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HÉLIO OLIVEIRA DO NASCIMENTO

DEVELOPMENT OF CHROMATOGRAPHIC METHOD FOR APPLICATIONS IN QUALITY CONTROL AND AUTHENTICITY OF DISTILLED BEVERAGES

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HÉLIO OLIVEIRA DO NASCIMENTO

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Thesis submitted to the Coordination of the Postgraduate Program in Chemistry, at the Federal University of Ceará, as a partial requirement for obtaining the title of Doctor in Chemistry. Concentration area: Analytical Chemistry.

Supervisor: Dr. Ronaldo Ferreira do Nascimento.

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Approved in 01/27/2023.

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ABSTRACT

Cachaça and whiskey distilled beverages are widely consumed in Brazil and their acceptance is closely related to quality control in their production. However, these beverages are frequent targets for adulteration, which alters the quality of these products and can lead to health problems for consumers. This work comprises three chapters where the first deals with a literature review on applications of the Solid-phase microextraction (SPME) technique in cachaça and whisky, the second was the development of a new methodology for the analysis of higher alcohols, n-butanol and total esters using gas chromatography coupled to barrier discharge ionization detector (GC-BID) and SPME via headspace (HS) as a sample preparation method for the analysis of 25 samples of cachaça and 26 of whiskeys, in the third chapter 16 samples of original and 14 counterfeit whiskeys were differentiated through principal component analysis (PCA) applied to data obtained by the HS-SPME-GC-BID method and aldehydes derivatized by 2,4-dinitrophenylhydrazine (2,4-DNPH) in high performance liquid chromatography coupled to diode array detector (HPLC-DAD). The results showed that the use of SPME in chromatographic analyzes of distilled beverages is a powerful tool that allows both the development of methods for quality control applications and the differentiation between original and counterfeit beverages.

Keywords: solid-phase microextraction (SPME); gas chromatography coupled to barrier discharge ionization detector (GC-BID); high performance liquid chromatography coupled to diode array detector (HPLC-DAD); cachaça; whisky; principal component analysis (PCA).

RESUMO

A cachaça e o uísque são bebidas destiladas amplamente consumidas no Brasil e sua aceitação está intimamente relacionada ao controle de qualidade em sua produção. No entanto, essas bebidas são alvos frequentes de adulteração, o que altera a qualidade desses produtos e pode levar a problemas de saúde para os consumidores. Este trabalho é composto por três capítulos onde o primeiro trata de uma revisão de literatura sobre aplicações da técnica de microextração em fase sólida (SPME) em cachaça e uísque, o segundo foi o desenvolvimento de uma nova metodologia para a análise de álcoois superiores, n-butanol e total ésteres utilizando cromatografia gasosa acoplada a detector de ionização por descarga de barreira (GC-BID) e SPME via *headspace* (HS) como método de preparo de amostras para análise de 25 amostras de cachaça e 26 de uísques, no terceiro capítulo 16 amostras originais e 14 uísques falsificados foram diferenciados por meio de análise de componentes principais (PCA) aplicada aos dados obtidos pelo método HS-SPME-GC-BID e aldeídos derivados por 2,4-dinitrofenilhidrazina (2,4-DNPH) em cromatografia líquida de alta eficiência acoplada a arranjo de diodos detector (HPLC-DAD). Os resultados mostraram que o uso de SPME em análises cromatográficas de bebidas destiladas é uma ferramenta poderosa que permite tanto o desenvolvimento de métodos para aplicações no controle de qualidade quanto para a diferenciação entre bebidas originais e falsificadas.

Palavras-chave: microextração em fase sólida (SPME); cromatografia gasosa acoplada a detector de ionização por descarga de barreira (GC-BID); cromatografia líquida de alta eficiência acoplada a detector de arranjo de diodos (HPLC-DAD); cachaça; uísque; análise de componentes principais (PCA).

FIGURE LIST

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1 USE OF SPME IN THE ANALYSIS OF CHEMICAL COMPOUNDS IN CACHAÇA AND WHISKY

ABSTRACT

Solid-phase microextraction is a sample preparation technique that has been widely used in various types of matrices, among which we can mention food matrices, such as distilled beverages, like cachaça and whisky. When applied to the analysis of volatiles in these matrices, SPME expands the potential for investigation of volatile organic composition. In this sense, this review studied the works of application of SPMEin cachaça and whiskey in the last 23 years. For the search in the literature, the google scholar, science direct and web of science database was used. 29 articles on the subject were found, in which most used the SPME via headspace (HS), with polyacrylate fiber or CAR/DVB/PDMS. Furthermore most of the compounds were separated and identified by one-dimensional or two-dimensional gas chromatography coupled to a mass spectrometry detector. In thisway, there is a great potential for the application of this technique for the analysis of distilled beverages, especially for cachaça and whiskey still little explored.

Keywords: cachaça, whisky, solid-phase microextraction, gas chromatography, mass spectrometry, volatile organic compounds.

1.1 Introduction

Cachaça is a typically brazilian drink with alcoholic graduation ranging from38 to 48% v/v at 20ºC, produced from the distillation of the must prepared from the fermentation of sugarcane *(Saccharum officinarum L.*)(Brazil, 2005). Brazil is the world's largest producer of cachaça in the world with 1131 registered establishments in 2020 and 7.22 million liters exported in 2021(MAPA, 2022). Whisky is a beverage with alcoholic graduation ranging from 38 to 54% v/v at 20ºC (Brazil, 2011). The main whisky-producing countries are Scotland, Ireland, Canada, the United States of America (USA), and Japan. The most consumed whiskeys in the world are European. They present a growing market, with theUSA and China as the leading consumer markets, while in the Mercosur countries, Brazilis the largest consumer (Europe, 2021).

The production of these spirits involves many steps, including fermentation where alcohol production takes place and distillation, where the alcohol content is raisedto a level close to 40% (Bortoletto, Silvello, Alcarde, 2018; Ratkovich et al., 2023). During these stages, several chemical compounds are produced in smaller quantities that can contribute to increasing the quality of the drink orbe a contaminant, so many of them are monitored and regulated by regulatory agencies (Brazil, 2005; Brazil, 2011). The study of chemical composition of distilled beverages, in addition to ensuring the quality of the beverage produced, can also provide subsidies to improve the production process (Serafim and Lanças, 2019).

Distilled beverages present a rich volatile organic composition like terpenes, alcohols, esters, carboxylic acids, ketones (Ferracane et al., 2022; Lima et al., 2022) and many inorganic elements (Pawlaczyk et al., 2019) traditionally analyzed by gas chromatography coupled to different detectors like Flame ionization detector (GC-FID) (Charapitsa et al., 2021), Mass spectrometry (GC-MS)(Ferracane et al., 2022) and recentlyby the Dielectric barrier discharge ionization detector (BID) (Nascimento et al., 2022).

When working with the analysis of distilled beverages, it is necessary to bearin mind that most works inject the sample without pre-treatment in the chromatograph, only a dilution is made in the equipment itself (using split ratio) or outside it through sample dilution (Barbosa et al., 2022). However, as there are different sample preparation methods for organic compounds, these methods appear in works with distilled beverages, such as the

traditional liquid-liquid extraction (LLE), solid-phase extraction (SPE), use of the Quick-Easy-Cheap-Effective-Rugged-Safe (QuEChERS) method for the determination of 9 pesticides and 16 HPAS in cachaça (Da Silva et al., 2019) and headspace sampling for analysis of volatiles in spirits (Nóbrega, 2003). More recently, miniaturized techniques of dispersive liquid-liquid microextraction (DLLME) have been successfully used in the analysis of whiskeys (Perestrelo et al., 2022) and solid-phase microextraction (SPME) in the analysis of whiskey and cachaça (Nascimento et al., 2022; Nascimento et al., 2023; Bigão et al., 2024; Ashmore et al., 2023).

When talking about miniaturized sample preparation techniques for the analysis of organic compounds in alcoholic beverages, solid-phase microextraction and its different configurations is the most used (Piergiovanni et al., 2022; Nolvachai et al., 2023). The different modalities of SPME are encompassed in the following techniques: SPME devices, needle-based devices, and coated stir bars (Paiva et al., 2021). SPME has the advantage of being environmentally friendly, simple, inexpensive, requires small amounts of sample, presents the possibility of automation, can be used in both gas chromatography and liquid chromatography, high sensitivity, and pre-concentration capability (Płotka-Wasylka, et al., 2015; Nolvachai et al 2023).

Although there are review articles on SPME applied to food matrices, environmental samples and bioanalyses (Godage, Gionfriddo, 2019) and another addressing the issue of green techniques for the analysis of wine, beer and spirits in MS detector (Piergiovanni et al., 2022), no exist focusingon the use of SPME in the analysis of chemical constituents in cachaça and whisky covering different detectors and analytes. Therefore, this article is a review of articles that applied SPME technique in the analysis of chemical compounds in cachaça and whisky beverages in the last 24 years.

1.2 Materials and Methods

The google scholar, science direct and web of science database were used to find the papers with the terms SPME and Spirits, SPME and whisky, SPME and cachaça, SPME and alcoholic beverages, SPME and distilled beverages, SPME and sugarcane spirits. In the analysis of alcoholic beverages in the period from 2000 to 2024. Several articles were found, but when was read the abstract and full text, it was selected 29 articles to continuethe articles studies. They are listed in Table 1.

1.3 Results and Discussion

According to data presents in Table 1, there is a great number of articles published in quality control and authenticity of distilled alcoholic beverages, but only 28 articles about cachaça and/or whiskey were published using SPME as sample preparation. Most works involved direct injection. Next, there will be a discussion of the articles found and relevantaspects in these works related to SPME and chromatography.

1.3.1 SPME extraction conditions

Considering the commercially available fibers, there are fibers with a single polymer that can be solid (polyacrylate-PA) or liquid (polydimethylsiloxane-PDMS and polyethylene glycol-PEG) and those in which there is solid polymer dispersed in PDMS (resulting in in CAR/PDMS and CAR/DVB/PDMS) or even CAR/DVB. The mechanismby which analytes are trapped can be absorption (PA and PDMS) or adsorption (mixed fibers and PEG). There are also some fibers with more than one thickness, such as PDMS,which can have a thickness of 7, 30 and 100 µm. The greater the thickness of the fiber, the lower the maximum temperature it supports, the greater the volatility and lightness ofthe extracted compounds must be. The order of polarity of commercially available SPME fibers increases towards polydimethylsiloxane, divinylbenzene, polyacrylate andcarbowax (Pawliszyn, 2012).

According to the Table 1, the works involving SPME for the matrices studied, most were carried out in the headspace mode, using commercial fibers, using mainly the fiber coated with polyacrylate (10) and CAR/PDMS/DVB (10), followed by DVB/PDMS (1), PDMS (1) and CAR/PDMS (1). A fiber with more than one type of coating ends up covering a greater polarity range and consequently extracting a greater number ofcompounds and the suitability of the extraction conditions must consider the physicochemical properties of the analytes such as boiling point, vapor pressure, polarity,and others (Hwang et al., 2020; Boyaci et al., 2015).

Table 1 - Articles about applying SPME in cachaça and whisky

Reference	Year	SPME type	SPME conditions	Chromatography	Sample	objective
Nascimento et al.,	2022	HS-SPME PA	20 min, 50ºC, without NaCl	GC-BID	whisky and cachaça	Validation
Silveira et al.,	2021	HS-SPME PA	10 min, 60ºC, 0,5% NaCl	GC/MS	cachaça and must	volatile profile differentiation
Oliveira et al.,	2020	HS-SPME DVB/CAR/PDMS	3 min,45ºC, without NaCl	CG/MS	cachaça	Validation
Santo et al.,	2020	SPME -HPLC	0,1% ACN, 0.5 min, 10 uL. min^{-1}	LC/MS	cachaça	Validation
Karp et al.,	2019	HS-SPME-CAR/PDMS	30 min, 30 ºC, wihout NaCL	GC/MS	cachaça	innovation in production
Amorim, Schwan, Duarte	2016	HS-SPME DVB/CAR /PDMS	25 min, 60 ºC, without NaCl	GC/MS	cachaça	innovation in production
Zacaroni et al.,	2017	HS-SPME DVB/CAR/PDMS	50 min, 45 ºC,without NaCl	GC/MS	cachaça	extraction optimization
Santiago et al	2016	HS-SPME DVB/CAR/PDMS	50 min, 45 ºC,without NaCl	GC/MS	cachaça	innovation in production
Menezes et al.,	2015	DI-CF-SPME-PA	60 min, 70 ºC, without NaCl	GC/MS	cachaça	Validation
Machado et al.,	2012	DI-SPME-PA	20 min, 50ºC, without NaCl	GC/MS	cachaça	Validation
De Souza et al.,	2009	HS-SPME PA	25 min, 60 ºC, without NaCl	GCxGCMS	cachaça	volatile profile differentiation
Cardeal, Marriott	2009	HS-SPME PA	25min, 60 ºC, 5% NaCl	GCxGCMS	cachaça	innovation in production

Cardeal et al.,	2008	HS-SPME PA	25min, 60 ºC, 5% NaCl	GCxGC/MS	cachaça	volatile profile differentiation
Nonato et al.,	2001	HS-SPME PA	25min, 60 ºC, 5% NaCl	GC-FID	cachaça	Validation
Bigão et al.,	2024	HS-SPME CAR/PDMS	60 min, 50ºC, without NaCl	GC/MS	whisky	volatile profile differentiation
Ashmore et al.,	2023	HS-SPME DVB/CAR/PDMS	40 min, 30ºC, without NaCl	GC/MS	whisky	volatile profile differentiation
Cody,Fukudome, Ubukata	2022	HS -SPME PDMS/DVB	60 min, 25ºC,30 % NaCl,	GC/HRTOFMS	whisky	volatile profile differentiation
Ferracane et al.,	2022	PDMS-SPMEarrow	5min, 30ºC, without NaCl	GCxGC/MS	whisky	Validation
Zhang et al.,	2022	HS-SPME DVB/CAR/PDMS	5min, 70ºC, without NaCl	GCxGC/MS	whisky	volatile profile differentiation
Waymark, Hill	2022	HS-SPME PDMS/DVB	10min, 35ºC, without NaCl	GC/MS	raw material to whisky	volatile profile differentiation
Daute et al.,	2021	HS-SPME DVB/CAR/PDMS	10 min, 60ºC, Saturated with NaCl	GC/MS	whisky	volatile profile differentiation
Stupak et al.,	2018	HS-SPME DVB/CAR/PDMS	20 min, 40ºC, without NaCl	GC/MS	whisky	volatile profile differentiation
Wiśniewska et al.,	2017a	HS-SPME DVB/CAR/PDMS	45min, 40ºC, without NaCl, 250 rpm	GCxGC/MS	whisky	comparison between techniques
Zacaroni et al	2017	HS-SPME DVB/CAR/PDMS	50min, 45ºC, without NaCl	GC/MS	whisky and bitters	volatile profile differentiation

Slabizki, Potouridis, Schmarr	2014	HS-SPME	No information	GC/MS	whisky	solve an analytical problem
Nie, Kleine-Benne	2012	Stir Bar Sorptive Extraction (SBSE)	25ºC, 800 rpm	GC/MS	whisky	validation
Nie, Kleine-Benne	2011	Stir Bar Sorptive Extraction (SBSE)	60 min, 25ºC, 1000 rpm	GC/MS	whisky, wine and fruit juice	volatile profile differentiation
Monica et al.,	2001	HS-SPME-PA	15 min, 37ºC,without NaCl	GC-FID	whisky	volatile profile differentiation

Both direct and headspace SPME mode have advantages and limitations in applications in different types of matrices. Headspace extraction mode has become one of the most used methods for the extraction of Volatile organic compounds (VOCs) and Semivolatile organic compounds (SVOCs). In this type of extraction, the fiber does not come into direct contact with the sample, which increases the useful life and reduce the number of interferents resulting from nonspecific adsorption. In addition, there is no needto use organic solvents, since adsorption and subsequent desorption occur exclusively by temperature small amounts of sample can be used to perform the extraction as the fiber does not need to be completely covered (Lancioni, et al., 2022).

Gionfriddo, Souza-Silva and Pawliszyn (2015), when investigating the behaviorof direct and headspace injection modes in complex matrices, found that direct immersion minimizes the occurrence of artifacts related to coating saturation and provides enhanced extraction of polar compounds. The authors suggest that direct immersion is better for *invivo* SPME strategies in quantitative metabolomics studies of complex plant-basedsystems. The works that used direct immersion of SPME in alcoholic beverages present in Table 1 were Menezes et al., (2015) that used a cooling system to improve extraction in a new method (DI-CF-SPME) for the determination of 16 HPAS in artisanalcachaças and the work by Machado and collaborators that analyzed ethyl carbamate in cachaça (Machado et al., 2012).

The most recent types of innovation in SPME coatings are metal organic frameworks (MOF), carbon-based nanostructured materials, and ionic liquids (IL), Molecularly imprinted Polymers (MIPs) and Biologically based sorbents (Paiva et al., 2021; Lancioni etal., 2022). Considering these types of coatings in SPME and the works present in Table 1, most of them were addressed traditional SPME, this scenario is very different when SPE/ SPMEis applied in the analysis of pesticides in surface and groundwater where there is an increasing use of fibers manufactured with new coatings than commercial ones (Sousa et al., 2021). Considering the work of table 1, only the work of Ferracane and collaborators applied a new device (SPME arrrow) to analyze the volatile profile of three types of whiskeys using GCxGC and MS detector. When comparing SPME arrow with conventional SPME, the authors found greater amounts of compounds extracted by SPME arrow, greater sensitivity by up to 6 times and greater precision (Ferracane et al., 2022).

The most parameters that were optimized in HS-SPME extraction, Table 1, were temperature (ranging from 25 to 70 ºC), extraction time (3 to 60 min) and addition of salt (NaCl)(ranged from 0% to 5%). In addition to these parameters, rotation and sample volume

also influence the extraction of volatile compounds from distilled beverages (Pati et al., 2021).

The increase in temperature favors the passage of molecules to the vapor state through thermal agitation, the salt through the salting out effect. The increase in time favors greater interaction between the analytes and the fiber, which may increase the amount extracted. However, this increase in the amount extracted is related to the physicochemical properties of the analytes of interest. An excess of temperature, salt or time can favor the desorption process instead of favoring the adsorption of the analyteson the fiber. In this way, it is necessary that the extraction conditions are optimized (Lord, Pawliszyn, 2000).

When talking about optimization of experiments, it can occur in a univariateway, when one parameter is modified and the others are kept constant, or multivariate way, when all parameters are varied at the same time. When multivariate optimization isused, the optimal condition is found faster and more precise (Pati et al 2021). In view of this, severalmethods of design of experiments (DoE) including full factorial, fractional factorial, Plackett–Burman, orthogonal array, central composite, Box–Behnken, Doehlert, and D- optimal designs are commonly used for sample preparation optimization (Mousavi, Tamiji, Khoshayand, 2018). Considering the works in Table 1, only 3 works carried out multivariate planning of the SPME extraction conditions (Nascimento et al., 2022; Zacaroni et al., 2017; Machado et al., 2012).

1.3.2 Type of chromatography

Regarding the gas chromatographs used, most studies used coupling to a mass detector (23), followed by a flame ionization detector (2). Some works used both detectorsand classified the compounds into minor compounds and major compounds respectively. In addition, there was a work with a dielectric discharge barrier detector was recently published (1).

MS detector stands out from the others due to its ability to detect and identifythe compounds eluted from GC. In addition, the mass detector features different ionization modes, mass analyzers and ion detectors. It is very used in works that evaluatethe volatile profile of samples and validation studies (Zoccali, Tranchida, Mondello, 2019). The other types of detectors are applied more to method validation, since in these cases there is an analytical standard to carry out the identification (Table 1).

A more in-depth profile of the volatile components of spirits can be obtainedby the combined use of SPME and two-dimensional comprehensive chromatography (GCxGC).

In this type of chromatography there are two capillary columns with differentstationary phases and the presence of a modulator that works as an interface between thetwo columns, in addition to having a sophisticated detection system. This results in a wide peak resolution capacity in complex mixtures in less time or equivalent to one- dimensional gas chromatography (Mostafa, Edwards, Górecki, 2012).

Considering the articles in Table 1 that used GCxGC, a range of 70- 200 compounds were identified in 3 works that analyzed cachaça (De Souza et al., 2009; Cardeal, Marriott, 2009; Cardeal et al., 2008) and more recentlythere was the publication of the work in whisky samples (Ferracane et al., 2022; Zhang et al., 2022; Wiśniewska et al., 2017a).

In addition to gas chromatography, SPME can also be coupled with Liquid chromatography and its different detectors. In this case, SPME in tube is used, a capillary column works as the SPME device between the loop and the injection needle. This technique brings numerous advantages such as reduced sample handling, low operating cost and short analysis time (Jalili, Barkhordari, Ghiasvand, 2020). Considering the works in Table 1, we can mention theanalysis of 10 pesticides in cachaça by SMPE in which liquid chromatography was coupled to a tandem mass detector (Santos et al., 2020).

A relevant fact in the articles shown in Table 1 was the growing combined use of chromatography data with the electronic nose device (e-nose) in whisky analysis like references [Ferracane et al., 2022; Stupak et al., 2018; Wiśniewska et al., 2017a). E-nose have the ability to identify substances with an active odor and when the sample is well prepared, they show good correlations with the results presented by the chromatography, moreover, it can be produced with relatively low-costmaterials (Zhang et al., 2022; Silvello, Alcarde, 2020).

1.4 Conclusion

There is a small amount of work that used the SPME technique for the analysis of distilled alcoholic beverages, given the existence of numerous sample preparation techniques for organic compounds and the wide use of SPME in different devices and applications in different matrices.

Among the articles that use SPME in samples of cachaça and whiskey, mostused the SPME via headspace (HS), with polyacrylate fiber or CAR/DVB/PDMS.Furthermore, most of the compounds were separated and identified by one-dimensional or two-dimensional gas chromatography coupled to a mass spectrometry detector.

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2 CHAPTER 2- NEW HS-SPME-GC-BID METHOD FOR THE DETERMINATION OF VOLATILE CONSTITUENTS IN DISTILLED BEVERAGES

ABSTRACT

To maintain quality control in the production of distilled beverages, it is necessary to perform several types of chromatographic analysis, where gas chromatography (GC) coupled with mass spectrometry (GC/MS) and flame ionization detector (GC-FID) have been used as traditional analysis techniques for volatile organic compounds. Morerecently the dielectric barrier discharge ionization detector (BID) has been coupled to GC(GC-BID) which is more sensitive than the FID and the thermal conductivity detector (TCD) for analyzing various volatile compounds. This study aims to develop a new method for determining higher alcohols, n-butanol, and ethyl acetate in cachaça and whisky samples using headspace solid-phase microextraction (HS-SPME) followed by GC-BID. The HS-SPME method was optimization with multivariate analysis. The analytical results were provided by the weighted linear Square (WLS) method for linearity correction, internal standard addition (ISA) as a calibration method for matrix effect correction, reproducibility, recovery and accuracy. The best conditions for extraction of the compounds by HS-SPME were obtained for 50ºC, 20 min and without addition of NaCl. The WLS regression allowed to obtaining satisfactory correlation coefficients (0.9632 to 0.9992). The matrix effect (ME) was verified for n-propanol, isobutanol and isoamyl in both matrices. The recovery values of the cachaça matrix ranged from 81.24 to 106.94 %, except for isoamyl alcohol, and ranged from 82.06 to 106.25 % for the whisky matrix, except for n-butane. The precision ranged from 0.66 to 7.38 % and 0.62 to 6.77 % for the cachaça and whisky samples, respectively. Limit of detection (LOD) values ranged from 0.26 to 0.51 mg L^{-1}, while limit of quantification (LOQ) values ranged from 0.78 to 1.55 mg L^{-1}. The validation process and statistical methods were successfully applied to quality assurance of the analysis of cachaça and whiskey samples, where n-propanol, ethyl acetate, isobutanol and isoamyl were detectedfor all samples within the maximum content for higher alcohols and ethyl acetate, except n-butanol. The method can be satisfactorily applied to quantify higher alcohols and estersin samples of alcoholic beverages.

Keywords: solid-phase microextraction; gas chromatography coupled to barrier discharge ionization detector; volatile organic compounds; cachaça; whisky; multivariate optimization.

2.1 Introduction

The most consumed alcoholic beverages in the world in 2018 were beer (42%), followed by wine (29%), spirits (23%), and other beverages (6%)(WHO, 2022). Cachaça is a typically Brazilian drink with alcoholic graduation ranging from 38 to 48% v/v at 20ºC, produced from the distillation of the must prepared from the fermentation of sugarcane *(Saccharum officinarum L.*)(BRAZIL, 2005). Brazil is the world's largest producer of cachaça in the world with 1131 registered establishments in 2020 and 7.22 million liters exported in 2021 (, 2021; IBRAC, 2022).

Whisky is a beverage with alcoholic graduation ranging from 38 to 54% v/v at 20ºC (BRAZIL, 2009; BRAZIL, 2011). The main whisky-producing countries are Scotland, Ireland, Canada, the United States of America (USA), and Japan. The most consumed whiskeys in the world are European. They present a growing market, with the USA and China as the leading consumer markets, while in the Mercosur countries, Brazil is the largest consumer (Europe, 2021).

Distilled alcoholic beverages are produced by the fermentation of the raw material, followed by distillation at high temperature; these products are prepared mainlyby fermentation in the liquid or solid state, which results in high alcohol content and othervolatile compounds after the distillation process (Melo et al., 2021; Lima et al 2022).

Cachaça and whisky samples contain a wide variety of constituents of different chemical classes, such as higher alcohols (n-propanol, isobutyl alcohol, and isoamyl alcohol), ethyl esters (ethyl acetate being the majority), acetates, fatty acids, ketones, monoterpenes, and phenols (He and Bayen, 2020; Silva, Silvello, Bortoletto, 2020). However, the relative proportions may vary markedlyaccording to the peculiarities of the raw material and the production process (Maia, Marinho and Nelson, 2020). These compounds can also be present under different conditions, such as varying concentrations, volatility and polarity, which affect the extraction process and the chromatographic profile (Câmara et al., 2007).

In this context, numerous studies have been carried out to improve the qualityof raw materials, the production processes, and the control of contaminant compounds. The quality of the chemical composition of alcoholic beverages is essential to maintain aproduct quality standard and ensure the population's food security. In addition, this analytical control provides competitiveness in the face of products of consecrated quality(Serafim and Lanças, 2019).

Regarding the analysis methods for the volatile organic constituents (VOCs) of distilled beverages, GC-FID (Lima et al., 2022) and GC/MS (He and Bayen, 2020) have been the most used techniques for quantitative analysis of the higher alcohols, n-butanol, total aldehydes (as acetaldehyde), total acids (as acetic acid), and total esters (as ethyl acetate) using the most common injection techniques (direct, split and splitless) (Maia, Marinho and Nelson, 2020, Pereira et al 2012; Etievant, Maarse, Berg, 1986).

Direct injection is the simplest and easiest technique for introducing samples into the GC-FID, in particular the analysis of VOCs in distilled beverages. However, the presence of large amounts of water reduces the life of the chromatographic column and results in signal disturbances in the FID (Etievant, Maarse, Berg, 1986). In addition, the presence of sugar compounds in the sample causes dirt in the liner due to the heating of organic matter. On the other hand, Headspace Solid-phase Microextraction (HS-SPME) is a solvent-free technique, which circumvents these problems and provides a better precision, accuracy and more information of the volatile composition in beverage samples (Jalili, Barkhordari, Ghiasvand, 2020).

Recently, the Barrier discharge ionization detector (BID) was developed for applications in GC-BID. It is a detector that provides higher sensitivity (100 times) than the TCD for the analysis of inorganic gases and two times more sensitivity than FID for the analysis of aliphatic compounds (Shinada et al., 2012). In addition, unlike FID, the BID can be used for water analysis in food and pharmaceutical matrices (Frink, Weatherly, Armstrong, 2014; Frink, Armstrong, 2016), and can be applied to a wide variety of analytes (Antoniadou, Zachariadis, Rosenberg, 2019). It finds application in the analysis of VOCs present in alcoholic beverages (Lopes, Fernandes, Nascimento, 2021), but there are no reports of analytical methods involving the application of GC-BID for the analysis of these compounds for our types of samples. In addition, the BID (Nondestructive detector) can be coupled to existing chromatographic and spectroscopic techniques, which have been used for analyses of authenticity and certification of whisky origin, through different statistical and chemometric analyses (Wiśniewska et al., 2015a).

Many analytical methods do not have constant precision over a wide range of concentration, when the homoscedasticity condition is not met and larger deviations are expected at higher concentrations than smaller deviations associated with lower concentrations. Thus, to obtain true quantitative results, weighted least squares linear

regression (WLS) is useful, as it is a very powerful statistical technique capable of solvingthe reported difficulties of heteroscedasticity (Miller and Miller, 2010).

Regarding calibration methods, the external standard (ES) method is the simplest; however, it can have many errors and is not useful when the sample has a matrix effect.

The standard addition method (SDA) fixes the matrix effect, but it is a very time-consuming method. The Internal standard method (IS) corrects signal variations, but it is difficult to find a chemically identical standard to the analyte absent in the sample. Matrixmatchet reduces some of the matrix effects, however, requires an analyte-free matrix (Miller and Miller, 2010).To get around these problems of the presence of matrix effect and absence of analyte-free matrix the combination of standard addition and internal standard method, named internal standard addition (ISA) it's an interesting option (Hewavitharana, Kassim, Shaw, 2018; Orazbayeva et al., 2017).

This work aims to develop a new method for determining higher alcohols, n-butanol, and ethyl acetate in cachaça and whisky using GC-BID by HS-SPME with multivariate optimization, using the WLS method for linearity correction, and ISA calibration and statistical methods for matrix effect correction, precision, recovery, and accuracy.

2.2 Materials and methods

2.2.1 Optimization of HS-SPME - GC-BID conditions

Considering that several factors interfere in the HS-SPME extraction, temperature, salt content, and extraction time, these parameters were chosen to carry outan experiment design, a full factorial design type in two levels with replicas at the centralpoint (2^3). The conditions varied at each factor were temperature of extraction: 50, 60 and70 ºC; Salt content (NaCl): 0, 2.5 and 5 % (m/v); Exposure time at headspace: 5, 15 and 25 min.

The HS-SPME extraction was performed using a Supelco (Bellefonte, USA) manual holder containing a fiber assembly with an 85 μm polyacrylate (PA) coating. Thefiber was conditioned in the injection port of a gas chromatograph at 280 °C for 30 min before use, and blank desorption was at 240 ºC for 3min. 1 mL of sample (ethanolic solution or beverage samples) was placed in a 7 mL glass vial with PTFE/silicone septa. The graphics were generated by R software (Grömping, 2014).

The sample`s extraction was performed by exposing the fiber to the headspace

kept at a selected temperature for a set time. Extraction was supported by magnetic stirring fixed at 500 rpm. A fiber blank injection was performed before each injection to prevent contaminants.

The standard analytes, n-propanol (99.5 %), ethyl acetate-AcOEt (99.5 %), isobutanol (99.0 %), n-butanol (99.4 %), isoamyl alcohol (98.5 %), amyl alcohol (99.9 %) and absolute ethanol (99. 5 %) were purchased from Sigma Aldrich (USA). The NaCl used was purchased from synth (Brazil). The ultrapure water was obtained in a milli-Q purification system. The heating plate was purchased from Fisaton, model 752A (Brazil).

2.2.2 Comparison of GC-BID and GC-FID

2010 plus gas chromatograph equipped with a split/splitless injector coupledwith a Barrier Discharge Ionization detector (GC-BID) (Shimadzu, Japan) connected to a gas purifier from Valco instrument Co. Inc. was used. The injector temperature was 240 °C in split mode (1:5). The chromatographic column used was Equity-5 (30 m x 0.25 mm I.D x 0.25μm of 5% phenyl - 95% dimethylpolysiloxane) supplied by Supelco (USA). Helium (99.9999%) was used as a carrier gas provided by Messer Gases (Brazil).

The carrier gas flow was 1.8 mL min^{-1}. The temperature program was as follows: 40 °C (hold 3 min), at 10°C min^{-1} increase to 65 °C, then a 50 °C min^{-1} increaseto 200 °C (hold 1 min). The detector temperature was 300 °C, and the flow rate was 50 mL min^{-1}. A micro syringe (Hamilton, Nevada, USA) was used to inject 1 µL of sampleto split injection (ratio of 1:35).

To compare, a GC-FID 17A (Shimadzu, Japan) was used with hydrogen 4.5 (99,995%) as carrier gas. The injector temperature and column flow rate were the same as those used in the GC-BID. The FID temperature was 250 ºC and for an efficient operation were used the (synthetic air 5.0 (99,995%) and hydrogen 4.5 (99,995%) at 30:1ratio mL min^{-1}. The column and temperature program in HS-SPME, and the split injection were as described above.

2.2.3 Validation of the HS-SPME-GC-BID method

The selectivity, linearity, recovery, precision, LOQ and LOD were obtained according to the following procedures:

The selectivity of the method was carried out through the injection of individual

and mix solutions spiked with 200 mg L^{-1} of 5 compounds in ethanol:water solution (40:60 v/v) and injection of ethanolic solution without the addition of the analytes.

Linearity was obtained through calibration curves of the compounds with 7 points ranging from 25 to 500 mg L^{-1} spiked in the solvent (ethanol: water solution, 40:60v/v), in the cachaça and whisky matrices. n-pentanol (amyl alcohol) was used as an internal standard at 390 mg L^{-1}. Therefore, were used the combined standard addition and

internal standard calibration methodologies (ISA) as previously adopted in soil (Hewavitharana, Kassim, Shaw, 2018) and biological fluids (Orazbayeva et., 2017).

The adequacy of the correlation coefficient was evaluated against the value established by regulatory agencies [ANVISA, 2017; INMETRO, 2021), and a statistical evaluation of the significanceof linear regression was performed using the F test (*Fisher-Scenedecor*). If F *calculated*

> F *tabulated* for 95% confidence, linear regression is significant, otherwise, if F *tabulated* > F *calculated*, there is no linear relationship between the x and y axes, even with an R-value close to 1 (Miller and Miller, 2010).

After checking the significance of the OLS regression, statistical tests were performed to assess the homoscedasticity or heterogeneity of the variances, using the Hartley F Test. The curves that presented heteroscedastic behavior had their slope (*bw*), intercept (*aw*), and correlation (*rw*) coefficients recalculated, considering the WLSmethod (Miller and Miller, 2010; Danzer e Currie,1998). The standard deviation of the angular (*Sb*) and intercept (*Sa*) of WLS regression were calculated according to the equations previously used (Asuero, Sayago, González, 2006).

The intra-day precision of the method was obtained by nine determinations of solutions prepared independently, at low, medium, and high concentrations (MAPA, 2011).

The LOD and LOQ values were performed through successive dilutions of the analyte standard solution (100 mg L^{-1}) in ethanol-water (40:60%, v/v) and injection into the GC-BID.

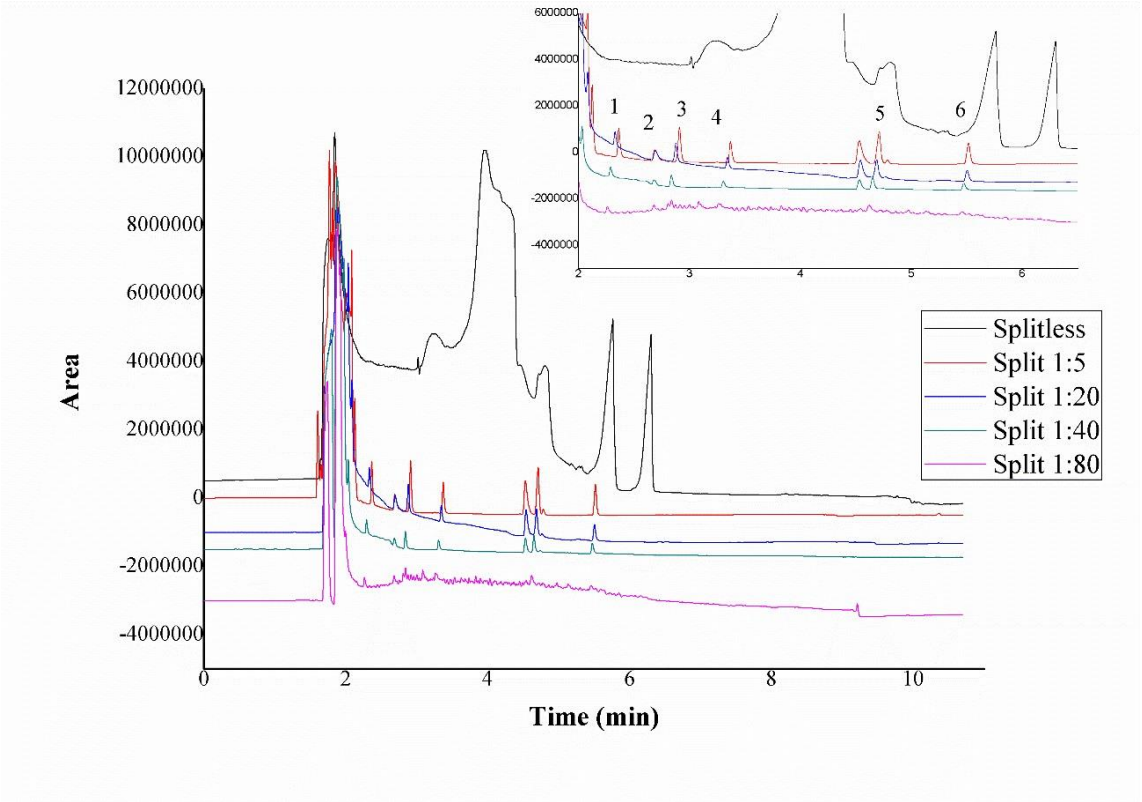
2.2.4 Cachaça and whisky samples analyses

Samples of cachaça and whisky were collected at a local market in 2021 (Fortaleza, Ceará, Brazil).

2.3 Results and discussion

The following split ratio conditions were tested: splitless mode, split ratio 1:5; 1:20; 1:40, and 1:80. Previous studies have shown that HS-SPME extraction conditions used were the best condition found when analyzing these compounds using 85µm polyacrylate fiber and quantification by GC-FID (Nonato et., 2001). Fig. 1 shows the chromatograms obtained with different split ratio conditions tested in GC-BID.

Figure 1 - Split ratio optimization



Legend: Chromatogram in black (splitless) compared with chromatograms obtainedin Split mode in GC-BID; Magnification = Split 1:5 (red), Split 1:20 (blue); Split 1:40 (green); Split 1:80 (pink). Elution order: n-propanol (1), Ethyl acetate (2), Isobutyl alcohol (3), n-butyl alcohol (4), Isoamyl alcohol (5), amyl alcohol (6).

As shown in Fig. 1, the conditions under which splitting of the injected sampleis applied prevent the solvent peak from overlapping the retention time of the analytes (Solvent Effect). However, a high split ratio causes a decrease in the analytical signal andresults in an inadequate quantification method. Therefore, the 1:5 split ratio was chosen to continue the studies.

Regarding the sample volume, Fig. 6 (supplementary material) presents thearea

data of the compounds of interest when the sample volumes were 1 and 3 mL. The increase in sample volume favors the extraction of n-propanol and ethyl acetate, while a smaller volume favors the extraction of isobutanol, n-butanol, isoamyl, and amyl alcohol. This behavior is probably because larger molecules need a longer equilibrium time or high vapor pressure. On the other hand, a larger sample volume results in a more significant solvent effect (on the chromatogram) which affects the peak width and the consequent need for a higher split ratio. Thus, 1 mL sample volume was chosen becauseit is compatible with the 1:5 split ratio and the requirements of the HS-SPME technique with agitation.

The elution order of the compounds, as shown in Fig. 1, as the carbon chain increased, the retention time also increased. In addition, branched-chain compounds suchas isobutanol and isoamyl are eluted before their linear chain analogues because they havehigher vapor pressure (n-butyl and amyl), as shown in Table 4.

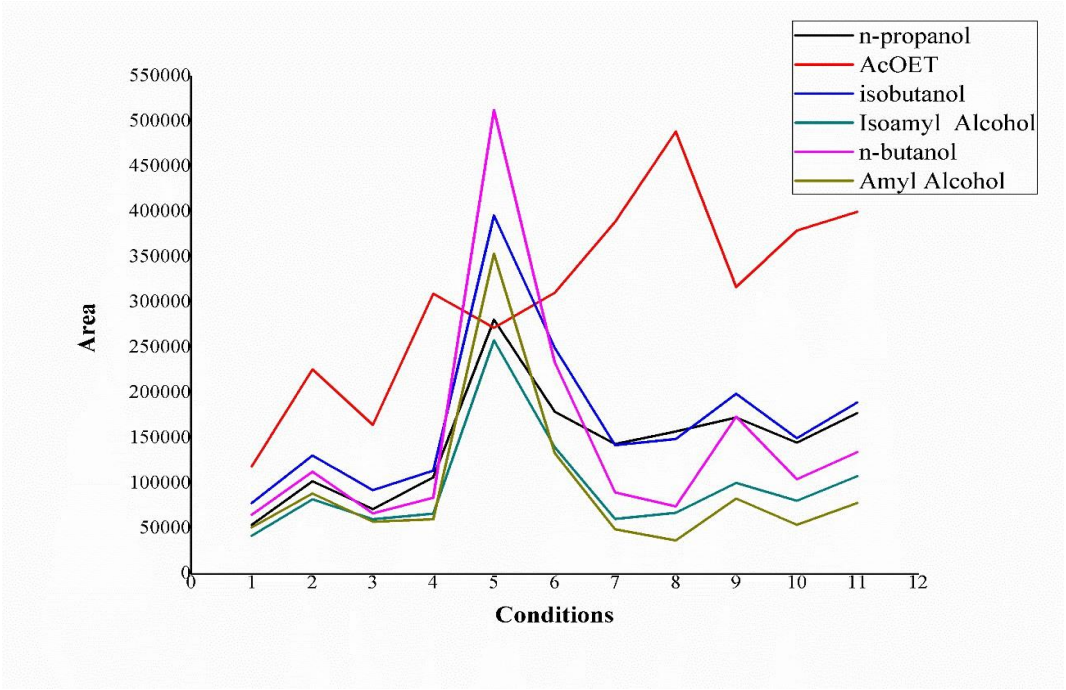
2.3.1 Optimization of sample preparation

Considering that the best conditions for extraction of these compounds by HS-SPME reported in the literature were found in the univariate way for cachaça (Nonato et al., 2001) and whisky (Lee et al., 2001), a multivariate optimization was performed according to the conditions described in item 2.1.

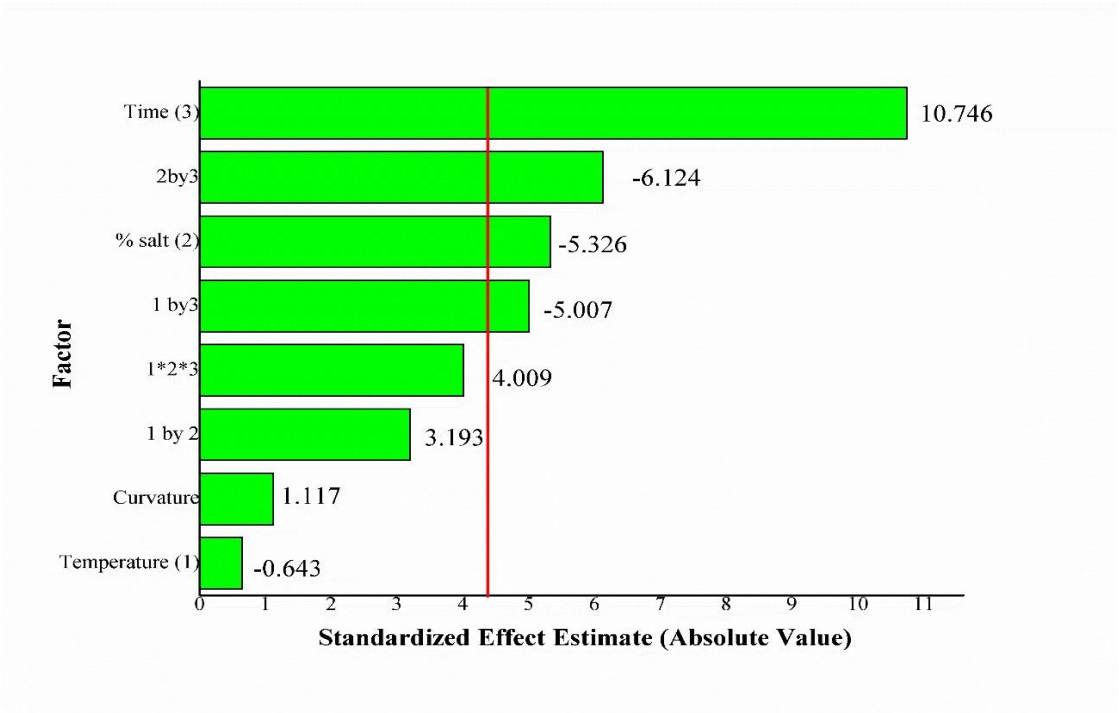
Fig. 2a shows the variation of the compound's areas over the 11 testsperformed. Based on this, the extraction profile of alcohols was similar, while for ethyl acetate, a different behavior was noted.

Figure 2 - Evaluation of the experimental design. 2a) Variation of area relative response. 2b) Pareto Chart.

a)



b)



Legend: Pareto chart of design of experiments applied for 95% confidence (p =0.05%): Factor 1 (temperature), factor 2: percentage of salt, factor 3 (time), curvature = curvature of the response surface; 1by2 (interaction between factors 1 and 2), 1by3 (interaction between factors 1 and 3), 1*2*3 (interaction between factors 1, 2 and 3).

Based on the data analysis, the sum of the areas of all compounds was chosento be the response of the experimental design, since most compounds presented a similarprofile, with an optimal extraction condition close to those of test 5. Even though test 5 has not been the best for ethyl acetate, the area of this compound in this condition is superior to that of n-butyl alcohol and is close to the magnitude of the other compounds.

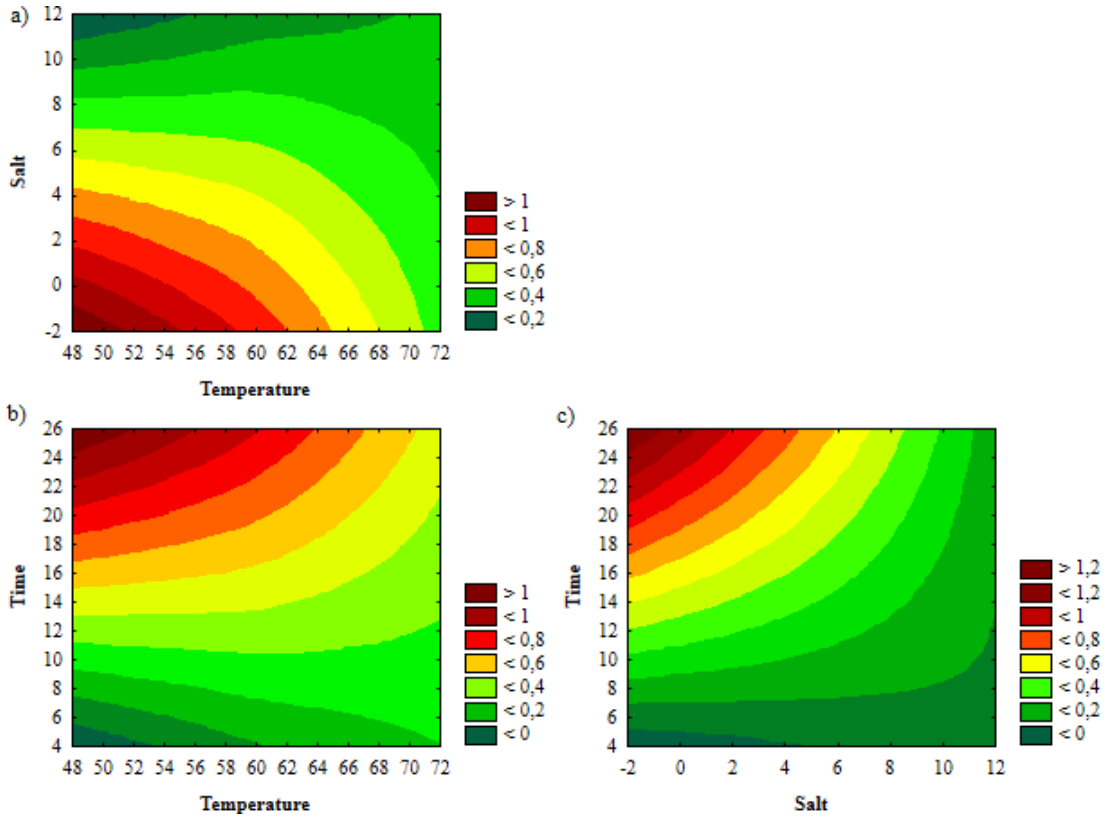
Table 5 shows the variance (ANOVA) data analysis for the model, where the significant parameters are shown in red, and the non-significant ones are shown in black. The values of the determination coefficient (r^2) and determination coefficient adjusted were high (> 0.95), in addition, the lack of fit of the model was not significant, which indicates that most of the data is explained by the model (Fukuda etal., 2018).

Fig. 2b is the Pareto chart generated from ANOVA Table, considering the factors studied and their interactions. According to Fig. 2b, the time was the only variable that positively influenced the extraction of compounds and the most significant. The longer the time, the greater the amount of extracted compounds. The salt content and temperature had negatively influenced the extraction of the compounds, and only the influence of the salt content was significant.

The interaction between temperature and salt content was not significant. In contrast, the others were significant and negative, showing that although the temperaturealone is not significant for the extraction of compounds, the interaction between it and time should not be neglected, as the concomitant increase of these two parameters reducesthe amount of compounds absorbed on the fiber. There were no significant interactions between the three factors.

Furthermore, the model's curvature was not significant, showing that a linear equation is sufficient to explain the relationship between observed and predicted data. Based on this evidence, the maximum extraction of the compounds can be reached whenthere is an increase in time combined with a temperature and salt content reduction.

Figure 3 - Contour surface. 3a) Salt x temperature interaction. 3b) Time x temperature interaction. 3c) Time x salt interaction



The analysis of Figures 3 above revealed that the best condition for extractingthe compounds under study is 50 ºC for 20 min without adding salt. In this condition, thedesirability function is greater than 0.8. This condition is different from that found by (Nonato et al., 2001) when performing a univariate optimization. The best condition found for the extraction by HS-SPME was 60 ºC for 25 min with the addition of 6.5 g of NaCl in 4 mL of samplein GC-FID. This highlights the importance of using a multivariate experimental design tofind the best extraction condition in HS-SPME in analyzing these volatile constituents indistilled beverages (Wiśniewska et al., 2015b; Zacaroni et al., 2017).

For the extraction process investigated there is a difference between the temperature of the solution and the temperature of the headspace due to heat exchanges with the environment; a thermometer was placed in the position where the SPME fiber isexposed to extract the compounds, and it was evaluated the relationship between the temperature of the solution and the headspace. The data from this experiment are shownin Fig. 7. It can be seen that the difference between the temperature of the solution andthe headspace increases as a higher temperature is used, since when the temperature of the solution is 70º, the maximum

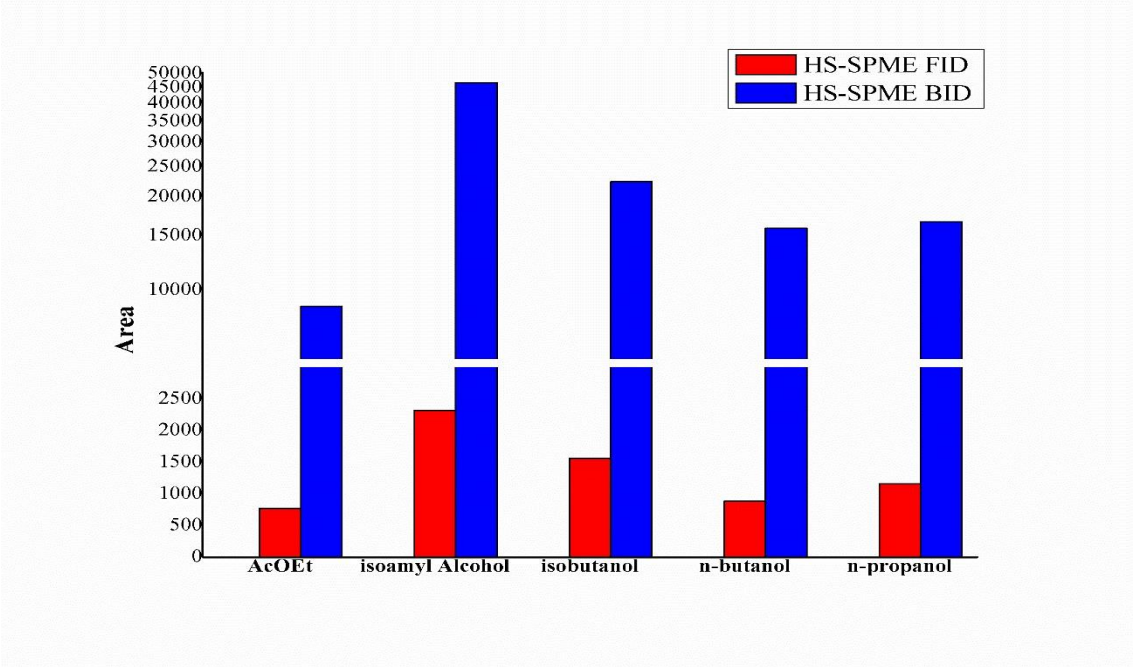
headspace temperature reaches is 57 ºC (81%). In comparison, at a temperature of 50 ºC, the headspace reaches 43ºC (86%). It was concluded that although the solution temperature was 50 ºC, the headspace temperature (in the experiments conducted) was 43ºC.

2.3.2 Comparison of GC-BID and GC-FID

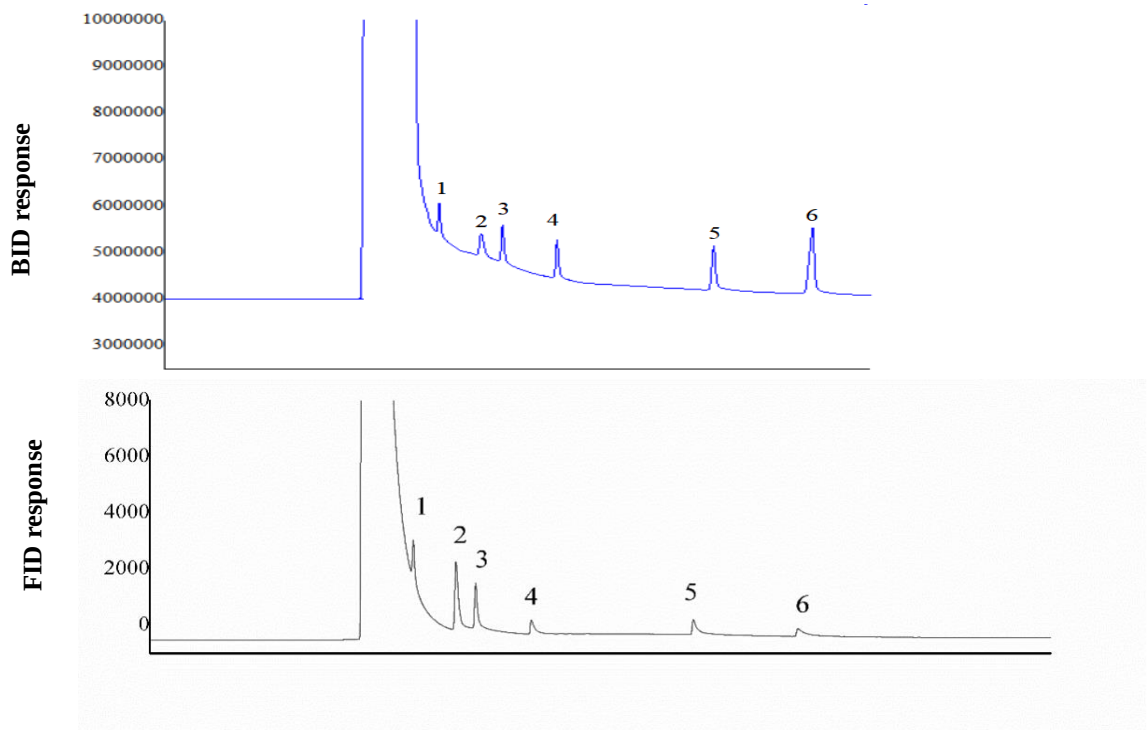
Considering that the traditional methodology for analyzing higher alcohols n-butanol and ethyl acetate uses direct injection in GC-FID, a comparison was made between the methods in the optimized condition HS-SPME and direct injection in both detectors, since the GC-BID is advertised as a more sensitive detector than GC-FID in the analysis of alcohols (Shinada et al., 2012). Fig. 4 shows the response comparison of the chromatogramsobtained for GC-BID and GC-FID for peak area (a) and chromatographic profile (b).

Figure 4 – Comparison of responses between GC-BID and GC-FID. 4a) Comparison of area response relative. 4b) Comparison of the chromatogram profiles

a)



b)



Legend: Chromatogram of GC-BID (blue) and GC-FID (red) obtained by HS-SPME. Elution order: n-propanol (1), Ethyl acetate (2), Isobutyl alcohol (3), n-butyl alcohol (4), Isoamyl alcohol (5), amyl alcohol (6).

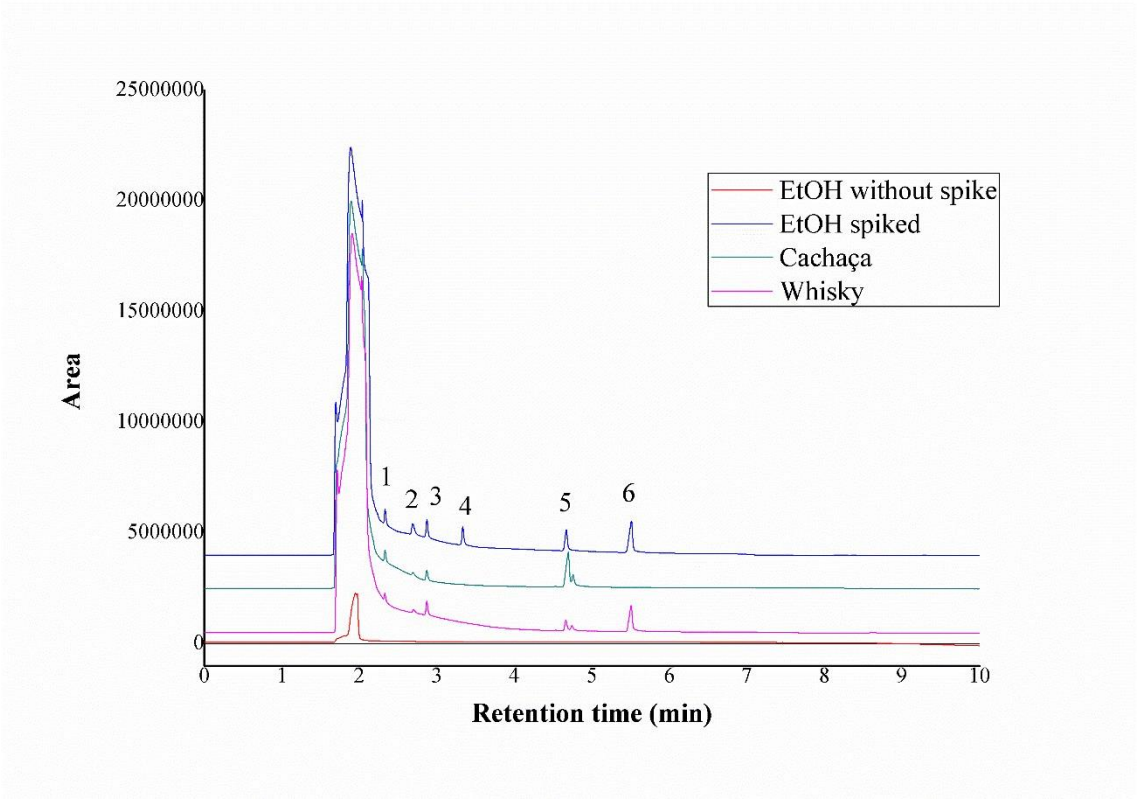
The BID has greater sensitivity for the compounds analyzed than the FID, given that the area values are 11.46 to 20.12 times greater than those obtained by GC- FID. A recent comparative study involving the response ratio (BID/FID) showed that theBID response is 6 times greater than the FID for n-butanol, ethyl acetate and isoamyl alcohol by direct injection (Antoniadou, Zachariadis, Rosenberg, 2019).

The comparison between the direct injection and HS-SPME methods, using the BID, showed that in HS-SPME the relative response value (peak area) was higher forall compounds, except n-propanol. The high response to propanol by direct injection maybe due to the BID ionization mechanism. In addition, the injected solvent was water: ethanol (40:60 v/v) and the BID response is dependent to water, and consequently the peak area of the n-propanol was strongly affected by the solvent effect, which was moresignificant in the HS-SPME than for direct injection (due to the higher split rate). For theFID, all the peak area compounds were higher in the HS-SPME method. Thus, the performance comparison between the detectors showed that the GC-BID is more advantageous than the traditional ones that use the GC-FID.

2.3.3 Merit Figures

Fig. 5 shows the chromatograms of the ethanolic solution (solvent) with and without addition of the analyte standard (200 mg L^{-1}), and the chromatograms of the undoped cachaça and whisky samples. The selectivity of the HS-SPME method was evaluated by analyzing the analyte chromatograms and mass spectra obtained by GC-MS,as recommended by literature (ANVISA, 2017).

Figure 5 - Selectivity of the HS-SPME-GC-BID method



Legend: Chromatograms obtained by HS-SPME-GC-BID. Elution order: n-propanol (1), Ethyl acetate (2), Isobutyl alcohol (3), n-butyl alcohol (4), Isoamyl alcohol (5), amyl alcohol (6).

The ethanol: water solution (40% v/v) was used to construct the curvecalibration in the solvent which is free from interferences to the analytes (n-propanol, AcOEt, isobutanol, n-butanol, isoamyl and amyl alcohol) as is shown in Fig. 5. However, the samples already have the analytes of interest, except for n-butanol and amyl alcohol, which are not present in either matrix. Furthermore, no interferent peaks from other compounds were observed in the matrix.

It was verified (Table 2) that the matrix effect (ME) affected the performanceof the analytical method and that GC-BID was not a very reproducible technique. Thus, the internal standard addition calibration (ISA) was applied for the correction, which provided a better performance from accuracy and precision in several different ways (Hewavitharana, Kassim, Shaw, 2018; Orazbayeva et al., 2017). Furthermore, it reduced the analysis time and provided a cheaper internal standard, without the need to use the deuterated standard (Orazbayeva et al., 2017).

Table 6 shows Hurtley's F test result to verify the homoscedasticity of the variances and its comparison with F test (tabulated). As shown in Table 6, all the calibration

curves showed heteroscedasticity behavior, with F calculated > F critical. In this way, calibration curves were generated using the WLS regression method. The parameters obtained by the WLS regression are given in Table 2.

The calibration curves present (Table 2) slopes ranging from 0.3241 to 0.9457and linear coefficients ranging from -0.2298 to +0.1046. The correlation coefficient ranged from 0.9632 to 0.9992.

The matrix effect (% ME) was calculated using the following Equation (1):

$$\% ME = \frac{(a_m - a_s)}{a_s} x\ 100 \qquad \text{Eq. 1}$$

a_m = slope of the matrix curve; a_s = slope of the solvent curve; ME = Metrix Effect.

The existence of a matrix effect is not considered when the value stays insidethe range of -20% < ME< 20% [39].

Cachaça and whisky matrices showed an increase in the analytical signal formost compounds, except for isoamyl alcohol in cachaça and ethyl acetate in whisky, which showed a decrease in the analytical signal in a small proportion (Table 2). However, % ME values were pronounced and outside the limits established by EuropeanCommission (Pihlström, et al. 2020) for n-propanol, isobutanol, and n-butanol in the two matrices. Thus, toquantify these compounds, only the curve in the matrix should be used, while for the others, both equations can be used (matrix or solvent curve).

Once established which equation should be used to quantify the compounds,the analysis of the statistical significance of the coefficients of the straight-line equation must be carried out. In this case, the significance was evaluated by comparing the coefficients with the respective confidence intervals. The coefficients, with zero confidence intervals, were considered non-significant and should be removed from the equation. In contrast, coefficients that do not have zero, were considered significant and kept in the equation (Miller, 1991). In this way, all the angular coefficients (slopes) were significant,while the linear coefficients of the calibration curves for n-propanol (in solvent and cachaça), isobutanol (in cachaça), and isoamyl (in cachaça) were not significant. The other angular coefficients were significant and must be kept in the quantification

Next, the data obtained from the calibration curves, the solvent (ethanol/water 40:60, v/v), and cachaça and whisky matrices are shown in Table 2.

Table 2 - WLS coefficients, line equations and ME (%) from HS-SPME-GC-BID method

	Matrix	**Slop (*b*w)**	***Sb*w**	**CI *b***	**Intercept (*a*w)**	**S*a*w**	**CI *a***	**ME (%)**	**R**	**Final Equation**
n-propanol	solvent	0.3241	0.0222	0.0598	0.0086	0.0092	0.0192		0.9884	y= 0.3241x
	cachaça	0.3976	0.0218	0.0615	0.0143	0.0079	0.0170	**22.66**	0.9926	y= 0.3976x
	Whisky	0.4299	0.0144	0.0563	0.0138	0.0051	0.0100	**32.65**	0.9972	y=0.4299x + 0.0138
AcOEt	solvent	0.5444	0.0200	0.0691	-0.0256	0.0036	0.0110		0.9966	y= 0.5444x - 0.0256
	cachaça	0.5875	0.0118	0.0385	-0.0108	0.0024	0.0067	**7.93**	0.9990	y= 0.5875 – 0.0108
	Whisky	0.5421	0.0096	0.0334	-0.0099	0.0026	0.0058	**-0.42**	0.9992	y= 0.5421 – 0.0099
Isobutanol	solvent	0.5284	0.0166	0.0459	0.0201	0.0045	0.0110		0.9975	y=0.5284 + 0.0201
	cachaça	0.6630	0.0415	0.1025	-0.0116	0.0128	0.0300	**25.47**	0.9903	y=0.6630x
	Whisky	0.6390	0.0160	0.0701	0.0270	0.0040	0.0091	**20.94**	0.9984	y= 0.6390x + 0.0270
n-butanol	solvent	0.4783	0.0283	0.0934	0.0471	0.0134	0.0271		0.9914	y= 0.4783 + 0.0471
	cachaça	0.6621	0.0254	0.0574	-0.0371	0.0085	0.0209	**38.43**	0.9963	y= 0.6621 – 0.0371
	Whisky	0.9457	0.0651	0.2079	-0.2298	0.0243	0.0526	**97.73**	0.9884	y= 0.9457 – 0.2298
Isoamyl Alcohol	solvent	0.7927	0.0404	0.1256	0.0670	0.0166	0.0351		0.9936	y= 0.7927x + 0.0670
	cachaça	0.7395	0.0923	0.5301	0.0666	0.0782	0.1582	**-6.71**	0.9632	y= 0.7395x
	Whisky	0.8339	0.0932	0.3903	0.1046	0.0357	0.0706	**5.19**	0.9702	y= 0.8339x + 0.1046

After evaluating the % ME and the significance of the calibration curve coefficients, precision (repeatability) and recovery test were calculated. Table 7 (supplementary material) shows the recovery and precision values of the HS-SPME-GC-BID method.

The analyte recoveries in the cachaça matrix ranged from 81.24 to 106.94 %for the compounds in the three concentrations studied. As recommended by the Brazilian Guide (MAPA, 2011), most values were acceptable, suggesting values from -20 to +10% for concentrations ≥10 μg/Kg. The exception was isoamyl alcohol at the lowestconcentration, which showed a recovery of 43.28%. The precision values of the cachaçamatrix ranged from 0.62 to 6.77 %, and all were within the recommended (MAPA, 2011), which suggests values of 7.3% for concentration range from 10 to 99.99 mg L^{-1} and 5.3 % for 100 to 999.99 mg L^{-1}

The analyte recoveries in the whisky matrix ranged from 82.06 to 106.25 % for the compounds in three concentration levels. As recommended by the Brazilian Guide(MAPA, 2011), most values were acceptable, suggesting values from -20 to +10% for concentrations ≥10 μg/Kg. The exception was n-butanol at the lowest concentration, which showed a recovery of 152.26%. The precision values in the cachaça matrix ranged from 0.66 to 7.38 %, and all were within the recommended by MAPA (2011), which suggests values of 7.3 % for concentration levels from 10 to 99.99 mg L^{-1} and 5.3 % for 100 to 999.99 mg L^{-1}.

For our study, the LOD values ranged from 0.26 to 0.51 mg L^{-1}, while LOQ values ranged from 0.78 to 1.55 mg L^{-1} (see Table 8). These values were lower than those reported by Pereira and co-workers that found 5.74 to 13.90 mg L^{-1} [14], and Granato and co-workers found 6.68 to 8.80 mg L^{-1} (Granato et al., 2014) for cachaça analysis using GC- FID by direct injection. On the other hand, our values were even lower than the LOD values reported in the literature for the whisky analysis by GC-MS. Fitzgerald and co- workers found 3.30 to 10.00 mg L^{-1} using headspace (Fitzgerald et al., 2000), and Chung and co-workers found 0.89 to 8.46 mg L^{-1} by direct injection (Chung et al., 2015). The individual values for the analytesare showed in Table 8.

The merit figures obtained revealed a great potential of application of the HS- SPME-GC-BID method in the analysis of volatile constituents in distilled beverages.

2.3.4 Application of the method in cachaça and whisky samples

Table 3 shows the content of higher alcohols, n-butanol, and ethyl acetate obtained by the HS-SPME-GC-BID method applied in cachaça and whisky samples.

Table 3 - Constituent content of cachaça and whisky by WLS regression (mg/100 mL ofAA)

	Cachaça					**Whisky**				
Sample	n-prop	AcOEt	isobut	isoam	Higher Alcohols	n-prop	AcOEt	isobut	isoam	Higher Alcohols
1	45.39	16.44	23.80	133.66	202.85	Nd	7.19	13.26	16.60	29.86
2	33.91	8.11	38.63	107.57	180.11	45.66	25.06	60.42	40.81	146.89
3	3.36	Nd	4.96	20.74	29.06	10.28	4.09	18.57	5.02	33.87
4	22.18	7.25	43.09	144.79	210.06	Nd	4.17	6.69	10.47	17.16
5	59.27	13.91	35.80	182.50	277.57	41.31	21.53	57.68	54.18	153.16
6	10.33	11.51	21.37	113.29	144.99	11.82	9.11	14.40	36.13	62.34
7	30.04	21.05	64.86	118.97	213.87	39.91	14.57	51.38	28.76	120.05
8	17.40	6.29	25.96	86.51	129.88	21.04	37.48	35.53	nd	44.09
9	46.04	10.49	40.08	108.83	194.95	13.19	4.32	19.33	7.26	39.79
10	25.82	7.61	35.46	119.77	181.05	35.26	47.50	72.03	49.27	156.57
11	12.83	3.72	6.08	21.67	40.59	22.16	22.15	42.21	19.84	84.22
12	9.35	11.44	19.81	88.22	117.37	24.05	69.55	158.53	152.72	335.31
13	36.31	8.77	45.47	109.03	190.81	12.93	58.83	76.93	157.59	247.46
14	10.81	9.24	24.77	39.23	74.81	20.58	13.36	33.35	11.01	64.93
15	38.14	8.09	34.07	157.83	230.04	35.93	46.87	73.33	158.62	267.88
16	41.34	11.21	42.24	124.79	208.37	36.87	20.28	50.80	29.16	116.83
17	41.61	31.52	28.06	104.74	174.41	32.89	21.86	51.77	34.14	118.80
18	40.31	26.61	47.55	153.80	241.67	25.70	26.29	33.71	21.13	80.54
19	17.19	8.18	22.74	59.84	99.77	4.80	18.05	20.30	30.43	55.54
20	21.84	11.10	54.56	108.49	184.89	13.14	22.15	92.86	197.67	303.67
21	46.57	14.53	32.79	170.51	249.86	29.56	29.06	41.35	45.42	116.34
22	28.07	8.48	27.53	101.09	156.70	30.94	31.49	51.09	15.08	97.11
23	33.39	16.95	33.81	62.38	129.59	15.91	nd	17.12	10.13	43.16
24	31.62	17.76	75.78	115.31	222.70	3.05	nd	6.64	2.87	12.56
25	70.19	19.96	30.61	136.25	237.05	32.47	15.28	41.61	16.82	90.91
26						23.44	19.10	40.78	31.79	96.01

Legend: n-prop = n-propanol; AcOEt =Ethyl acetate; Isobut= Isobutyl Alcohol; isoam=isoamyl Alcohol; nd=not detected.

The values of higher alcohols found in the cachaça samples ranged from 29.06 to 277.57 mg/100 mL AA, with an average of 172.92 and presented isoamyl alcoholas the majority (Table 3). All samples showed values within the maximum allowed by Brazilian legislation, which is 360 mg/ 100 mL of AA (BRAZIL, 2005). These values were similar to the maximum values of 310.43 mg/100 mL of AA in samples of cachaça collected in local markets in Paraiba-Brazil (Oliveira et al., 2020). Studies carried out by Alcarde et al., (2009) detected only one sample with higher alcohols outside the limit established by Brazilian legislation in 30 samples investigated. Low values for higher alcohols, found in some samples, showed a concern with the product quality, which were similar to results noted by Agnol, Friedrich and Fundo (2011).

The formation of higher alcohols occurs during fermentation and is strongly dependent of factors, such as fermentation conditions, amount of yeast cells in the must, fermentation temperature, and final alcoholic content of the sugarcane wine (Lima et al., 2022). Temperatures above 32ºC, pH below 4.0 and the presence of oxygen also favor the production of higher alcohols (Vilela et al., 2007).

Artisanal produced cachaça (distilled in alembics) has higher alcohol concentrations than those industrially produced (distilled in columns). The higher ethanol concentration in the vapor phase in alembics results in lower efficiency in separating these constituents from ethanol (Whitby, 1992). Another factor that interferes with the content of these constituents is the aging time of the drink (Miranda and Martins 2008) or the type of wood (Bortoletto and Alcarde, 2013).

Higher alcohols greatly influence the formation of flavors and aromas characteristic of spirits, but very high amounts can change odor characteristics consideredpleasant to highly unpleasant. An example of this is isoamyl alcohol, which, when presentin large quantities in spirits, is associated with the smell of banana Isoamyl alcohol with “malt” aromas, "whisky”, “wine”, “banana” and “sweet (Oliveira et al., 2020).

N-butanol was not detected in the cachaça samples studied, which follows the requirements of Brazilian legislation (< 3 mg/100 mL of AA) (BRAZIL, 2005). The presence of n- butanol in cachaça samples compromises the drink quality (Lima et al., 2022). Caetano and co-workers [2021], when analyzing 24 artisanal cachaças of Salinas-MG-Brazil, found (in three sample)levels of n-butanol above those established by Brazilian legislation (BRAZIL 2005, Brazil 2011). The ethyl acetate content ranged from undetected (nd) to 31.52 mg/100 mL

AA, within limits established by Brazilian legislation (<200 mg/100 mL of AA). The major esters in distilled beverages are ethyl acetate, followed by ethyl lactate, both have a fruit flavor that, in high concentrations, becomes unpleasant. Brazilian legislation's maximum content of total esters refers only to ethyl acetate (BRAZIL, 2005). The profile observed by the samplesin this work is consistent with that observed in the literature, where Brazilian spirits generally present ethyl acetate content within the specifications (Lima et al., 2022). Alcoholic beverages distilled in alembics (from cooper) have higher ester contents because copper catalyzes esterification reactions at high temperatures (Murray, 2022). The aging of beverages causes an increase in the ethyl acetate content, due to the esterification reactions between the acidsand alcohols of the beverages, and the ester extraction from the wood (Miranda and Martins, 2008). From a comparative study between glass containers and oak barrels, the authors observed a more significant increase (3-fold) increase in ethyl acetate content in beverages stored in oak barrels for 360 days.

The highest alcohol values found in the whiskey samples ranged from 12.56 to 355.31 mg/100 mL AA, with an average of 112.89 mg/100 mL AA, and isoamyl (or isobutyl) alcohol was the majority (Table 3). Twenty-two samples showed values withinthe maximum allowed by Brazilian legislation (<300 mg/ 100 mL AA) (Brazil, 2005; Brazil, 2011), and only 3 samples had alcohol content higher than this value. Câmara et al., (2007) and Caldeira et al., (2007), when analyzing commercial whisky samples by HS- SPME, found isoamyl alcohol as the major.

N-butanol was not detected in the whisky samples analyzed, which follows the requirements of Brazilian legislation (< 3 mg/100 mL of AA) (Brazil, 2005; Brazil, 2011). The ethyl acetate content ranged from undetected (ND) to 69.55 mg/100 mL AA, within limits established by Brazilian legislation (<150 mg/100 mL AA) (Brazil, 2005; Brazil, 2011). Caldeira et al., (2007), when analyzing commercial whiskey samples, they found high concentrations of methyl esters, with ethyl decanoate being the majority. The authors also proposed that the ethyl octanoate and isoamyl acetate esters were the substances responsible for the characteristicflavor taste (sweet, fruity and fresh, and banana, respectively). In contrast, the alcohols that contributed to the whisky flavor were isoamyl (fruit notes) and isobutyl (bitter, harsh).

2.4 Conclusion

The HS-SPME-GC-BID method was optimized in a multivariate way, and the best conditions for extraction of the compounds were obtained for 50ºC, 20 min and without addition of NaCl.

The method validation was successful and allowed to find satisfactory valuesof determination coefficients (0.9632 to 0.9992) for the calibration curves. The matrix effect evaluation revealed values outside the established for n-propanol, isobutanol, and n-butanol in the cachaça and whisky matrices. The analyte concentration was verified ina number of ways, such as combined standard addition and internal standard calibration (ISA), which presented heteroscedastic behavior, weighted least squares linear regression (WLS) and statistical significance of the coefficients were evaluate. In addition, it was possible to obtain recovery and accuracy values acceptable for most compounds in both matrices, except for isoamyl alcohol in cachaça and n-butanol in whisky at the lowest concentration, which showed recovery values of 43.28 and 152.26 %, respectively. The LOD values were lower than those reported in the literature to analyze of the compoundsstudied in the two matrices.

The HS-SPME/GC-BID method was applied in the determination of higher alcohols, ethyl acetate, and n-butanol in 25 samples of cachaça and 26 samples of whiskyand contributed to evaluating the quality of alcoholic beverages commercialized in the Brazilian market.

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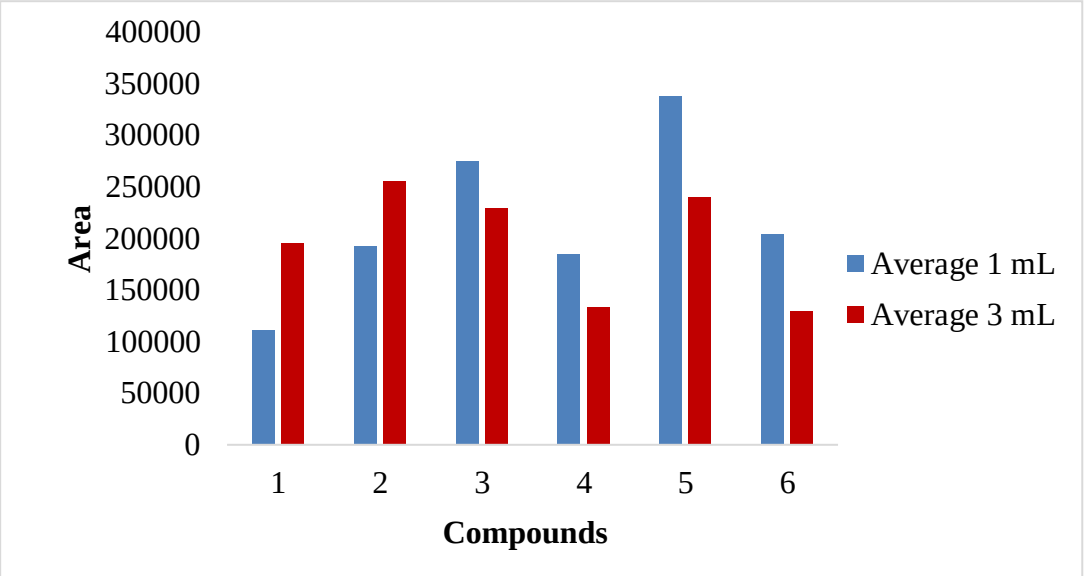
2.5 Supplementary material of "New HS-SPME-GC-BID method for thedetermination of volatile constituents in distilled beverages"

Figure 6 - Optimization of sample volume



Legend: n-propanol (1), Ethyl acetate (2), Isobutyl alcohol (3), n-butyl alcohol (4), Isoamyl alcohol (5), amyl alcohol (6).

Figure 7 - Measurement of headspace temperature

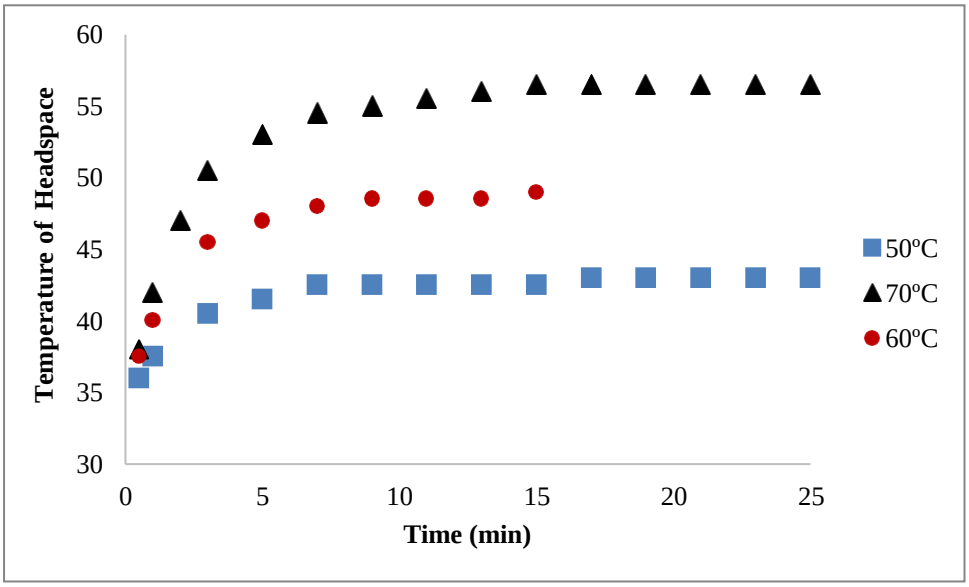


Table 4 - Physicochemical properties of compounds analyzed by GC-BID

Compounds	**Boiling point (ºC)**	**MM (g/mol)**	**Vapor pressure** (mmHg)
n-propanol	97.2	60.10	97.2
Ethyl acetate	70.4	88.11	93.2
Isbutyl alcohol	108.0	74.12	10.4
n-butanol	117,7	74.12	7.00
Isoamyl alcohol	132.5	88.15	2.37
amyl alcohol	137.5	88.15	2.20

Source: Hazardous substances data bank (HSDB) https://pubchem.ncbi.nlm.nih.gov/ accessed at 23/05/2022.

Table 5 - ANOVA of full factorial design applied to optimize SPME extraction

Factor	**SS**	**DF**	**MS**	**F**	**P**
(1)Temperature (ºC)	3.416E+09	1	3.416E+09	0.4133	0.586179
(2) Salt contente (% de sal)	2.345E+11	1	2.345E+11	28.3673	0.033491
(3) time (min)	9.536E+11	1	9.536E+11	115.3641	0.008557
1x2	8.429E+10	1	8.429E+10	10.1978	0.08565
1x3	2.072E+11	1	2.072E+11	25.0674	0.037654
2x3	3.100E+11	1	3.100E+11	37.5043	0.025642
1x2x3	1.328E+11	1	1.328E+11	16.0708	0.05696
Lack of fit	1.032E+10	1	1.032E+10	1.2482	0.380108
Pure error	1.653E+10	2	8.27E+09		
Total SS	1.953E+12	10			

Legend: R-sqr: 0,98625; R-adjust: 0,95417; MS Pure Error=826566E4; significative values are red.

Table 6 - Homogeneity of variances tests (F test Hurtley)

	Matriz	F calc	F tab	Results
n-propanol	solvent	15.696	8.38	**Heteroskedastic**
	cachaça	23.079	8.38	**Heteroskedastic**
	Whisky	93.722	8.38	**Heteroskedastic**
AcOEt	Solvent	190.593	8.38	**Heteroskedastic**
	cachaça	255.115	8.38	**Heteroskedastic**
	Whisky	66.063	8.38	**Heteroskedastic**
isobutanol	Solvent	58.299	8.38	**Heteroskedastic**
	cachaça	128.061	8.38	**Heteroskedastic**
	Whisky	378.043	8.38	**Heteroskedastic**
n-butanol	Solvent	31.171	8.38	**Heteroskedastic**
	cachaça	169.55	8.38	**Heteroskedastic**
	Whisky	572.335	8.38	**Heteroskedastic**
Isoamyl alcohol	Solvent	31.202	8.38	**Heteroskedastic**
	cachaça	66.111	8.38	**Heteroskedastic**
	Whisky	188.724	8.38	**Heteroskedastic**

Table 7 - Recovery and coefficient of variation of HS-SPME-GC-BID method

			Cachaça			
	Recovery (%)			**coefficient of variation (%)**		
Concentration mg L^{-1}	***100***	***300***	***500***	***100***	***300***	***500***
n-propanol	97.86	102.59	101.84	3.76	0.62	1.84
AcOEt	81.31	89.49	100.94	6.77	1.43	6.08
isobutanol	81.24	94.25	96.88	6.50	5.99	3.17
n-butanol	105.88	106.82	95.78	4.90	3.92	4.66
Isoamyl alcohol	43.28	106.94	105.63	3.19	4.89	4.55
			Whisky			
	Recovery (%)			**coefficient of variation(%)**		
Concentration mg L^{-1}	***100***	***300***	***500***	***100***	***300***	***500***
n-propanol	106.25	83.79	98.67	3.96	5.21	3.31
AcOEt	87.91	98.92	101.73	5.41	7.38	4.86
isobutanol	106.23	92.04	102.42	7.23	5.17	2.49
n-butanol	151.16	88.48	82.06	4.68	4.85	1.62
Isoamyl alcohol	102.10	93.16	98.49	6.78	4.84	0.66

Table 8 - LOD values of volatile organic compounds in alcoholic beverages

Reference	Matrix	Method	n-prop	AcOEt	n- but	isobut	Isoamyl
This work	cachaça/whisky	HS-SPME-GC-BID	0.51	0.45	0.36	0.29	0.26
Pereira et al., 2012	cachaça	DI-GC-FID	13.9	10.5	8.63	5.74	7.6
Granato et al., 2014	cachaça	DI-GC-FID	6.68	-	-	6.68	8.0
Fitzgerald et al.,2000	whisky	HS-SPME-GC-MS	4.4	10	6.9	6	3.3/3.4
Chung et al., 2015	Whisky/ others	DI-GC-MS	8.46	-	0.77	-	0.89

Legend: LOD values reported in the literature for different methods. The unit of values is mg L^{-1}. n-prop = n-propanol; AcOEt =Ethyl acetate; Isobut= Isobutyl Alcohol; isoam=isoamyl Alcohol; nd=not detected.

3 CHAPTER 3 - CHEMOMETRIC TOOL ASSOCIATED TO CHROMATOGRAPHY DATA TO ASSESS THE QUALITY AND AUTHENTICITY WHISKEYS

ABSTRACT

Whisky is a distilled beverage consumed worldwide, with high added value that is often the target of adulteration. There are several quality control methods in the production of these distillates, among which the chromatographic methods stand out. These methods associated with chemometric tools provide powerful tools for the assessment of authenticity and differentiation between types of whiskey. This work proposes a new methodology that combines chromatographic data with chemometric tools to differentiate between original and counterfeit whiskeys. For this purpose, 14 counterfeit and 16 original whiskeys were analyzed by two chromatographic methods. One that analyzes volatile compounds by Solid-phase microextraction (SPME) via headspace (HS) - gas chromatography coupled to barrier discharge ionization detector (BID), HS-SPME-GC-BID, and another to carbonyl compounds derivatized by 2,4-dinitrophenylhydrazine (2,4-DNPH) in high performance liquid chromatography coupled to diode array detector (HPLC-DAD). After obtaining the data, principal component analysis (PCA) was applied. The analytical merits figures of the HS-SPME-GC-BID method were reported in this work. The analytical merits figures of the 2,4-DNPH-HPLC-DAD method were selectivity, linearity, recovery, and precision. The evaluation of the homoscedasticity of the variances revealed heteroskedastic data and the coefficients were estimated by the weighted linear squares (WLS) method. Correlation coefficient varied from 0.9904 for furfural to 0.9999 for acetaldehyde. The evaluation of significance coefficients shows that linear and angular coefficients were significant. Recovery of the method ranged from 80.84 to 107.21 % for the compounds, except for acrolein in the highest concentration and precision values ranged from 0.99 to 3.94 %, except for acrolein at highest concentration. The PCA application allowed the differentiation between the two types of samples where original samples had high content of analytes while the whiskey counterfeit samples in general had low levels of the investigated analytes. The analytical merits figures, selectivity, linearity, recovery, and precision of the 2,4-DNPH-HPLC-DAD method were satisfactorily obtained. The PCA application allowed the differentiation between 16 original and 14 counterfeit whiskies.

Keywords: gas chromatography coupled to barrier discharge ionization detector; high performance liquid chromatography coupled to diode array detector; chromatographic data; whisky adulteration; principal component analysis.

3.1 Introduction

Whisky is a beverage with an alcoholic strength ranging from 38 to 54% v/v at 20 ºC, obtained through the aged simple alcoholic distillate of cereals, totally or partially malted, with the addition of potable ethyl alcohol of agricultural origin, as well as water for alcohol and caramel reduction for color correction (BRAZIL, 2009; EC, 2019). Whisky has great worldwide acceptance, with high added value and presents different forms of production, this contributes to this drink being the target of adulteration. The most common types of adulteration consist of diluting with water and ethanol, adding caramel and mixing with other spirits (Tosato et al., 2018; Mackenzie and Aylott, 2004; Kamiloglu, 2019).

Different spectroscopic and chromatographic techniques are used to differentiate between original and counterfeit whiskeys, these techniques are often associated with chemometric methods to facilitate the distinction between sample types (Power et al., 2020; Rezende et al., 2022). Considering that chromatography is widely used for quality control of distilled beverages, this technique can still be useful in confirming authenticity analyzes and providing evidence of the adulteration process used, since it always undergoes new changes (Stupak et al., 2018). Analysis of samples of counterfeit and original whiskeys by GC-FID revealed a higher content of higher alcohols in adulterated beverages due to the addition of cheap alcoholic beverages, produced without adequate quality control (Lad, Tiwari, Sharma, 2021). Liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI-MS) was used to assess the authenticity of aged beverages (Tosato et al., 2018). The HS-GC-MS technique shows better results when compared with UV-Vis, FTIR-T, TIR-AT in the evaluation of the authenticity of different types of whiskeys using the partial least-squares discriminant analysis (PLS-DA) (Wiśniewska et al., 2017b).

The volatile compounds more common in whisky are terpenes, alcohols, esters, carboxylic acids, ketones (Ferracane et al., 2022) and many inorganic elements (Pawlaczyk et al., 2019), where the majority compounds are n-propanol, isobutyl and isoamyl alcohol), n-butanol, acetaldehyde, acetic acid and ethyl acetate. These compounds are traditionally analyzed by gas chromatography, coupled to flame ionization detector (GC-FID) (Charapitsa et al., 2021), and mass spectrometry (GC-MS) (Ferracane et al., 2022) through direct injection and use of split mode injection are regulated (BRAZIL, 2011). Recently, the dielectric barrier discharge ionization detector (GC-BID) was developed for gas chromatography applications (Shinada et al.,2012). The performance between BID and FID was compared for higher alcohols, n-butanol and ethyl acetate, and the BID more was more sensitive, and allowed the

quantification of these compounds in cachaça and whiskey with low detection limits (Nascimento et al., 2022).

Regarding the aldehydes present in distilled beverages, the acetaldehyde is the majority compound that gives the beverage pungent characteristics. There are also other aldehydes present in smaller amounts in distilled beverages (formaldehyde, furfural, 5-hydroxymethylfurfural (5-HMF), acrolein, and others), which are contaminants and become part of composition of beverage depending on the produced mode (Daute et al., 2021). Some aldehydes can be analyzed by GC or HPLC, but the methodology that uses HPLC-DAD preceded of a derivatization reaction with 2,4 dinitrophenylhydrazine (2,4 DNPH) for quantification of aldehydes and ketones has been widely used for different types of distilled beverage samples (Nascimento et al., 1997).

Therefore, is proposed a new strategy that uses chemometric tools associated with chromatographic data to differentiate original and counterfeit whiskeys using the HS-SPME-GC-BID for alcohols and esters, and 2,4 DNPH-HPLC-DAD method for aldehydes.

3.2 Materials and methods

For the application of chemometric methods, samples of original (16) and counterfeit whiskeys (14) were analyzed by two chromatographic methods. Originals samples were black&white, old parr 12 and 18 years, gold label, red label, black label, natu nobilis, passport, wall street, buchanas 12 years, J&B, old eight, drurys, jack daniels, teacher and white horse.

The HS-SPME-GC-BID method developed by Nascimento et al., (2022). was used to obtain the levels of higher alcohols, n-butanol and ethyl acetate. To obtain the aldehyde contents was used, in which a derivatization of the formaldehyde, acetaldehyde, acetaldehyde, furfural, 5-hydroxymethyl furfural (5-HMF) and acrolein is carried out with 2,4 dinitrophenylhydrazine (Nascimento et al., 1997).

3.2.1 HS-SPME - GC-BID

The H-SPME extraction was performed at 50ºC, 20 min and without salt addition. A system of Supelco (Bellefonte, USA) holder containing a fiber assembly with a 85 µm polyacrylate (PA) coating was used. The fiber was conditioned in the injection port of a gas chromatograph at 280 °C for 30 min prior to use and blank desorption was at 240 ºC for 3min. 1 mL of sample (ethanolic solution or whisky sample) was placed in 7 mL glass vial with

PTFE/silicone septa.

The extraction of samples was performed by exposure of the fiber to the headspace, kept at a selected temperature for a set time. Extraction was supported by magnetic stirring fixed at 500 rpm. A fiber blank injection was performed before each injection to prevent contaminants.

n-propanol (99.5 %), ethyl acetate-AcOEt (99.5 %), isobutanol (99.0 %), n-butanol (99.4 %), isoamyl alcohol (98.5 %), amyl alcohol (99.9 %) and absolute ethanol (99. 5 %) were from Sigma Aldrich (USA). The NaCl used from synth (Brazil). The ultrapure water was obtained in a milli-Q purification system. The hot plate from Fisaton, model 752A (Brazil).

A 2010 plus gas chromatograph equipped with a split/splitless injector coupled with a Barrier Discharge Ionization detector (GC-BID) (Shimadzu, Japan) connected to a gas purifier from Valco instrument Co. Inc. was used. The injector temperature was 240 °C in split mode 1:5. The chromatographic column used was Equity-5 (30 m×0.25 mm×0.25μm of 5% phenyl–95% dimethylpolysiloxane) supplied by Supelco (USA). Helium (99.9999%) was used as a carrier gas and was supplied by Messer Gases (Brazil).

The flow of carrier gas was 1.8 mL min^{-1}. The temperature program was as follows: 40 °C (hold 3 min),10°C min^{-1} to 65 °C, then 50 °C min^{-1} to 200 °C (hold 1 min). The detector temperature was 300 °C and the flow rate was 50 mL min^{-1}.

3.2.2 HPLC-DAD analyses

Formaldehyde, Furfural, Acrolein, Perchloric Acid from VETEC. 5-hydroxymethylfurfural (5-HMF), acetaldehyde 99.9% and 2,4-dinitrophenylhydarzine were purchased from Sigma-Aldrich. Chromatographic grade methanol and acetonitrile from J.T. Baker. Absolute ethanol ≥ 99.5 % and Sulfuric acid from Merck. The ultrapure water was obtained in a milli-Q purification system.

The chromatographic analysis was performed in a high-performance liquid chromatography (HPLC), Shimadzu, model 20 A, coupled to a diode array detector (DAD). A reversed-phase column C18, 25 cm x 4.6 mm x 5 μm, was used (purchase from Rigol). The injection volume was 20.0 μL.

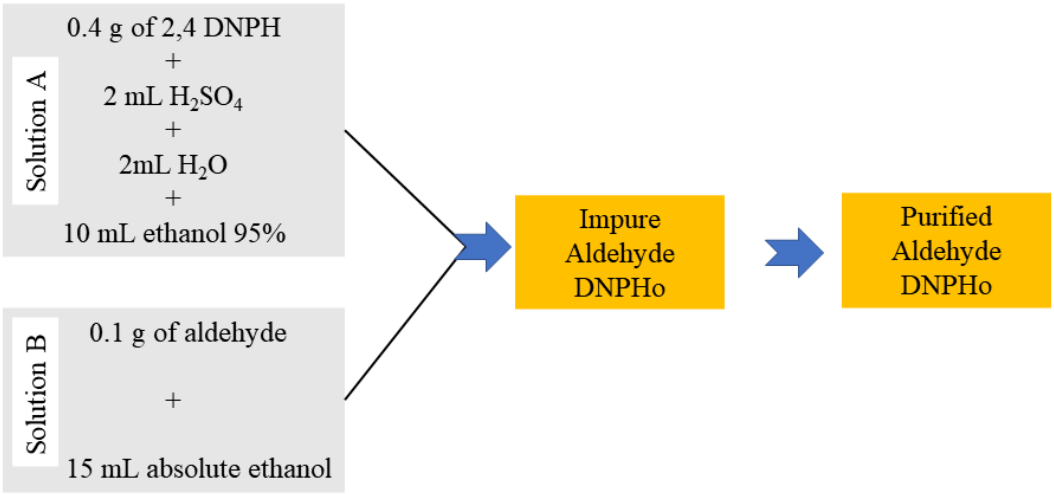
The mobile phase used in the analysis of 2,4-DNPH hydrazones (2,4-DNPHo) was methanol-water, with elution in gradient mode as follows: from 70 to 80% methanol in 7 min, from 80 to 90% in 2 min, from 90 to 70 % in 3 min, remained at 70% for 5 min, with total time of 17.0 min. The flow rate was 1.0 mL min^{-1} at 360 nm.

3.2.3 Derivatization with 2,4-DNPH

3.2.3.1 Obtaining derivatives 2,4 DNPHo of aldehyde standards.

A 2,4-DNPH solution was prepared with 0.4 g in 2 mL of sulfuric acid, adding 2 mL of water dropwise with stirring, with stirring until complete solubilization, and diluted at 10 mL of 95% ethanol (*Solution A*). The hydrazones were obtained as described: 0.1 g of the aldehyde standard were dissolved in 15 ml of ethanol (*Solution B*). The freshly prepared 2,4-DNPHo and subsequent recrystallization, were made according to the reference Nascimento et al., (1997) as shown in the following Figure 8.

Figure 8 - Flowchart of the procedure for the derivatization of aldehyde standards.



For quantitative analysis, a stock solution (1000 mg L^{-1}) of DNPHo in acetonitrile was prepared. The working solutions, from which the points of the calibration curve were prepared, were obtained by diluting the stock solution in an ethanol/water solution (45:55 v/v).

The solvent curve was prepared in 7 levels in triplicate with concentrations ranging from 20 to 120 mg L^{-1} for acetaldehyde and from 5 to 25 mg L^{-1} for formaldehyde, furfural and 5-HMF, for acrolein 0.25 to 12.5 mg L^{-1} by diluting the stock solution in 45% ethanolic solution (v/v).

3.2.3.2 Derivatization of samples

For the derivatization of whisky samples, initially, a 4% (w/v) solution of 2,4 DNPH was prepared by dissolving 0.4 g of the compounds in 100 mL of grade chromatographic

acetonitrile. Another solution of 1.0 mol L^{-1} perchloric acid was prepared dissolved in ultrapure water. To these solutions were added to amounts of beverage in the following proportions: 4 ml of sample: 1 ml of 4% (m/v) 2,4 DNPH solution: 0.5 ml of 1 mol L^{-1} perchloric acid solution.

3.2.4 Merit figures investigated by HS-SPME-GC-BID and HPLC-DAD methods

The figures of merit investigated by the HS-SPME-GC-BID method are described in (Nascimento et al., 2022). Although the HPLC-DAD method is already known, linearity, selectivity, precision, accuracy were investigated.

The selectivity was obtained through the injection of the individual standards to know the retention time and UV/VIS spectrum. In addition, the chromatogram of the ethanolic solution was compared with the matrix derivatized without doping the standards. The alcoholic content of the samples injected was 45%.

The homoscedasticity of the variances was evaluated according to the procedure adopted by (Ziegel, 2004; Danzer and Currie, 1998). Linearity was estimated for two calibration curves by external standardization at 7 levels (solvent), with each level injected in triplicate in the range of 5 to 60 ppm for acetaldehyde, 0.5 to 25 ppm for the furfural, 5-HMF, and formaldehyde and from 0.25 to 12.5 for the acrolein. The evaluation of the significance of the regression coefficients were evaluated using the t test, according to the reference (Danzer and Currie, 1998).

3.2.5 PCA analysis

The data of content of carbonyl and volatile compounds obtained by the HS-SPME-GC-BID and 2,4-DNPH-HPLC-DAD methods were used to perform a principal component analysis using the statistical software.

3.3 Results and Discussion

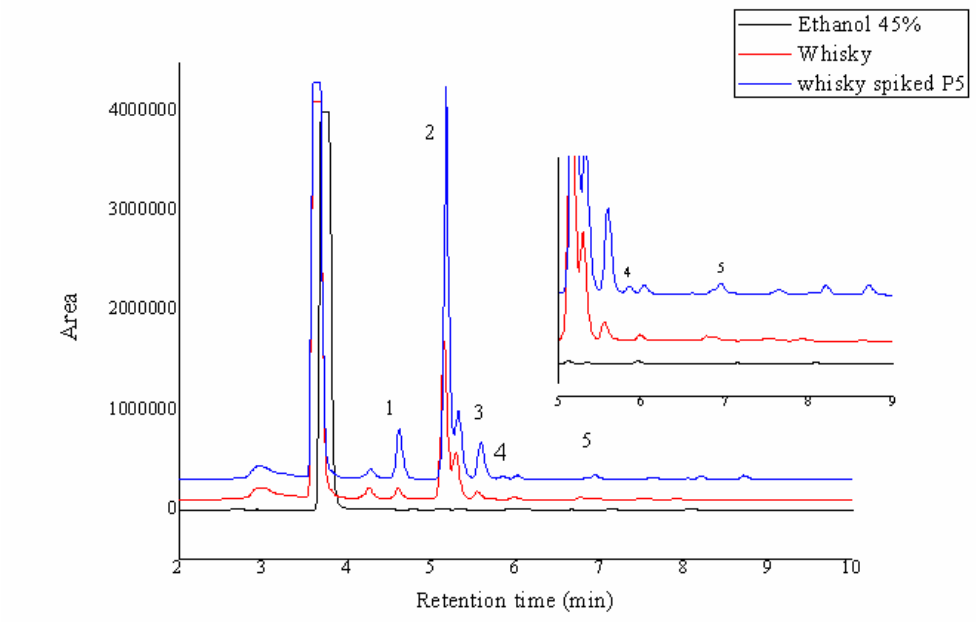
The following results are described in two topics: quality control of the HPLC-DAD method and chemometric applications.

3.3.1 Figures of merits of 2,4 DNPH-HPLC-DAD method

The first figure of merit investigated for selectivity is shown in Fig. 9. shows the chromatograms of derivative 45% ethanolic solution (solvent) and whisky matrix with and without doping. The Fig. 9 shows the matrix doped with P5 of analytical curve. The selectivity of the 2,4 DNPH-HPLC-DAD method was evaluated by analyzing these chromatograms and by the UV VIS spectra of the as recommended by (ANVISA, 2017).

The ethanolic solution 45% used to construct the curve in the solvent does not present peaks referring to the formaldehyde, furfural, 5-HMF and acrolein, and have little amount of acetaldehyde (Fig. 9). However, the matrix already has the analytes of interest, confirmed by analysis of retention time when doing doping and UV/VIS spectrum. Furthermore, no interference peaks from other compounds were observed to the same retention time of the analytes in the matrix.

Figure 9 - Selectivity for the 2,4 DNPH-HPLC-DAD



Legend:

Chromatograms of the Ethanol 45%, whisky and whisky spiked derivative solutions. Compounds: formaldehyde (1), acetaldehyde (2), furfural (3), 5-HMF (4) and acrolein (5).

After evaluating the selectivity, the linearity was evaluated, starting with the analysis of the homoscedasticity of the variances using the Hartley F tests, whose results are shown in the following Table 11.

Although there are several tests for evaluating the homoscedasticity of variances,

when working with high values such as area, in chromatography, the recommendation is to use the Hartley F test (MAPA, 2011). As can be seen in Table 11, Hartley's test revealed heteroscedastic data for most compounds in the two tested curves. Thus, the linear regression coefficients were calculated using the weighted least squares method (WLS), according to the equations proposed by (Ziegel, 2004).

Table 9 presents the values of intercept and angular coefficient of each linear regression, calculated by the WLS method and the values of the significance test applied to the calibration curves.

According to Table 9, the slop values ranged from 14065.53 to 87862.59 while the intercept values ranged from 25360.05 to 339614.36 and R value ranged from 0.9904 to 0.9999. These values showed a slight difference when compared to those calculated by the OLS method (Table 12 supplementary material). Therefore, the WLS coefficients were adopted and the significance of the coefficients of the calibration curves was evaluated.

Table 9 - Values of the regression coefficients and significance test applied of the WLS method

	slop (b)	Sb	t Calc	t tab	intercept (a)	Sa	t calc	t tab	R	Final Equation
Formaldehyde	87862.59	2494.059	35.2284	2.571	339614.36	9260.20	36.6746	2.571	0.9980	y=87862.59x + 339614.36
Acetaldehyde	84455.30	416.460	202.7909	2.571	80234.62	5031.11	15.9477	2.571	0.9999	y=84455.30x + 80234.62
Furfural	78036.85	4882.423	15.9830	2.571	159773.65	25205.07	6.3389	2.571	0.9904	y=78036.85x + 159773.65
5-HMF	14065.53	490.095	28.6976	2.776	37603.30	1197.23	31.4085	2.776	0.9970	y=14065.53x + 37603.30
Acrolein	24938.10	621.309	40.1364	2.571	25360.05	4596.55	5.5172	2.571	0.9985	y=24938.10 +25360.05

According to the significance test applied, all angular and linear coefficients were considered statistically significant and should be kept in the quantification equation of the compounds (Miller and Miller, 2018).

After this step, the recovery and accuracy of the method were evaluated, as shown in Table 13.

The recovery values of the whisky matrix ranged from 80.84 to 107.21 % for the compounds, except for acrolein in the lowest concentration (110. 74 %). Most values were acceptable as recommended by the brazilian guide (MAPA, 2011), suggesting values from -20 to +10% for concentrations ≥10 mg/Kg. Most precision values of the whisky matrix were within the recommended (ranged from 0.99 to 3.94 %) (MAPA, 2011), which suggests values of 7.3% for concentration range from 10 to 99.99 mg $L^{-1.}$ The exception was the precision values of acrolein at highest concentration (8.38 %).

Table 10 shows the content of compounds analyzed in samples of original (O1-O16) and counterfeit whiskeys (F1-F14).

On the original samples the formaldehyde content ranged from not detected to 3.78 mg/100 mL of AA (0-17.01 mg L^{-1}), with an average of 1.34 mg/ 100 mL of AA, these results were higher than those found by Burini and coli that found content of formaldehyde ranging from 0.66 to 1.35 mg L^{-1} (Burini and Coli, 2004). Acetaldehyde content ranged from 2.27 to 54.88 mg/100 mL of AA (10.22 – 246.96 mg. L^{-1}), with an average of 34.35 mg/ 100 mL of AA. These values were above the maximum allowed by brazilian legislation (<20 mg/ 100 mL of AA) for 75 % of the samples (BRAZIL, 2011). Jeong et al., (2015) found similar content (29.30 mg L^{-1}) for acetaldehyde and lower (0.82 mg L^{-1}) for formaldehyde in whisky [26] and Chung et al found content of acetaldehyde range from 0.58 to 3.59 mg L^{-1} Chung et al., (2015) and Lachenmeier, Sohnius (2008) found values of not detected to 77 mg L^{-1}. Furfural content ranged from not detected to 2.27 mg/100 mL of AA (0 -10.22 mg L^{-1}), with an average of 0.93 mg/ 100 mL of AA. These values were higher to that found by Perestrelo et al., (2022) when analyzing this compound in whiskey, they found a variation from 0.026 to 0.037 mg L^{-1} and similar than those found by Fritzgerold et al., (2000) when analyzing Irish whiskey ranging from not detected to 7.3 mg L^{-1}. 5-HMF content ranged from 1.08 to 32.05 mg/100 mL of AA, with an average of 4.40. Brazilian legislation recommends a maximum of 5 mg/ 100 mL of AA for the sum of furfural and 5-HMF (BRAZIL, 2011), these values found were in accordance with the recommended by the legislation recommendation for all original samples, except for AO4, AO14 and AO15. Acrolein content ranged from not detected to 0.66 mg/100 mL of AA, with an average of 0.18.

On the counterfeit whisky the formaldehyde content ranged from not detected to 1.77 mg/100 mL of AA, with an average of 0.98. The acetaldehyde content ranged from 0.91 to 12.86 mg/100 mL of AA, with an average of 8.86. Furfural content ranged from not detected to 0.76 mg/100 mL of AA, with an average of 0.08. The 5-HMF content ranged from 0.86 to 37.51 mg/100 mL of AA, with an average of 16.91. The acrolein content ranged from not detected to 0.16 mg/100 mL of AA, with an average of 0.01.

Counterfeit samples had lower levels of formaldehyde, acetaldehyde, furfural and acrolein than original samples. 5-HMF content was 3.8 times more elevated in the counterfeit. Although the samples presented acetaldehyde content within the legislation, other parameters such as the minimum content of higher alcohols, present in Table 14, were below the recommended by the brazilian legislation for most samples (BRAZIL, 2011). Counterfeit samples had lower levels of volatile compounds, but the alcoholic strength was like the original samples. According with Lad and collaborators the dilution with other cheap spirits results in higher levels of higher alcohols (Lad, Tiwari, Sharma 2021).

Table 10 - Aldehyde content in whiskey samples in mg/100 mL of AA

Sample	Formaldehyde		Acetaldehyde		Furfural		5-HMF		Acrolein	
	average	DP	average	DP	average	DP	average	DP	average	DP
AF1	**1.31**	0.02	**0.91**	0.04	**ND**	ND	**37.51**	0.48	**ND**	ND
AF2	**1.08**	0.03	**2.59**	0.09	**ND**	ND	**26.78**	1.00	**ND**	ND
AF3	**0.30**	0.00	**2.09**	0.09	**ND**	ND	**30.77**	0.04	**ND**	ND
AF4	**0.75**	0.08	**2.43**	0.16	**ND**	ND	**17.50**	0.24	**0.16**	0.01
AF5	**1.77**	0.11	**3.02**	0.05	**ND**	ND	**15.78**	0.40	**ND**	ND
AF6	**0.28**	0.01	**3.52**	0.06	**ND**	ND	**10.89**	0.43	**ND**	ND
AF7	**1.10**	0.01	**12.86**	0.29	**0.04**	0.02	**9.21**	0.35	**ND**	ND
AF8	**0.68**	0.02	**4.16**	0.05	**ND**	ND	**13.95**	0.14	**ND**	ND
AF9	**0.33**	0.05	**1.84**	0.17	**ND**	ND	**15.75**	0.25	**0.01**	0.002
AF10	**1.73**	0.08	**2.79**	0.11	**0.26**	0.01	**13.41**	0.26	**ND**	ND
AF11	**1.76**	0.14	**2.71**	0.21	**0.76**	0.26	**14.21**	0.54	**ND**	ND
AF12	**1.68**	0.05	**4.37**	0.08	**ND**	ND	**14.71**	0.57	**ND**	ND
AF13	**ND**	ND	**8.18**	0.16	**ND**	ND	**0.86**	0.03	**ND**	ND
AF14	**0.94**	0.005	**2.62**	0.05	**ND**	ND	**15.47**	0.38	**ND**	ND
AO 1	**1.13**	0.03	**17.79**	0.21	**0.69**	0.06	**1.08**	0.25	**0.13**	0.01
AO2	**1.50**	0.04	**47.09**	0.30	**1.31**	0.04	**3.65**	0.09	**ND**	ND
AO3	**3.78**	0.10	**54.88**	1.93	**ND**	ND	**2.47**	0.07	**ND**	ND
AO4	**2.04**	0.11	**43.61**	1.90	**2.27**	0.11	**2.79**	0.16	**0.60**	0.01
AO5	**0.71**	0.02	**17.93**	0.33	**ND**	ND	**2.31**	0.10	**0.46**	0.11
AO6	**2.94**	0.07	**54.98**	0.47	**1.20**	0.07	**2.81**	0.20	**0.66**	0.05
AO7	**1.11**	0.05	**32.69**	1.62	**0.46**	0.01	**1.98**	0.05	**ND**	ND
AO8	**1.04**	0.04	**49.87**	1.54	**0.46**	0.02	**2.51**	0.07	**0.19**	0.02
AO9	**2.27**	0.05	**56.32**	0.71	**0.69**	0.01	**1.68**	0.25	**0.27**	0.03
AO10	**1.38**	0.01	**44.46**	0.20	**1.31**	0.03	**3.09**	0.64	**0.35**	0.01
AO11	**0.69**	0.02	**2.27**	0.04	**0.75**	0.05	**1.16**	0.19	**ND**	ND
AO12	**ND**	ND	**20.10**	0.16	**1.30**	0.05	**2.08**	0.07	**ND**	ND
AO13	**0.97**	0.01	**38.16**	1.57	**1.21**	0.03	**1.68**	0.14	**ND**	ND
AO14	**1.93**	0.13	**31.48**	0.77	**1.69**	0.07	**32.05**	1.65	**ND**	ND
AO15	**ND**	ND	**24.64**	0.50	**0.98**	0.07	**5.87**	0.20	**0.17**	0.01
AO16	**ND**	ND	**13.36**	0.16	**0.54**	0.03	**3.25**	0.03	**ND**	ND

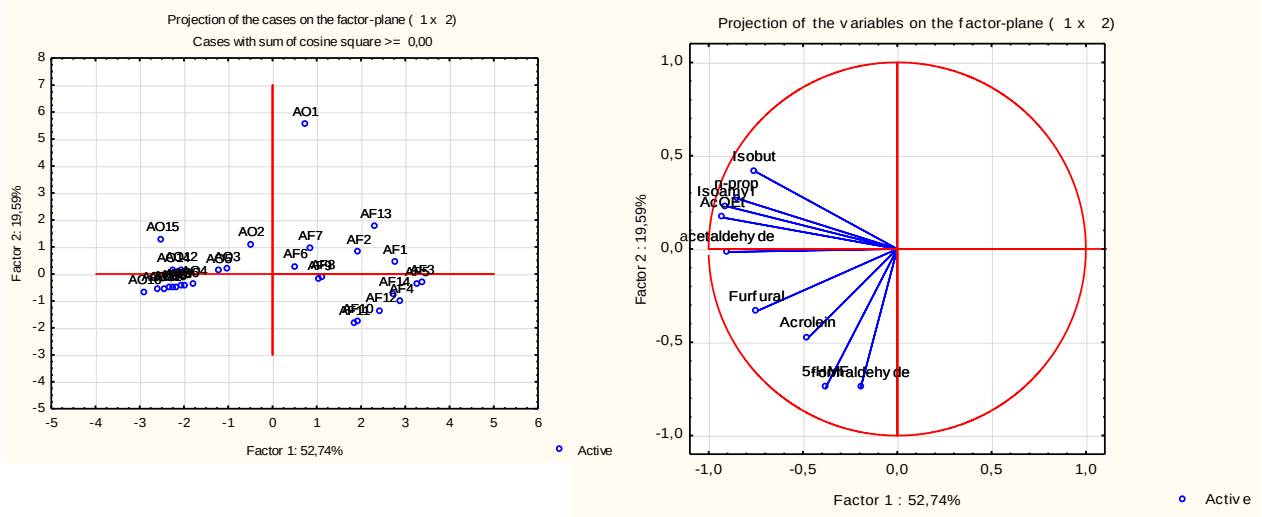
Legend: ND=not detected.

3.3.2 Application of chemometric techniques for differentiation between original and counterfeit whiskeys

In Figure 10, PC1 explains 52.74% of the data and PC2 explains 19.52% of the data, totaling 72.26% of the data variance. Figure 10a shows counterfeits samples on the right, while the original on the left. Samples O1 differ from the other originals. On the other hand, Figure 10b show that original samples have high content of compounds analyzed. What distinguishes counterfeits samples is the absence or low content of most of the analyzed compounds.

The distinction between original and counterfeit samples was also obtained by Teodoro and collaborators used PCA and PLS-DA on data obtained by paper spray mass spectrometry (PS-MS) to distinguish samples scotch whiskey with good results despite the samples being heterogeneous (Teodoro et al., 2017), the same chemometric tools were used by Cantarelli et al., (2015) distinguish whiskey samples of high commercial values. Li et al., (2017) successfully used hierarchical cluster analysis (HCA) to differentiate whiskey samples containing carbonyl compounds (acetaldehyde, formaldehyde, and furfural) (Fang et al., 2017).

Figure 10 - PCA plot of scores (a) and loadings (b) of the whiskey samples analyzed by 2,4-DNPH-HPLC-DAD and -H-SPME-GC-BID methods



3.4 Conclusion

The analytical figures of the 2,4-DNPH-HPLC-DAD method were satisfactorily obtained through selectivity, linearity, recovery, and precision. The evaluation of the homoscedasticity of the variances revealed heteroskedastic data and the coefficients were estimated by the WLS method. Correlation coefficient varied from 0.9904 for furfural to 0.9999 for acetaldehyde. The evaluation of significance coefficients shows that only acrolein had the not significant intercept, for other compounds both coefficients were significant. Recovery of the method ranged from 80.84 to 107.21 % for the compounds, except for acrolein in the lowest concentration and precision values ranged from 0.99 to 3.94 %, except for acrolein at highest concentration.

The application of the PCA allowed the differentiation between the two types of samples and within each group, where originals whiskey samples had a high content of compounds and counterfeits samples had low levels of the investigated analytes.

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3.5 Supplementary material of "Chemometric tool associated to chromatography data to assess the quality and authenticity whiskeys"

Table 11 - Homoscedasticity tests of variances for the 2,4 DNPH-HPLC-DAD method

	Hartley F		
	F calculated	F tabulated	**Results**
Formaldehyde	1095.15	20.8	heteroskedastic
Acetaldehyde	518.93	20.8	heteroskedastic
Furfural	492.60	20.8	heteroskedastic
5-HMF	75.58	20.8	heteroskedastic
Acrolein	2886.74	20.8	heteroskedastic

Table 12 - Values of the regression coefficients calculated by the OLS method

Analytes	**slop**	**standard deviation**	**intercept**	**standard deviation**	**R**
Formaldehyde	103304.20	5860.41	179473.19	76865.48	0.9905
Acetaldehyde	85463.72	1308.14	49633.78	44180.00	0.9993
Furfural	84418.19	4895.95	117151.24	64215.65	0.9901
5-HMF	15167.45	596.47	24440.58	8360.46	0.9962
Acrolein	29916.76	1319.86	2904.47	8655.69	0.9942

Table 13. Precision and recovery values of the 2,4-DNPH-HPLC-DAD method

	Recovery		**Precision**	
	P4	**P6**	**P4**	**P6**
Formaldehyde	84.39	106.40	1.27	1.46
Acetaldehyde	100.44	102.53	1.12	3.85
Furfural	88.40	107.21	3.60	3.94
5-HMF	105.06	103.71	3.35	1.25
Acrolein	80.84	110.74	3.40	8.38

Table 14 - Content of alcohols and ethyl acetate obtained by the SH-SPME-GC-BID method in mg/100 mL of AA

Sample	n-prop	AcOEt	n-but	isobut	isoamyl
AF1	nd	nd	nd	3.39	nd
AF2	0.54	nd	nd	6.49	nd
AF3	nd	nd	nd	nd	nd
AF4	nd	nd	nd	nd	nd
AF5	nd	nd	nd	nd	nd
AF6	26.47	nd	nd	32.09	21.53
AF7	2.7	nd	nd	10.59	4.63
AF8	0.43	nd	nd	3.14	nd
AF9	0.26	nd	nd	1.61	nd
AF10	nd	nd	nd	nd	nd
AF11	nd	nd	nd	nd	nd
AF12	nd	nd	nd	nd	nd
AF13	nd	nd	nd	1.86	nd
AF14	nd	nd	nd	nd	nd
AO 1	25.45	10.8	nd	39.73	16.13
AO2	41.24	42.54	nd	81.25	60.52
AO3	14.37	2.09	nd	23.87	8.93
AO4	34.94	25.01	nd	47.76	55.31
AO5	33.26	58.12	nd	100.22	148.57
AO6	36.41	27.24	nd	57.98	20.47
AO7	42.8	16.91	nd	57.81	36.68
AO8	21.81	47.83	nd	97.56	192.83
AO9	47.47	18.1	nd	64.88	65.36
AO10	30.82	22.47	nd	39.75	27.42
AO11	28.41	15.87	nd	47.17	39.66
AO12	3.28	4.94	nd	18.32	22.21
AO13	28.77	40.2	nd	88.91	179.4
AO14	25.13	62.19	nd	170.94	178.68
AO15	38.49	18.41	nd	58.69	42.36
AO16	27.15	20.43	nd	48.81	25.98

Legend: nd= not detected; n-prop = n-propanol; AcOEt= acetate de etila; n-but = n-butanol; isobut =isobutanol, isoamyl = isoamyl alcohol.

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