, but showed lower ethanol yield. These were

following by the group of WCY and DCY. The study by Duarte; Dias; Oliveira; Teixeira*; et al.* (2010) on fermented fruits produced from the pulps of *gabiroba, umbu, cupuassu, jaboticaba* and cacao inoculated with *S. cerevisiae* UFLA CA 1162 revealed that higher alcohols are present in large concentrations in all fermented fruits (226.01 to 774.78 mg 100 mL aa). Consistent with observations from this study, the amount of higher alcohols is dependent on the yeast strain and is responsible for the secondary aroma of beverages (DELAHUNTY; EYRES; DUFOUR, 2006; DUARTE; DIAS; OLIVEIRA; TEIXEIRA*; et al.*, 2010).

1-Hexanol is considered to be favorable at concentrations between 0.5 to 10 mg 100 $mL^{-1}$ aa (D'ACAMPORA ZELLNER *et al.*, 2008; GONZÁLEZ *et al.*, 2010). In this study, the concentration of 1-hexanol in fermented banana was lower using WCY (mean of 5.33 mg 100 $mL^{-1}$ aa) and it could be considered a positive characteristic for these samples.

The concentration of ethyl acetate varied among the fermented banana musts, independently of the fermentation conditions. The presence of acetate esters in low doses (50-80 mg $L^{-1}$), especially ethyl acetate, contribute to the aroma and have a positive impact on the quality of the product (HERNÁNDEZ-GÓMEZ; ÚBEDA; BRIONES, 2003; DUARTE; DIAS; OLIVEIRA; TEIXEIRA*; et al.*, 2010). The average values found in the present study for fermented banana *Terra* were in this range. This results are similar to other research using banana *Nanica* (32.84 mg 100 $mL^{-1}$ aa) (GUIMARÃES FILHO, 2003) and fruit such as gabiroba (22.96 mg 100 $mL^{-1}$ aa).

However, different fruits provide different results. For example, the amount of ethyl acetate found in the present study was lower when using cacao (295.17 mg 100 $mL^{-1}$ aa) which is consistent with other studies (DUARTE; DIAS; OLIVEIRA; TEIXEIRA*; et al.*, 2010). The results of the present study also showed that the VOCs in fermented banana musts were also found in other types of beverages made with different fruits.

The ethyl (S)-2-hydroxypropanoate, at low concentrations (10 mg 100 $mL^{-1}$ aa), contributes to the stabilization of distillate flavour and softens the harsh flavour characteristics in beverages (SOUFLEROS; MYGDALIA; NATSKOULIS, 2005). This ester was detected at low concentrations in fermented banana musts (3.07 to 16.68 mg 100 $mL^{-1}$ aa) mainly when WCY was employed.

Although some volatile fatty acids are minor compounds in fruit spirits, their odour might have undesirable effects on the aroma when found in amounts above the thresholds (0.7 mg $L^{-1}$) (DELAHUNTY; EYRES; DUFOUR, 2006). Hexanoic acid was

found at a concentration below the thresholds in the group employing WCY (2.7 mg 100 $mL^{-1}$ aa). It was also described as being naturally present in raw banana fruit, in the range of 0.238 to 0.438 mg $Kg^{-1}$ (SELLI *et al.*, 2012).

Oliveira; Cardello*; et al.* (2005) showed a negative correlation between the ethanol content and VOCs in sugarcane spirits, particularly in regard to acidity, ethyl acetate, 1-propanol, 2-methyl-1-propanol and 3-methyl-1-butanol. These correlations illustrate that a variation in the ethanol content of the fermented sugarcane must is an important factor for the standardization of the ethanol/volatiles ratio and the quality of the commercialized beverage produced in the industrial processes. In particular, clarifying the conditions of the fermentation processes will lead to improvements in the manufacturing of banana spirits with an acceptable amount of higher alcohols.

## 3.4 Conclusions

The present study shows that the kinetic parameters of alcoholic fermentation of banana *Terra* was influenced by yeast strain, enzymatic treatment and centrifugation. The maximum ethanol yield (85.97% and 86.39%) and efficiency (97.98%) were obtained using enzymatic treatment, WCY and centrifugation.

The SPME-GC/MS proved to be a useful, simple method that can be applied to evaluate aromatic compounds in beverages. A total of 22 VOCs were detected in fermented banana musts. The typical VOCs profile was determined especially by yeast strain, characteristics of the fermentation conditions and the type of fruit. The fermented banana *Terra* presented a volatile composition similar to that of other alcoholic beverages.

Although further studies are needed, our results suggest that work combining selected strains with commercial yeasts could be used in order to control the amount of VOCs, including higher alcohols.

# 4. CHAPTER 3: IDENTIFICATION OF AROMA-ACTIVE VOLATILES IN BANANA *TERRA* SPIRIT USING MULTIDIMENSIONAL GAS CHROMATOGRAPHY WITH SIMULTANEOUS MASS SPECTROMETRY AND OLFACTOMETRY DETECTION


## Abstract

Fruit spirits have been produced and consumed throughout the world for centuries. However, the aroma composition of banana spirits is still poorly characterised. We have investigated the aroma-impact compounds of the banana *Terra* spirit for the first time, using multidimensional gas chromatography (MDGC and GC×GC) in a multi-hyphenated system – i.e., coupled to flame ionisation detection (FID), mass spectrometry (MS), and olfactometry (O). Solid-phase microextraction (SPME) was used to isolate the headspace aroma compounds of the banana spirit. The detection frequency (DF) technique was applied and aroma regions, detected in the first column separation at > 60% Nasal Impact Frequency (NIF), were screened as target potent odour regions in the sample. Using a polar/non-polar phase column set, the potent odour regions were further subjected to MDGC separation with simultaneous O and MS detection for correlation of the aroma perception with MS data for individual resolved aroma-impact compounds. GC-O analysis enabled identification of the respective volatiles from 18 aroma-impact regions; for example, those comprising flower, whisky, green, and others. Compounds were tentatively identified through MS data matching and retention indices in both first and second dimensions. The principal volatile compounds identified in this work, that are responsible for the characteristic aroma of the banana spirit, are 3-methylbutan-1-ol, 3-methylbutan-1-ol acetate, 2-phenylethyl acetate and phenylethyl alcohol. This is the first such study to reveal the major aroma compounds that contribute to banana spirit aroma.


*Keywords:* Olfactometry; Aroma; multidimensional gas chromatography; gas chromatography - olfactometry; comprehensive two-dimensional gas chromatography; Banana spirit.

**Resumo**

As aguardentes de frutas tem sido produzidas e consumidas em todo o mundo Entretanto, os compostos responsáveis pelo aroma da aguardente de banana ainda são desconhecidos. Este trabalho, teve como objetivo investigar os compostos voláteis de importância aromática da aguardente de banana *Terra* pela primeira vez usando cromatografia gasosa multidimensional (MDGC e GC×GC) em um sistema acoplado com detectores de ionização por chama (FID), espectrometria de massa (MS) e olfatometria (O). Os voláteis foram isolados via *heaspace* por microextração em fase sólida (SPME). As regiões de importância aromática foram identificadas pela técnica de detecção por frequência (DF) por $^{1}$D GC/FID/O empregando 60 % de Frequência de Impacto Nasal (NIF) e confirmadas pela análise por GC×GC/FID. A análise por GC/O permitiu identificar 18 regiões com atividade odorífera com descrição do aroma tais como floral, uísque, frutado dentre outros. Empregando um conjunto de colunas de fase polar e apolar, as principais regiões odoríferas foram submetidas à análise de separação por MDGC com detectores simultâneos, MS e O para identificação individual dos compostos de aroma. Os compostos foram tentativamente identificados pela comparação com os dados do espectro de massas e o índice de retencão em ambas dimensões. Os principais compostos voláteis identificados, neste trabalho, responsáveis pela característica aromática da aguardente de banana *Terra* foram 3-metilbutan-1-ol, etanoato de 3-metilbutan-1-ol, etanoato de 2-feniletila e 2-feniletanol. Esta é a primeira vez em que um estudo revela a importância do conhecimento acerca dos principais compostos odoríferos que contribuem com o aroma de aguardente de banana, apesar de outros compostos contribuintes ainda precisarem ser identificados.

*Palavras-chave*: Olfatometria. Aroma. MDGC. GC/O. GC×GC/FID, Aguardente de banana.

## 4.1 Introduction

Banana (*Musa spp.*) is an important food crop which grows extensively in tropical and subtropical regions and is widely consumed throughout the world. However, loss and waste in fruit production represent a significant cost in the market; developing alternative products for banana is imperative. The distillates industry has demonstrated large interest in producing novel alcoholic beverages from residue or unusual raw materials (SAMPAIO *et al.*, 2013). Considering the excellent sensory properties, the manufacture of banana-based beverages is of interest to the industry, especially because of the pleasant flavour and amount of sugar. Many studies have shown the aromatic profile of banana (AURORE; PARFAIT; FAHRASMANE, 2009; ZHU, 2009; SELLI *et al.*, 2012; HSIEH *et al.*, 2013). The highest rated compounds, which appear to contribute to the aroma of this fruit, were esters, followed by aldehydes. The main esters were isoamyl acetate and 2-pentanol acetate, that contribute to fruit notes; n-hexanal and 3-methylbutanal were the aldehydes with important odour-active compounds in banana, providing herbal-green-grassy aroma (SELLI *et al.*, 2012; HSIEH *et al.*, 2013). The abundant sugar content in banana fruit suits the preparation of fermented-distilled beverages (spirits). In this way, banana spirit has been produced using the banana residue, which is the banana in the last stage of maturation without commercial value, which enables the acquisition of different flavours and, potentially, attracts new markets (SAMPAIO *et al.*, 2013).

Spirit beverages are characterised by their alcohol content in addition to the amount of a variety of volatile organic compounds (VOCs). These are affected by many factors, such as the raw materials; the fermentation conditions, including the yeast strain; the distillation process; the aging of the product; and, the type of wood used for storage (OLIVEIRA; ROSA*; et al.*, 2005). The composition and concentration levels of these compounds vary widely for each spirit. The complexity of the molecular composition arises from the factors outlined above. The large number of compounds, comprising a wide range of concentrations, requires analytical methods having good performance, i.e., separation power and sensitivity (NONATO *et al.*, 2001). Frequently, compounds at trace levels have greater influence on the sensory properties of alcoholic products than those present in high concentrations, according to relative odour impact (PLUTOWSKA; WARDENCKI, 2008). However, in order to understand the contribution of each VOC to odour quality, it is not sufficient just to

know whether these compounds are present or absent, one also must have knowledge of how they are perceived at given concentrations (DELAHUNTY; EYRES; DUFOUR, 2006). In addition, the identification of these compounds may be used to determine the flavour characteristics of the spirit, to detect illicit compounds and to identify inconsistent manufacturing practices (SAMPAIO *et al.*, 2013).

Despite the fact that distillate spirits, especially fruit spirits, have been produced for centuries, there are few published studies about their characteristic aroma composition across the many different products. Furthermore, no study has, until now, focused on identifying key aroma compounds of banana spirit by gas chromatography-olfactometry (GC-O).

Use of the human nose as a detector is necessary because perception thresholds may be far lower than those of instrumental detectors (DUSSORT *et al.*, 2012). It is also able to sense aroma attributes in the detected odour (DELAHUNTY; EYRES; DUFOUR, 2006). In complex samples, the identification of odour compounds using conventional GC-O in conjunction with flame ionisation detectors (FID) or mass spectrometry (MS) can be limited due to peak co-elution in the odour regions. Incorrect identification may result in a trace odourant being masked by a large odourless or weak odour active compound (DELAHUNTY; EYRES; DUFOUR, 2006; EYRES; MARRIOTT; DUFOUR, 2007).

Comprehensive two-dimensional gas chromatography (GC×GC) has proven to be a valuable tool to characterise very complex food volatile compositions. However, the combination of olfactometry and GC×GC may become complicated for judges, because of the slow breathing cycle of a human assessor compared to the rapid eluting peaks from the system. Multidimensional GC (MDGC) is able to resolve a number of selected co-eluting compounds in aroma regions, whilst permitting olfactory assessment of the individual compounds. It employs heart-cutting (H/C) and cryo-trapping (CT) devices for isolation and transfer of target solute(s) from a first dimension column ($^{1}$D) to a second dimension column ($^{2}$D), comprising a different column phase and being equipped with a sniff port outlet (EYRES; MARRIOTT; DUFOUR, 2007; CHIN; EYRES; MARRIOTT, 2012; PARAVISINI *et al.*, 2015)

To determine odour compounds, the method used for isolation of the analytes from the matrix is particularly important. The appearance of ‘aromagrams’ in GC-O depends largely upon the sample preparation procedure, which might affect the composition of the isolated compounds (PLUTOWSKA; WARDENCKI, 2008). Several

techniques have been applied to aroma extraction in food applications, particularly in alcoholic beverages. Headspace solid-phase microextraction (SPME) has been widely used as an effective technique to probe the headspace of a material, in combination with GC-O to study and characterise aroma-active compounds (MADRERA; GOMIS; ALONSO, 2003). This technique avoids possible contamination of the extract by non-volatile sample components and yields useful qualitative data due to its simplicity and ease of sample preparation (MAIKHUNTHOD; MARRIOTT, 2013). A mixed divinylbenzene/carboxen/polidimethylsiloxane (DVB/CAR/PDMS) fibre with 2 cm has been used in most cases found in the literature, which ensures satisfactory yield of the largest amounts of odour compounds from alcoholic beverages (PLUTOWSKA; WARDENCKI, 2008).

The purpose of this work is to identify the aroma-active compounds from banana *Terra* spirit by screening potent odours using GC-FID/O with the detection frequency (DF) technique. Odour regions were profiled using GC-FID/O and targeted for complete separation and further identification using heart-cut MDGC with simultaneous olfactory and MS detection. Headspace SPME is used as a sampling procedure to extract some aroma compounds. Compounds were tentatively identified using GC-MS and retention index (*I*) matching in GC-FID/O and MDGC-MS/FID/O. The identification was also performed using some standard odourant compounds.

## 4.2 Materials and Methods

### 4.2.1 Sample obtaining and chemical standards

The Banana *Terra* Spirit (40% ethanol) was manufactured in the *Microbiology Industrial Laboratory* of *Faculdade de Farmácia* of *Universidade Federal de Minas Gerais* (Brazil). The conditions for alcoholic fermentation employed enzymatic treatment of, and wet commercial yeast of *Saccharomyces cerevisiae* (*S. cerevisiae*) applied to, banana must. The distillation was performed using traditional copper alembics of 5 L. The fermented banana must was transferred to the vessel up, to 2/3 of its capacity. During the distillation, samples of approximately 50 mL were recovered and the ethanol amount of each was determined in order to obtain three fractions according to their ethanol content: “head” (>54 % v/v), “heart” (36-54 % v/v),

and “tail” (<36 % v/v). The “heart” of the banana *Terra* spirit was put into glass bottles and stored in the dark until analysis.

Standard analytical reagents were used as follows: ethanol (J.T. Baker, Mexico), 3-methylbutan-1-ol (Vetec, Brazil), 1-hexanol, (Fluka, Germany), 3-methylbutan-1-ol-acetate, acetic acid, 2-phenylethyl acetate, phenylethyl alcohol (Aldrich, Germany) and sodium chloride (Merck Chemical Co., Merck KGaA, Germany). Ultrapure water was obtained from a Milli-Q purification system (Millipore, Milford, MA).

For the preliminary screening, the following 1 cm SPME fibres were tested: 85 $\mu$m polyacrilate (PA), 65 $\mu$m polydimethylsiloxane/divinylbenzene (PDMS/DVB), 85 $\mu$m carboxen/divinylbenzene (CAR/PDMS) and 50/30 $\mu$m polydimethylsiloxane/carboxen/divinylbenzene (PDMS/CAR/DVB) (Supelco, Bellefonte, PA, USA). A 2 cm fibre of PDMS/CAR/DVB, was also evaluated.

### 4.2.2 Sample preparation and SPME procedure

For sample preparation, a 4.0 mL aliquot of banana *Terra* spirit, diluted with ultrapure water and brought up to a volume of 50.0 mL in a volumetric flask, was used. The extraction of volatile compounds was carried out using a headspace solid phase microextraction (SPME) performed manually. The fibre was chosen previously following evaluation of the intensity (numbers of peaks) of the aroma extract components from the sample. Four types of fibre were evaluated; PA, PDMS/DVB, CAR/DVB and PDMS/CAR/DVB. Following the optimization of the extract, a 1 and 2 cm PDMS/CAR/DVB fibre (Sigma-Aldrich St. Louis, MO, USA) was tested. The best result was used in the present study. All fibres were previously conditioned following the manufacturer’s instructions. For SPME, an 8.0 mL aliquot of banana spirit solution, with the addition of 2.0 g of sodium chloride, was used in a 20 mL Pyrex vial. The sample was equilibrated for 5 min at 60 ˚C, before starting the extraction by exposing the fibre to the sample headspace for 25 min at 60 ˚C. The total procedure was performed under magnetic stirring. SPME conditions follow previous studies. The fibre was thermally desorbed in the GC inlet port for 2 min at 240 ˚C under splitless conditions (NONATO *et al.*, 2001).

A $C_8$-$C_{20}$ alkane mixture (Sigma–Aldrich, St. Louis, MO) was used to determination of linear retention indices (*I*). The tentative identification of VOCs was

performed by comparing the mass spectra against library database records (NIST 08 Mass Spectrum library), and retention index data.

### 4.2.3 GC/FID/O and detection frequency analysis

An Agilent 6890 GC (Agilent Technologies, Nunawading, Australia) retrofitted with a Gerstel ODP II model olfactory detection port (Gerstel Inc., Linthicum, MD 21090, USA) was used for GC-FID/O analysis. The capillary column comprised a SolGel-WAX phase (30 m × 0.32 mm × 0.50 $\mu$m film thickness; SGE, Ringwood, Victoria, Australia). The column inlet was connected to a split/splitless injector. The effluent from the column outlet was split to the FID and the olfactory port, respectively, using a Y-union and two deactivated fused silica (DFS) capillaries. One capillary (150 cm length × 0.15 mm I.D.) was directed to the FID; the other capillary (75 cm × 0.15 mm I.D.) was directed to the olfactory port. The signal/noise ratio of alkane mixture decreased 10 times after using the Y-union connection, which provided a 10:1 split between the sniff port and the FID. The GC inlet was set at 240 ℃ with splitless sampling for 2 min. The hydrogen carrier flow rate was 3 mL $min^{-1}$. The oven was programmed starting at 50 ℃ (held for 2 min), increased by 3 ℃ $min^{-1}$ up to 80 ℃, then increased by 10 ℃ $min^{-1}$ up to 240 ℃ (held for 2 min). The FID and the GC-O transfer line temperatures were 250 and 200 ℃, respectively. The olfactory port was supplied with a 15 mL $min^{-1}$ constant flow of humidified air. A series of alkanes (C8-C20) was run to establish retention indices for compound confirmation in olfactometry analysis.

Odour impact compounds were evaluated using the detection frequency (DF) technique (POLLIEN *et al.*, 1997). Seven judges were trained in a three stage program. The first stage used a commercial aroma standard, which is commonly used in the training of panellists in sensory analysis of wine and alcoholic beverages. This stage consisted of evaluating the sensory perception of the judges and also standardizing the descriptive vocabulary. All the panellists were asked to describe the odours they perceived as precisely as possible. In the second stage, they were trained using the diluted solution of the standard odours. Following the training session, the panelists were directed to sniff the standard solution using the GC/FID/O (FIGURE 4.**1**). The final stage of training consisted of sniffing the real sample, continuing to use the GC/FID/O. At the end of this training stage, the similarity of the

results was evaluated to ascertain that each judge gave repeatable results and the panel agreement was satisfactory. Following this full training, the sample was sniffed three times by the seven trained panellists (4 females, 3 males) to determine the aroma-impact regions. During each analysis, the descriptions of odours perceived by the panellists were recorded and calculated as DF (POLLIEN *et al.*, 1997). In instances where the same aroma peaks and their consistent descriptors were detected by four or more panellists (i.e., DF ≥ 4; i.e., ≥ 60% positive detection by panellists), these regions were further selected as significant aroma-impact regions. The duration of the GC-O sniff run was limited to 30 min to prevent panellist fatigue (DELAHUNTY; EYRES; DUFOUR, 2006).

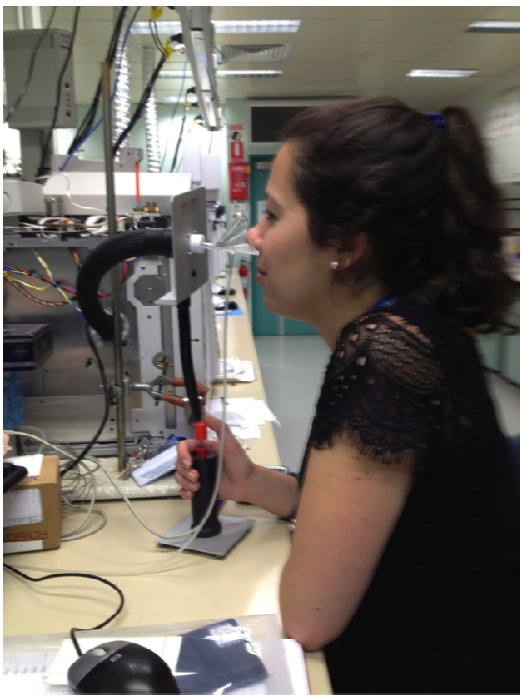

FIGURE 4.1 Picture of a panellist sniffing on the system of GC/FID/O
From: own Author, 2014

### 4.2.4 GC×GC/FID

GC×GC-FID analyses were carried out using an Agilent 7890 GC system (Agilent Technologies, Burwood, Australia) equipped with FID and retrofitted with a longitudinally modulated cryogenic system (LMCS) from Chromatography Concepts (Doncaster, Australia). The LMCS was operated at -10 ℃ with a modulation period of

7.5 s. The FID was operated at 250 ℃ at an acquisition rate of 100 Hz. The first dimension ($^1$D) column was a polar HP-FFAP (free fatty acid phase; nitroterephtalic acid modified polyethylene glycol, Agilent) capillary column (30 m x 0.25 mm x 0.25 $\mu$m). An Rxi-5 SilMS (5% phenyl dimethylpolysiloxane, Agilent) capillary column (1 m x 0.18 mm x 0.18 $\mu$m) was installed as a second dimension column ($^2$D). Injections were performed at 240 ℃ using splitless mode (2 min). Hydrogen was used as a carrier gas at 1.5 mL min$^{-1}$ flow rate. The GC oven was held at 40 ℃ for 2 min and increased to 240 ℃ by 3 ℃ min $^{-1}$. A series of alkanes ($C_8$-$C_{20}$) was used to establish the $^1$D retention indices ($^1I$) for each peak.

### 4.2.5 Heart-Cut MDGC/MS/O analysis

This analysis was carried out using the method proposed and validated according to Plutowska; Wardencki (2008) as shown in FIGURE 4.**2** and FIGURE 4.**3**. The GC-FID/O and H/C MDGC-MS/O analysis was performed on a Bruker System SCION 456-GC (Bruker Corporation, Fremont, CA) retrofitted with a SGE liquid carbon dioxide cryogenic trap (CT) device, a SGE olfactory port (ODO II model), an Agilent G2855A Deans switch (DS) device, and an Agilent G3180B 2-way effluent splitter (ES) (FIGURE 4.**2**). A polar HP-FFAP capillary column (30 m x 0.25 mm x 0.25 $\mu$m) was installed as a $^1$D column and the $^2$D column was a DB-5 MS column (Agilent; 30 m x 0.25 mm x 0.25 $\mu$m). The CT was positioned near the beginning of the $^2$D column to trap H/C fractions transferred to this column. The $^1$D column inlet was connected to a split/splitless injector (pressure applied P1). The outlet of $^1$D column, the inlets of $^2$D column, and inlet of a deactivated fused silica capillary (DFS) that terminated at FID, were connected to the DS device (pressure applied P2). The DS directs effluent flow from $^1$D, and is transferred to either: (a) FID/O via a DFS transfer line (1.0 m x 0.1 mm to the FID detector and 1.0 m x 0.1 mm to the olfactometry port), which was split (at approximate ratio of 1:1) using a Y-connector, or (b) the $^2$D column. The $^2$D column outlet was split (at an approximate ratio of 1:1) by the ES device (pressure applied P3) with flow directed to both the MS detector via a DFS transfer line (0.55 m x 0.1 mm), and to the olfactometry port via another DFS transfer line (0.50 m x 0.1 mm) in order to evaluate the odour activity of compounds resolved via MDGC separation.

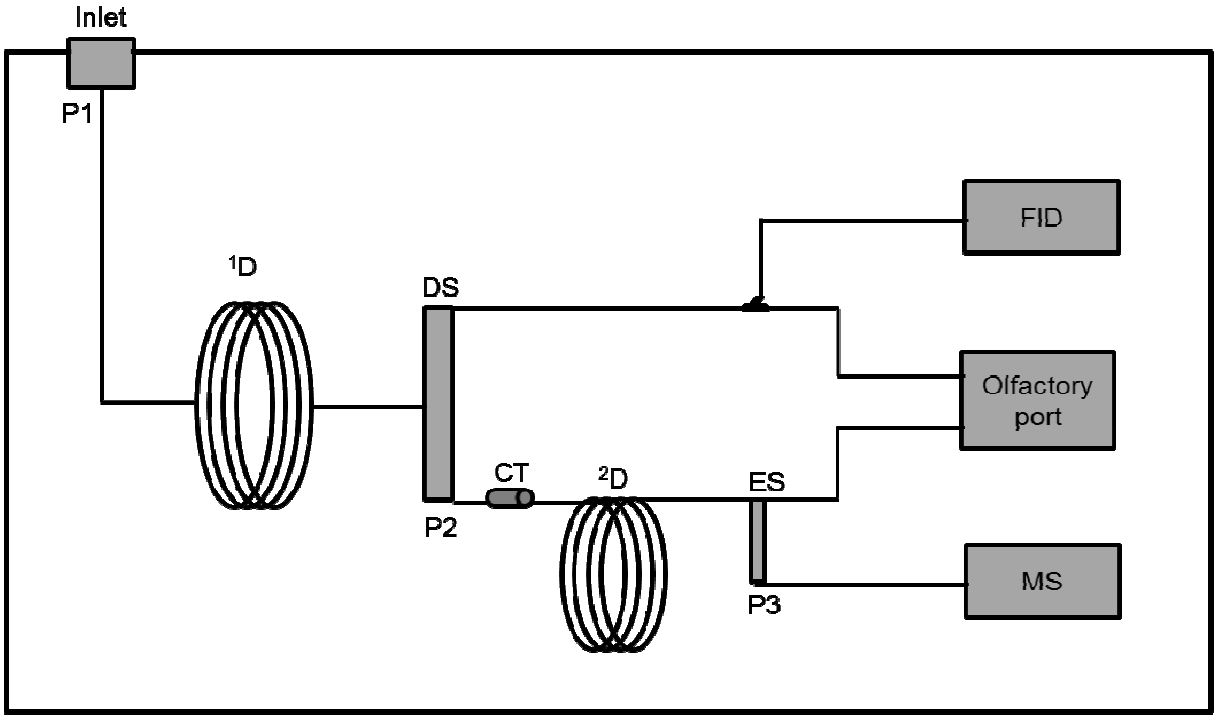


FIGURE 4.2 - System configuration of the integrated GC/FID and MDGC/MS with olfactometry detector. DS: Deans switch; ES: Effluent splitter; CT: Cryotrap; P1, P2, P3: Pressure applied
From: own Author, 2014

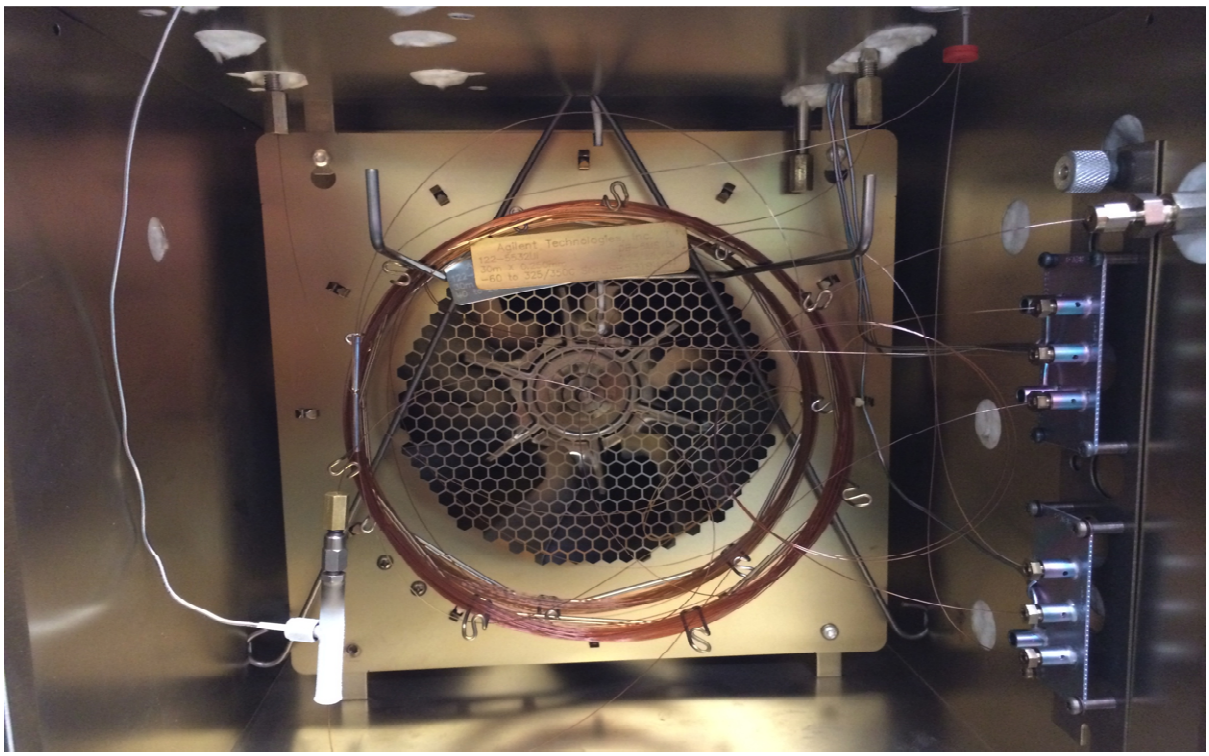

FIGURE 4.3 - Picture of the system configuration of the integrated GC/FID and MDGC/MS with olfactometry detector
From: own Author, 2014

Two experienced panellists performed olfactory evaluation of the H/C regions to locate odour-active peaks detected in either $^{1}$D or $^{2}$D. All the analysis was done in triplicate for each H/C. Retention times and odour descriptors were recorded during the analysis. The olfactory port was supplied with a 15 mL/min constant flow of humidified air. The system was operated under constant pressure mode to maintain the pressure balance between the two columns. Helium was used as a carrier gas with a constant inlet pressure of 51.5 psi (P1) and a constant auxiliary pressure for DS (P2) of 42.5 psi with ES P3 of 25 psi. To perform H/C MDGC operation, a 2-step sequence was programmed for the H/C event as reported previously (PLUTOWSKA; WARDENCKI, 2008). The inlet P1 was altered to 5 psi immediately after H/C of the target region as well as to simultaneously back-flush $^{1}$D, to ensure the sensory analysis during $^{2}$D was not affected by analytes eluting from $^{1}$D. The cooling CT was started 1.5 min before the transfer of the target region H/C to effectively cryo-focus the H/C compounds; cryogen supply was then closed to permit re-mobilisation of the trapped solutes into the $^{2}$D. The oven programming was restarted for each H/C target region using different programmed time events for the first separation according to the selected H/C fraction, described at Section 4.2.3.

The GC injection method was the same as used in the GC/FID/O for the Agilent system. The oven was programmed from 50 ℃ (hold 2 min), at 3 ℃ min$^{-1}$ to 80 ℃, then at 10 ℃ min$^{-1}$ to 240 ℃ (hold 8 min) to ensure all compounds eluted. The FID was kept at 250 ℃ with an acquisition rate of 20 Hz. The quadrupole MS was used with electron ionisation (70 eV) in full scan mode (mass range 45-350 *m/z*), and ion source temperature at 230 ℃. Second dimension index data were determined by transferring all alkanes to the second column, where they were heart-cut and cryotrapped and then eluted using the same procedure as target heart-cut regions.

The Bruker SCION workstation software (version 2.0) was used for data analysis. Compounds were tentatively identified by comparison of their mass spectra to the NIST library (NIST v. 2.0 Mass Spectral library) and retention index data.

## 4.3 Results and discussion

Four fibre coatings, PA, PDMS/DVB, CAR/DVB and PDMS/CAR/DVB were used to determine which coating was more appropriate for the determination of volatile aroma present in banana *Terra* spirit. Considering the number and intensity of the

peaks, a 2 cm PDMS/CAR/DVB fibre provided the best results (Complementary Figure - attached).

The GC-olfactometry study was carried out first with single dimension GC-FID/O to characterise the odourant zones of banana *Terra* spirit. FIGURE 4.**4** indicates the workflow for the study. The number of odours detected in GC-O was large and the often unresolved peaks were noticed to be associated with one or more compounds for which descriptors were reported. The intensities of odour active compounds were also relatively strong, indicating 18 regions in excess of 60% DF level (FIGURE 4.**5**). The GC×GC-FID contour plot representation (FIGURE 4.**6**) illustrates the complexity of the sample, the many co-eluting compounds which will arise for $^{1}$D GC analysis, and the components in $^{2}$D space clearly resolved by GC×GC. So, the requirement is to effectively deconvolute the overlapping peaks in the unresolved $^{1}$D chromatogram by applying the MDGC approach, and to determine the odour perception that is attributable to the resulting resolved components. In addition, the combination of two or more odourants can have an effect on the final aroma (of variable intensity) and give a different nuance as compared to the separate odourants. This may coincide with a complex region of the chromatogram where a large number of compounds co-elute (EYRES; MARRIOTT; DUFOUR, 2007; FALCÃO *et al.*, 2008). These co-eluting peaks have been resolved in the integrated systems proposed, such as GC×GC-TOFMS and MDGC-MS/O (EYRES; MARRIOTT; DUFOUR, 2007; PLUTOWSKA; WARDENCKI, 2008; MAIKHUNTHOD; MARRIOTT, 2013). The second part of this work comprises MDGC analysis (FIGURE 4.**4**) with heart-cut and cryo-trapping devices for the isolation and transfer of target solute(s) from $^{1}$D to $^{2}$D. The dimensions comprise two different phases, and were equipped with a sniff port to sensorially identify the relevant odours present in banana spirit.

Although the columns used in GC-O analysis and $^{1}$D MDGC were not exactly the same (SolGel-WAX and HP-FFAP, respectively), their similarity/polarity is sufficient to achieve appropriate correlation by running the alkane reference standards.

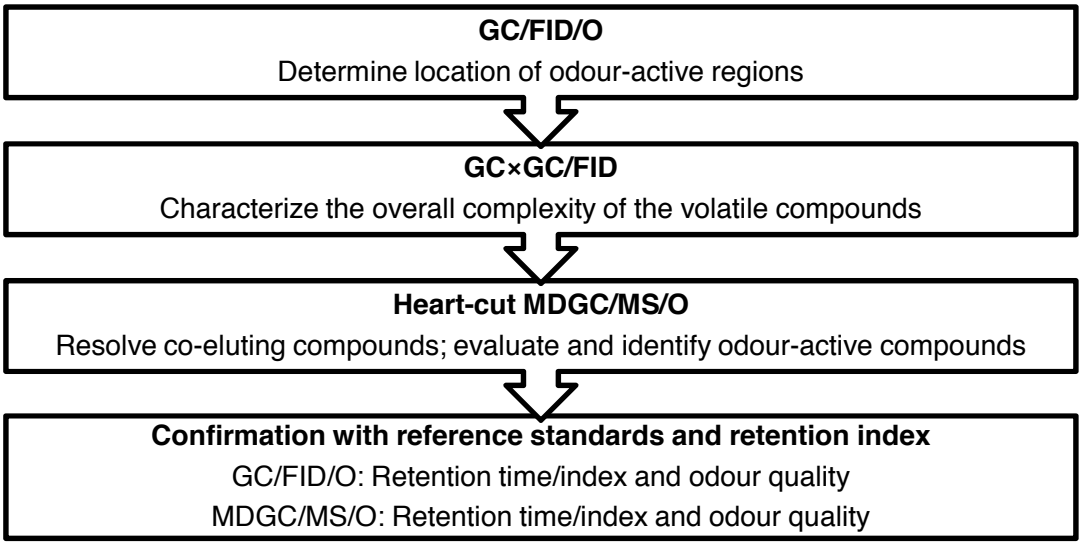


FIGURE 4.4 - Flow diagram of the strategy used to identify character-impact odourants in banana *Terra* spirit using GC×GC/FID, GC/FID/O and MDGC/MS/O systems

### 4.3.1 GC/FID/O and detection frequency analysis

The results of the GC-FID/O (chromatogram and aromagram) study in banana *Terra* spirit are given in FIGURE 4.5. The aroma-active compounds, detected according to the 60% Nasal Impact Frequency (NIF) cut-off value (i.e., detected by at least four panelists) as the threshold indicator, were selected as significant odourants for further identification. Eighteen odourant zones were selected from this spirit. The aroma peaks detected in $^{1}$D GC separation were screened as target potent odour regions in the sample (TABLE 4.**1**). The aromagram (FIGURE 4.**5**b) shows greater intensities for several aroma regions in overall odour intensity perceived by assessors.

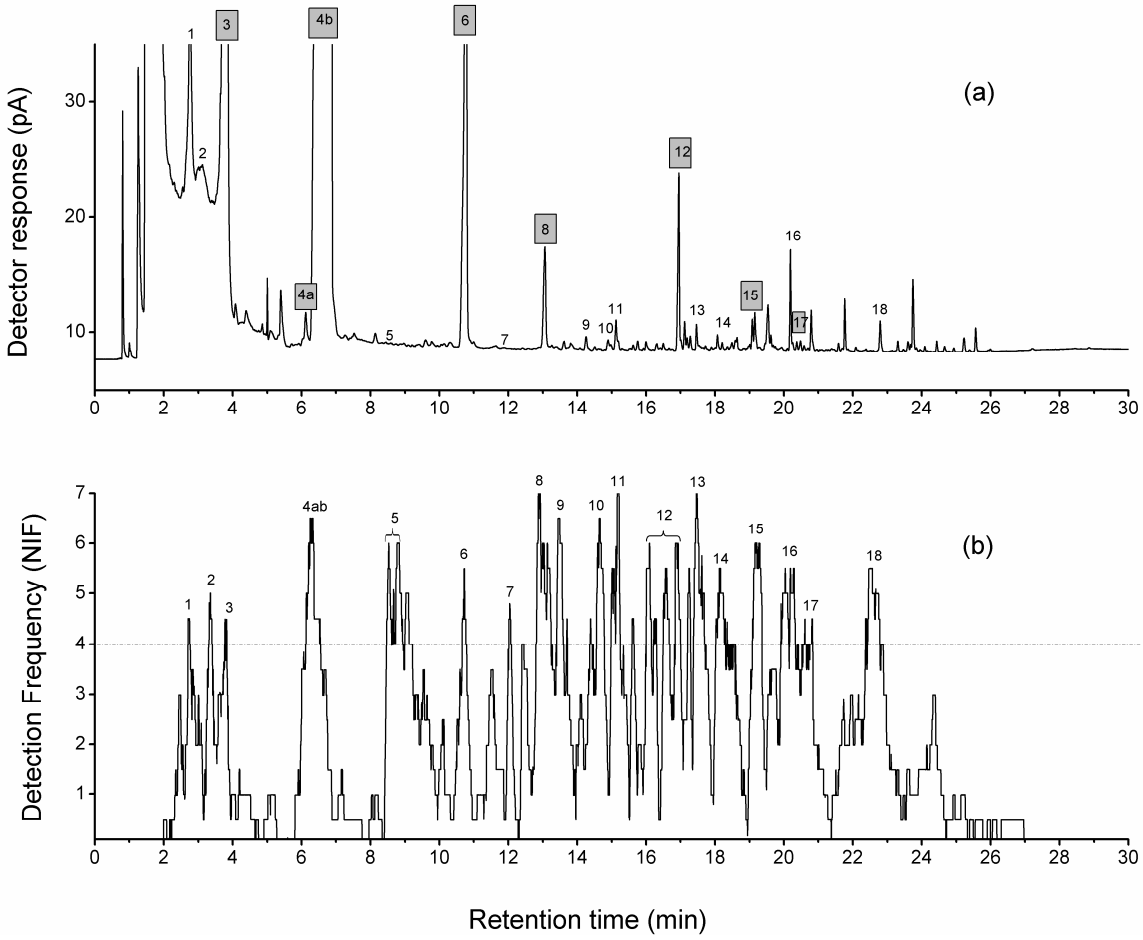


FIGURE 4.5 GC results for Banana Terra spirit. (a) GC/FID data with selected regions (represented by numbers) corresponding detected odours. (b) The SNIF aromagram
The dashed line indicates the level deemed to be positive indicators of odour activity at the 60 % NIF response. Only peaks above the line are reported in TABLE 4.**1**. Highlight numbers indicate regions selected for further heart-cut MDGC/MS/O analysis.

Similar to other distilled spirits, this spirit sample is composed of many volatile compounds that contribute to the aroma and flavour unique to this spirit (FIGURE 4.**5**a). Major volatile compounds are usually formed during the fermentation process, and their formation is influenced by the selected raw material and fermentation conditions. On the other hand, minor volatile components are mainly derived from the raw material and distillation method used (CORTÉS *et al.*, 2011; VILLIÈRE *et al.*, 2012). All these compounds are important quality indicators for the beverage, and must be present in appropriate concentrations to provide pleasant flavour and aroma to the distillate. Often, when present in excessively high concentrations, many of them

provide an unpleasant characteristic to the final product (DUSSORT *et al.*, 2012; SAMPAIO *et al.*, 2013).

The aroma-active compounds detected by DF and GC-FID/O analysis are crucial characteristic aroma compounds that determine the special flavours of banana spirit (TABLE 4.**1**). Specific regions correspond to different perceptions; e.g., regions 5 and 7 were described as a mushroom odour, which contributed significant sensory perceptions even though they appeared only as low intensity peaks in the corresponding GC-FID response. Others, such as the peak at a retention time of 24 min (FIGURE 4.**5**), did not accompany a detectable sensory perception, but possess a high intensity signal response.

Some regions could be discussed using the $^{1}$D *I* on the polar phase obtained from GC-O even if the peaks could not be identified. The aroma detected in the present study, by at least six judges in region number 5 at *I*=1301 and described as mushroom, has also been found to have the same *I* and odour description (mushroom, ferment) in cider (VILLIÈRE *et al.*, 2012). This fact could be attributed to oct-1-en-3-one, compound found in the bibliography for this odourant zone (CORDERO *et al.*, 2014). This compound was not found in banana fresh fruit before the present study (SELLI *et al.*, 2012; HSIEH *et al.*, 2013).

An odour perceived as caramel was detected at *I*=1895. It could be related to the volatile fraction obtained from the thermal degradation of sugar responsible for the typical caramel odour. This degradation could be intensified by the distillation process, which is use to made spirits. In a recent study furans, lactones and acids were reported as odour-active compounds resulting from the thermal breakdown of sugars predominant in the volatile odourant fraction of burnt sugar caramel. This attribute was perceived but could not be identified (PARAVISINI *et al.*, 2015).

TABLE 4.1 - Major aroma-active regions detected in Banana *Terra* spirit using GC/FID/O

| Region nº. | Retention time (min) | $I_{polar}$ (Solgel-Wax) | Odour Descriptors | Detection frequency |
|---|---|---|---|---|
| 1 | 2.761 | 1054 | Sweet | 4 |
| 2 | 3.390 | 1100 | Plastic | 5 |
| 3 | 3.810 | 1118 | Banana | 4 |
| 4a | 6.118 | 1212 | Whisky, Burn, malt | 5 |
| 4b | 6.871 | 1238 | Whisky, Burn, malt | 7 |
| 5 | 8.752 | 1301 | Mushrooms* | 6 |
| 6 | 10.758 | 1358 | Flower, grassy | 6 |
| 7 | 12.060 | 1395 | Mushrooms* | 5 |
| 8 | 13.061 | 1432 | Spicy, green | 7 |
| 9 | 13.618 | 1454 | Potato | 6 |
| 10 | 14.896 | 1506 | Green Pepper, spicy | 7 |
| 11 | 15.125 | 1519 | Crayons | 7 |
| 12 | 16.950 | 1632 | Bug, plastic, green | 6 |
| 13 | 17.460 | 1668 | Sweat | 7 |
| 14 | 18.083 | 1715 | Flower | 5 |
| 15 | 19.154 | 1803 | Flower, rose | 5 |
| 16 | 20.188 | 1895 | Caramel | 6 |
| 17 | 20.479 | 1924 | Rose, honey | 4 |
| 18 | 22.793 | 2103 | Dental | 6 |

* Minor intensity peak
# number of panellists recording this odour, out of 7 panellists

The odour described as a potato was described at $\underline{I}$=1454. The *I* of the polar phase of this peak gave some clues as to the presence of methional, which has a *I* reference at 1458 and a characteristic potato odour consistent with the zone perceived in this study. Also, Falcão *et al.* (2008), using GC-O, perceived the odourant zone described as cooked potato in samples of wines. However, methional was detected in just one wine using a sulphur compounds detector, due to this compound having a low perception threshold. Villière *et al.* (2012) described the

same odour at *I*=1480 in ciders, which could also be due to the presence of methional.

### 4.3.2 GC×GC analysis

The volatile complexity of banana *Terra* spirit is characterised by GC×GC/FID, as shown in FIGURE 4.**6**.

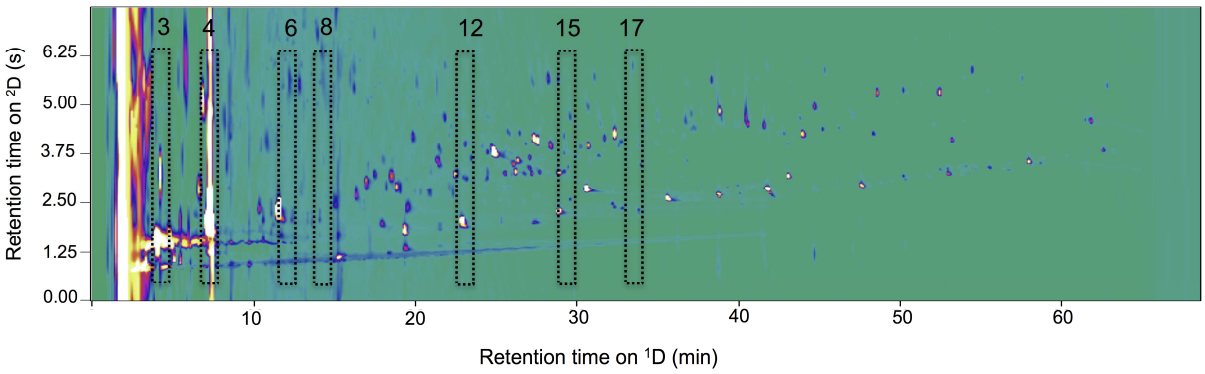


FIGURE 4.6 - GC×GC/FID contour plot of volatile compounds from banana *Terra* spirit using SPME

The $^{1}$D GC/FID/O peaks were correlated with $^{1}$D peaks in GC×GC-FID using the *I*. The corresponding two-dimensional contour plot obtained for the sample shows the superior resolution found using GC×GC, which provides enhanced peak capacity with a highly abundant number of volatile compounds. Also, this reveals the true complexity of this sample. For example, the highlighted region 15 in FIGURE 4.**6** corresponds to several overlapping compounds in $^{1}$D GC (such as the one at retention time19.15 min, shown in FIGURE 4.**5**a) but this co-elution was well resolved in GC×GC. Vertically correlated contour peaks co-elute on the $^{1}$D column and are separated on the $^{2}$D. The sensitivity enhancement due to the zone compression effect increases peak responses; therefore, smaller peaks can be better delineated compared with the single column experiment. Although GC×GC-FID is able to resolve the co-eluting peaks, it is not ideal for evaluating perceived odour due to inefficient sniffing and interpretation speed by the GC×GC-O panellists. If multiple compounds are shown to overlap in GC×GC with a number of compounds, arising

from a peak in the $^{1}$D GC analysis, then the application of H/C MDGC for the study of aroma-impact regions remains of interest for resolving and further characterising specific compounds (EYRES; MARRIOTT; DUFOUR, 2007; CHIN; EYRES; MARRIOTT, 2012; MARRIOTT *et al.*, 2012). The odour regions which will later be subjected to H/C MDGC analysis are highlighted by a dotted boxes and numbers. Also, the *I* for the regions was calculated and compared to $^{1}$D GC-O

### 4.3.3 Heart-Cut MDGC/MS/O analysis

The banana *Terra* spirit presented a relatively large number of components, so co-elution is likely to occur. This may lead to false or imprecise identification of the components responsible for the odour activity. Thus, the H/C MDGC-MS/O system was used to investigate co-elution within each odour region by taking suitable heart-cuts from $^{1}$D and transferring each to $^{2}$D according to the developed protocol. Seven regions, identified by number, were selected to perform the heart-cut analyses, leading to identifications listed in TABLE 4.**2**. These regions were chosen because of the intensity of sensory perception and higher concentration of the compounds. Other regions were also tentatively heart-cut, but were not successful, probably because most of the odourants were too diluted in the extracts to get a proper sensory perception and mass spectrometric signal (CAMPO; CACHO; FERREIRA, 2006). TABLE 4.2 also includes retention index data (*I*) for both $^{1}$D and $^{2}$D, along with *I* data for the available authentic standards. Positive identification was achieved using H/C MDGC-MS/O through comparisons of linear *I* and mass spectra with the linear *I* and mass spectra of standard reference compounds analysed under identical experimental conditions, and also by testing the characteristic odour of the compounds.

TABLE 4.2 - Identification of main odourants from the heart-cut regions with [1]D and [2]D by MDGC/MS/O in Banana *Terra* spirit

| Region n°. | Odour Description | H/C (min: sec) | $I_{polar}$ [a] Solgel-Wax | $I_{polar}$ [b] HP-FFAP | $I_{polar}$ [c] HP-FFAP | $I_{reference}$ [d] Wax or FFAP | Identified Compounds | $I_{apolar}$ [e] DB-5 | $I_{apolar}$ [f] DB-5 Standard | $I_{reference}$ [d] DB-5 |
|---|---|---|---|---|---|---|---|---|---|---|
| 3 | Banana | 8:50-9:00 | 1118 | 1114 | 1119 | 1117 | 3-methylbutan-1-ol- acetate | 861 | 876 | 876 |
| 4a | Malt, whisky | 12:40-12:80 | 1212 | 1256 | 1256 | 1109/1208 | 2-methylbutan-1-ol | 797 | * | 739 |
| 4b | Whisky, burnt | 12:80-13:10 | 1238 | 1370 | 1256 | 1205 | 3-methylbutan-1-ol | 767 | 764 | 736 |
| 6 | Flower, grassy | 16:50-16:80 | 1358 | 1370 | 1371 | 1360 | 1-hexanol | 879 | 868 | 851 |
| 8 | Spicy, green | 17:50-17:90 | 1432 | 1419 | 1417, 1425 | 1436 | ethyl octanoate | 1194 | * | 1198 |
| 12 | Bug, plastic, green | 21:30-21:50 | 1632 | 1644 | 1630 | 1636 | ethyl decanoate | 1392 | * | 1398 |
| 15 | Flower | 24:00-24:50 | 1803 | 1832 | 1832 | 1829 | 2-phenylethyl acetate | 1230 | 1265 | 1260 |
| 17 | Flower, rose | 25:40-25:70 | 1925 | 1939 | 1944 | 1925 | phenylethyl alcohol | 1124 | 1121 | 1118 |

[a] Retention index calculated for [1]D in GC-FID/O in SolGel-Wax column.
[b] Retention index calculated for [1]D in GC×GC-FID in HP-FFAP column.
[c] Retention index calculated for [1]D in MDGC-MS/O in HP-FFAP column.
[d] Retention index reference values reported online in Flavornet database (ACREE; ARN, 2013).
[e] Retention index calculated for [2]D in MDGC-MS/O in DB-5ms column, using target heart-cut analysis.
[f] Retention index calculated for [2]D in MDGC-MS/O in DB-5ms column with authentic standard, using target heart-cut analysis.

FIGURE 4.**7** Presents the chromatograms of each of the seven target regions, selected by H/C placed in the $^{2}$D column. Notably, in most cases, several compounds co-elute in the $^{1}$D GC result, and these peaks were well resolved in the $^{2}$D column for each region. Effective separation allows evaluation of the individual odour activity of each component. Moreover, note that in some regions (i.e., FIGURE 4.**7** - Regions 4b, 6, 8, 12 and 15) the better separation on $^{2}$D shows a relatively large number of peaks, with improved chromatographic response signals.

For the compounds identified using H/C MDGC the higher alcohols were the more abundant class in terms of total volatile composition in the banana *Terra* spirit, followed by ethyl esters and acetates. These alcohol compounds can originate during alcoholic fermentation by yeast and are dependent upon several factors such as raw materials, distillation process, etc. (OLIVEIRA; ROSA*; et al.*, 2005; MORAKUL *et al.*, 2012), and can originate from the fruit composition (SELLI *et al.*, 2012; HSIEH *et al.*, 2013). The higher alcohols, typically methylpropan-1-ol, (isobutyl alcohol), 3-methylbutan-1-ol (isoamyl alcohol), and 2-methylbutan-1-ol were responsible for the greater proportion of the aroma of the alcoholic spirit. This group of compounds is positively involved in the sensory quality of the distillate if they are not present in excessive concentrations (CORTÉS *et al.*, 2011; SAMPAIO *et al.*, 2013). These same higher alcohols are also reported to be the most abundant volatile compounds in many other spirits, including *cachaça*, rum and orujo (OLIVEIRA; ROSA*; et al.*, 2005; CORTÉS *et al.*, 2011; PINO *et al.*, 2012). The 3-methylbutan-1-ol was also originally found in the aroma profile of ripened fruits of Dwarf Cavendish banana cultivars (SELLI *et al.*, 2012) and in flavour characterisation of banana fruit described as fruity-wine note (HSIEH *et al.*, 2013). In this study, the 3-methylbutan-1-ol, and 2-methylbutan-1-ol, were present in regions 4a and b (TABLE 4.**2**) as co-elution regions, which present an aroma described as ‘whisky’, ‘malt’ and ‘burnt’. It could be separated and identified using H/C MDGC-MS/O as 3-methylbutan-1-ol and 2-methylbutan-1-ol, which were co-eluted using $^{1}$D analysis.

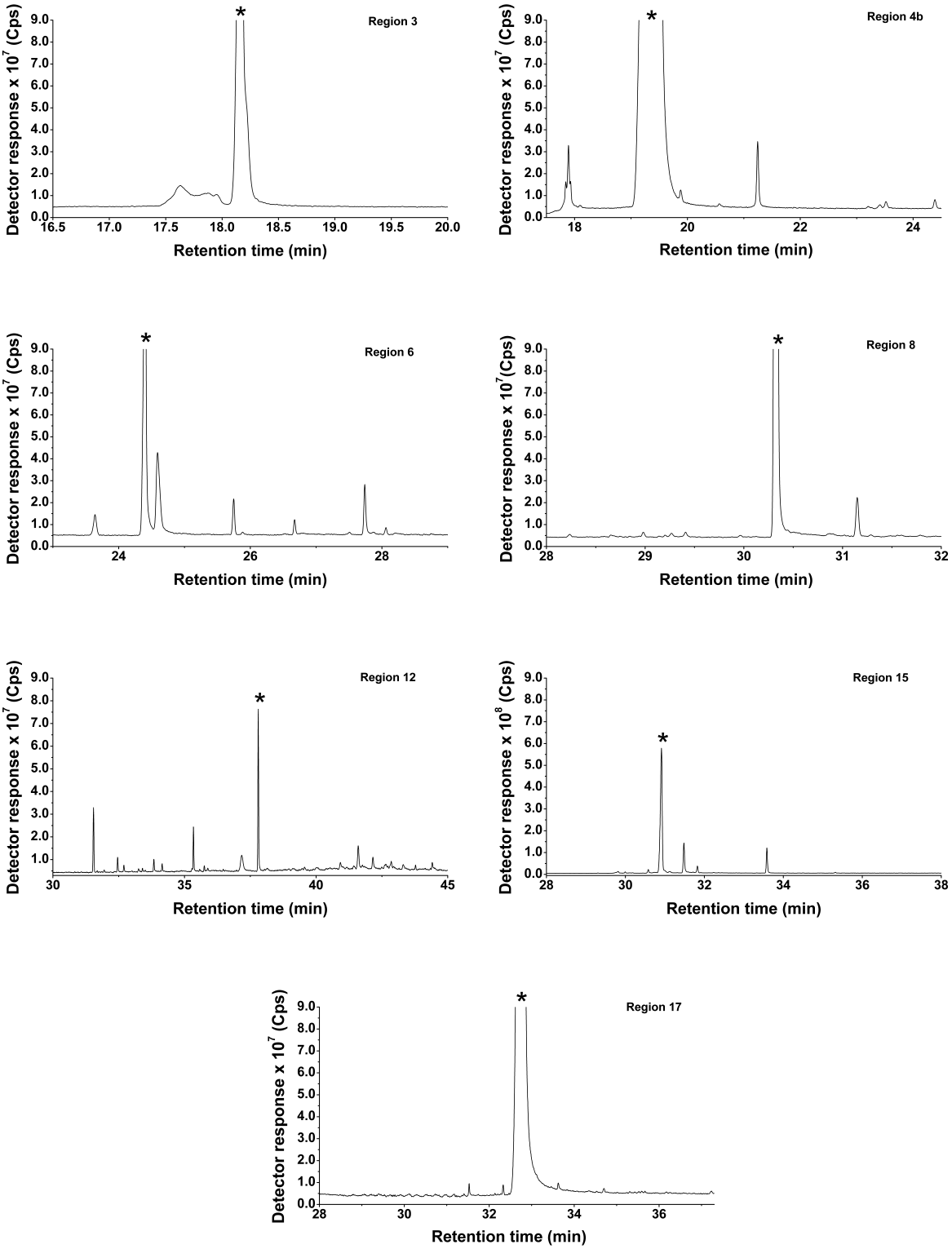


FIGURE 4.7 Chromatograms of the heart-cut MDGC/MS/O process for the selected regions in Banana *Terra* spirit

*Signifies the peaks of the component responsible for odour activity. The selected regions are indicated on FIGURE 4.**5**, and tabulated in TABLE 4.**2**.

1-Hexanol is one of the C6 alcohols, which originates in the raw material (banana fresh fruit) and has a positive influence on aroma (HSIEH *et al.*, 2013). At concentrations up to 12 mg $L^{-1}$, it contributes herbaceous notes to the spirit aroma (CORTÉS *et al.*, 2011), but at increased concentrations may contribute negatively to the product aroma with characteristics of "coconut-like", "harsh" and "pungent" (SAMPAIO *et al.*, 2013). In this sample, 1-hexanol was related to flower and grassy aroma perceptions.

Another relevant finding was that 2-phenylethanol, which plays an important role in the fruit and spirit's bouquet, which contributes floral, rosy nuances to the aroma (CORTÉS *et al.*, 2011). This alcohol is frequently found in spirit beverages, it derives from amino acid (L-phenylalanine) catabolism via a metabolic pathway reaction of *S. cerevisiae* during anaerobic carbonic fermentation (DUARTE; DIAS; OLIVEIRA; VILANOVA*; et al.*, 2010; CACHO *et al.*, 2012; PINO *et al.*, 2012). For this sample, 2-phenylethanol was clearly identified and related to a rosy aroma.

After the alcohols, the esters were clearly the dominant constituent in the banana spirit. The most significant acetate esters present in distillates are 3-methylbutan-1-ol-acetate and 2-phenylethylacetate (NIKIĆEVIĆ *et al.*, 2011; CACHO *et al.*, 2012). The 3-methylbutan-1-ol-acetate is responsible for fruity notes such as "banana" and "fruity" aroma and the 2-phenylethylacetate gives a flowery and fruity aroma with honey notes to spirits. Also, 3-methylbutan-1-ol-acetate was found in the aroma of the banana profile (DUARTE; DIAS; OLIVEIRA; VILANOVA*; et al.*, 2010; CORTÉS *et al.*, 2011; CACHO *et al.*, 2012; SELLI *et al.*, 2012; HSIEH *et al.*, 2013). They were positively identified in banana *Terra* spirit and contribute a pleasant aroma. The synthesis of acetate esters has been related to the reaction of acetyl-CoA with higher alcohols and also to the different enzymatic reactions by *S. cerevisiae* during fermentation as well as to reaction with acetic acid during distillation process (PERESTRELO *et al.*, 2006; CACHO *et al.*, 2012; SELLI *et al.*, 2012).

The presences of ethyl esters of fatty acids derived from the yeast are released by distillation and they also could be formed by the reaction of ethanol with fatty acids. During the aging of distillates, these ethyl esters increase their concentration adding pleasant fruity odours (CORDERO *et al.*, 2008). Ethyl octanoate and ethyl decanoate are considered important contributors of fruity and green notes to the aroma of alcoholic distillates (WANIKAWA *et al.*, 2002; NIKIĆEVIĆ *et al.*, 2011). They

were also reported as important aroma constituents in several fruit species. Both compounds were identified by comparison with MS library and odour descriptors. Ethyl octanoate was associated with a green note flavour and is generally associated with fruity or fat descriptors. Ethyl decanoate has been described as bug, plastic and green (TABLE 4.2), while the literature identifies this with grape, oily or brandy perceptions (NIKIĆEVIĆ *et al.*, 2011; VILLIÈRE *et al.*, 2012).

The correlation of coincident sensory and instrumental data significantly improved the understanding of aroma-active volatile attributes in the banana *Terra* spirit. Amongst the identified aroma volatiles, 3-methylbutan-1-ol-acetate, 3-methylbutan-1-ol, 1-hexanol and phenylethyl alcohol were reported previously as the aroma-impact compounds in the banana fruit analysed using GC-O and GC-MS (KOEK *et al.*, 2008; ZHU, 2009; HSIEH *et al.*, 2013). They are also components well known as by-products of yeast fermentation (OLIVEIRA; ROSA*; et al.*, 2005; MORAKUL *et al.*, 2012). Transformation of the aroma attribute within the banana product has been generated, particularly the occurrence of more ethyl esters as major aroma-contributing volatiles, which is probably caused by the spirit manufacturing process (i.e., fermentation and distillation).

However, all these experiments clearly show the complexity of the sample. Further investigations are recommended, mainly to study other aroma regions that still remain unidentified and that additional fractionation steps should be included. Also, it is important to evaluate the impact of different varieties of banana on the aroma perception of the alcoholic product.

## 4.4 Conclusion

This work reports for the first time the aroma profile of banana *Terra* spirit and also describes the most characteristic flavour in the beverage. Analysis by GC/FID/O highlighted 18 aroma impact regions; the present work addressed a number of these regions for further analysis using MDGC separation with parallel O and MS analysis. The identification of some aroma regions revealed the chemical compounds that were responsible for the sensory markers of the banana spirit, and improved the odour description of the product; it can potentially be used to guarantee beverage quality. The principal volatile compounds identified in this work and that were

responsible for the characteristic aroma of banana spirit are 3-methylbutan-1-ol, 3-methylbutan-1-ol- acetate, 2-phenylethyl acetate and phenylethyl alcohol.

It is considered useful for further works to determine the identified compound content in different types of produced fruit spirits. Further investigations are required to reveal the other volatile regions that contribute to the aroma in banana spirit; these regions either gave poor correlation with prior odour profiles, resulting in reduced quality MS library matches, or standards were not available to confirm component identities. The MDGC/MS/O system generally proved to be a strategic method to ensure a correct isolation and identification of some odorant compounds. In addition, associating separation systems as GC×GC and H/C MDGC/MS/O could be an interesting way to identify other unknown odorants in banana spirits.

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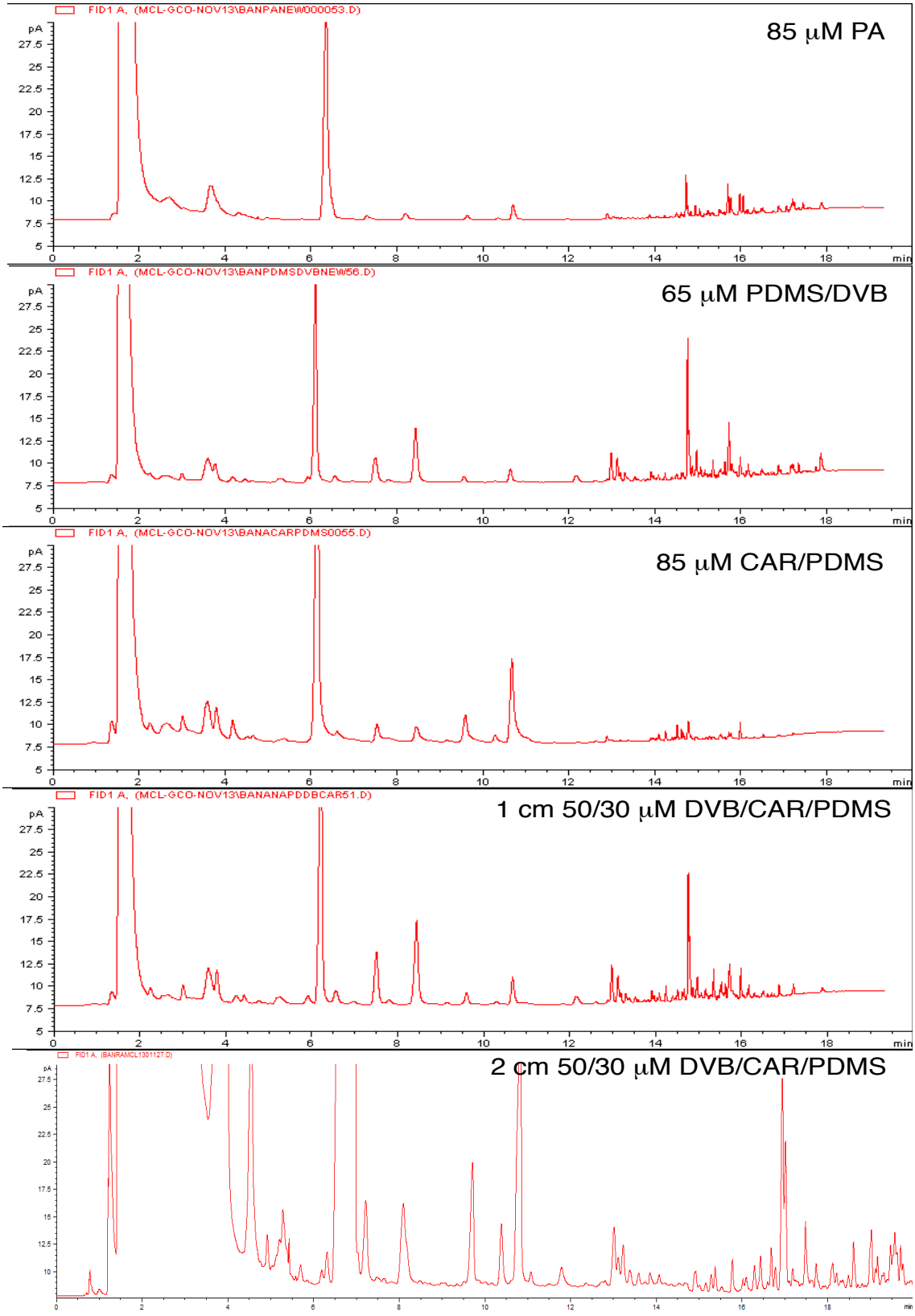
# APPENDIX




Complementary Figure, Chapter 4 – Chromatograms from banana *Terra* spirit using different fibres

## CONSIDERATIONS FOR FUTURE WORKS

Recombination of odour active compounds in the banana spirit to match the original aroma of the beverage and subsequent sensory evaluation can be used to validate the selection of odour active compounds as a final step in aroma analysis.

Quantification of VOCs in banana *Terra* spirit.

Further studies are needed to improve the identification of some powerful non-identified aroma compounds in banana and other fruit spirits. However, the complexity of the volatile fraction of such spirits, and the likely low concentration level at which these odourants are present has made the identification of such compounds difficult.

More extensive investigation is required to reveal the mechanisms of the development of the VOCs that contribute to the notes in banana spirits.

Cumulative SPME sampling can be applied to improve method detectability in a single GC/O or to isolate some other target compounds. It can produce a more representative global aroma profile and ensure correct isolation and identification of trace and ul-trace odourants which could not be identified in the present study.