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Article

Evaluation of Glycerol Profiles in Sugarcane Spirits (*Cachaças*)

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Um novo método para quantificação de glicerol em aguardentes de cana de açúcar (cachaças) foi proposto baseado na derivatização das amostras com cloreto de benzoíla seguido por extração em fase sólida e análise via cromatografia líquida de alta eficiência com detector de arranjo de diodos. Os limites de detecção e de quantificação para a análise de glicerol são de 0,25 e 0,74 mg L^{-1}, respectivamente. A exatidão é de 97,5%, a precisão de 93,5% e a linearidade r^2 = 0,998 para o intervalo de 0,56 mg L^{-1} a 112,0 mg L^{-1} de glicerol. Experimentos com quarenta e oito amostras de cachaças envelhecidas e não envelhecidas e sete extratos etanólicos de madeira mostraram que o processo de envelhecimento contribui para o aumento da concentração de glicerol. A concentração média de glicerol nas amostras de cachaças envelhecidas foi cerca de 10 vezes maior em relação às das cachaças não envelhecidas.

A new method for glycerol quantification in sugarcane spirits (*cachaças*) is proposed, based on sample derivatization using benzoyl chloride followed by solid-phase extraction and high performance liquid chromatography with diode array detection analysis. The limits of detection and quantification for glycerol analysis are 0.25 mg L^{-1} and 0.74 mg L^{-1}, respectively. The accuracy is of 97.5%, the precision 93.5% and the linearity r^2 = 0.998 in the range of 0.56 mg L^{-1} to 112.0 mg $L^{-1}$of glycerol. Experiments with forty eight samples of aged and non-aged *cachaças* and seven alcoholic extracts of wood showed that the aging process contributes to increase the glycerol concentration. The average concentration of glycerol in aged *cachaças* were about 10-fold higher than in non-aged *cachaças*.

Keywords: glycerol analysis, sugarcane spirits, alcoholic wooden extracts

Introduction

Cachaça, the typical Brazilian sugarcane spirit, is the alcoholic beverage obtained from distillation of fermented sugarcane juice. It has an annual production around two billion liters, from which about 1% is exported.[1-3] The spirit is appreciated for its aroma and flavor,[4] that are derived from fermentation, distillation and, particularly, from aging in wooden casks. Next to reactions occurring between species already present in the spirit, various other chemical compounds are extracted from the wood into the beverage, including aldehydes,[4] organic acids,[5] esters,[6] sugars,[7] coumarins and phenolic compounds.[8] The current Brazilian legislation allows the addition of sugars, expressed as sucrose, up to 30 g L^{-1}, being mandatory to inform on the label when the concentration is equal or higher than 6 g L^{-1}.[9]

The presence of glycerol in wines and its contribution to the sweet taste and the viscosity are well known.[10,11] Glycerol is the second most abundant alcohol and is produced by the yeasts cells during sugar fermentation as it maintains the osmoregulation and the redox balance of the yeasts cells.[12] Although the distillation process, glycerol (boiling point of 290 °C) is still present in the spirits such as the *cachaça* and the distilled of grape marc (*bagaceira*), however, at lower concentrations than those reported for wines.[13]

In many countries, the concentration of glycerol is considered a marker of wine and other beverages, since this compound contributes positively to the sensorial properties of the beverage.[14] The glycerol addition is not a common practice among Brazilian producers of *cachaças*, nevertheless, glycerol profiles for a significant number of *cachaças* have

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not been reported in the literature hitherto and knowledge thereof could be useful to prevent the practice of glycerol addition.[15-17] Therefore, the aim of this work was to investigate glycerol profiles in a selected group of sugarcane spirits (non-aged and aged in different wooden casks) and alcoholic extracts of wood species used for casks by application of a new methodology which takes advantage of glycerol derivatization using benzoyl chloride followed by solid-phase extraction and high performance liquid chromatography with diode array detection (HPLC-DAD) analysis.

Experimental

Materials and methods

Reagents

Analytical standards: glycerol was obtained from Sigma-Aldrich (USA), nonanoic acid was obtained from Acros Organics (USA), tetradecanoic and hexadecanoic acids were purchased from Fluka (UK), succinic, decanoic and dodecanoic acids as well as *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were obtained from Sigma-Aldrich (USA). Acetonitrile, ethanol, 2-propanol (J. T. Baker, USA) and dichloromethane (Tedia, USA), were all of HPLC grade. Water was deionized using a Milli-Q system (Millipore, USA). Benzoyl chloride (p.a. ≥ 99.5%) was purchased from Fluka (UK).

Samples

The 48 samples of sugarcane spirits (*cachaças*) studied in this work, all of them free of added sugar and distilled in pot stills (alembics), were provided by producers from various regions throughout Brazil. In order to evaluate the effects of aging on glycerol concentrations, seven alcoholic extracts of wood species used for casks production were analyzed. The extracts were prepared according to Silva *et al*.,[6] using as blank, a non-aged sugarcane spirit sample, unsweetened and with a non-detectable glycerol concentration, provided by Industrias Müller de Bebidas (Pirassununga, Brazil). Different kinds of wood species were used in the preparation of the alcoholic extracts: two samples of oak, provided by Prof John Piggot from the Department of Bioscience at Strathclyde University (Glasgow, Scotland), a sample of amburana (*Amburanacearensis*), a sample of jatobá (*Hymenaeacourbaril*), two samples of jequitibá branco (*Carinianaestrellensis*) and a sample of amendoim (*Pterogyne* sp.), provided by Francisco Antonio Rocco Lahr from the Laboratório de Madeiras e Estruturas de Madeiras da Universidade de São Paulo (São Carlos, Brazil). These wood species are commonly used for casks in which aging of *cachaças* occurs.

Glycerol quantifications

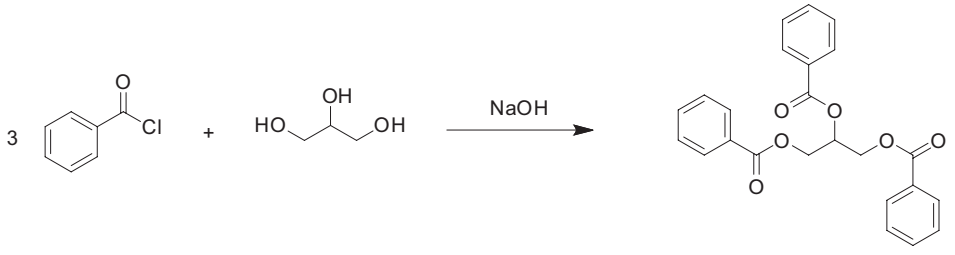
The method used for glycerol quantification was adapted from Miyagi *et al*.[18] for sugar analysis. It involves a derivatization procedure applying benzoyl chloride, followed by solid-phase extraction with C18 cartridges and HPLC-DAD analyses.

Derivatization of samples

The glycerol standard stock solution was prepared by dissolving 28 mg of glycerol in 50 mL of deionized water. In order to avoid ethanol interference during the derivatization procedure, 5.0 mL of sugarcane spirits were dried under nitrogen and then dissolved in 3.0 mL of deionized water. In 2 mL polypropylene flasks were added: 600 µL of re-dissolved samples (or standard solution), 180 µL of monobasic potassium phosphate (1.0 mol L^{-1}), 45 µL of undiluted benzoyl chloride and 135 µL of NaOH (8.0 mol L^{-1}). The reaction between glycerol and benzoyl chloride is performed as illustrated in Scheme 1. The flasks were agitated during 70 s in a vortex system (Lab Dancer Vortex Mixer, IKA, Germany) and then left for 20 min in order for the reaction to take place. Next, 135 µL of H_3PO_4 16% was added. All processes were performed at room temperature (25 ± 2 °C).

Solid-phase extractions

SampliQ C18 cartridges, 500 mg/6 mL (Agilent, USA), were used with a Visiprep SPE negative pressure system (Supelco, USA) in order to clean up the samples.



Scheme 1. Glycerol benzoylation reaction (Schotten-Baumann conditions).[19]

To remove bubbles, the SPE cartridges were rinsed with 10.0 mL of acetonitrile and 10.0 mL of deionized water. After the derivatization reaction, the entire contents of the polypropylene flasks were quantitatively transferred to the SPE-cartridge, kept in the SPE vacuum system until dryness. Next, the cartridges were rinsed with 10.0 mL of deionized water for the clean-up of the sample. The residue of interest retained in the cartridges was extracted with 5.0 mL of dichloromethane, then the samples were dried under nitrogen and afterwards dissolved with 2.4 mL of 2-propanol.

HPLC analyses

The samples were filtered through a 0.45-μm Teflon membrane (PALL, USA) prior to the chromatographic analyses, which were performed in a HPLC (Shimadzu, Japan), Model-10AD, equipped with an injector (Shimadzu, Japan) (20 μL loop) and a UV-Vis photodiode array spectrophotometric detector (SPD-M6A, Shimadzu, Japan). The column was a reversed-phase Shim-pack C18 (Shimadzu, Japan) with a 5-μm particle bed, measuring 250 × 2 mm i.d. coupled to a GVP-ODS guard column measuring 50 × 2 mm i.d. (Shimadzu, Japan).

The mobile phase was composed of a mixture of water and acetonitrile and the elution was done in the gradient mode (0.1-15.0 min: 40% of acetonitrile, 15.0-20.0 min: 80% of acetonitrile, 20.0-27.0 min: 80%, 27.0-32.0 min: 40% of acetonitrile, 32.0-40.0 min: 40% of acetonitrile) with a flow rate of 0.4 mL min^{-1}. The diode array detector was configured to monitor the absorbance at 230 nm.

LC-MS/MS analyses

For control of the benzoyl chloride derivatization procedure, glycerol standards and a couple of sugarcane spirits samples were analyzed by liquid chromatography with mass spectrometry in tandem detection (LC-MS/MS). The chromatographic separation was carried out using the same apparatus described above. The electrospray mass spectra were collected in the positive ion mode for the identification of the target compounds using a Bruker Daltonics ion trap mass spectrometer, model Esquire 4000 (Bremen, Germany).

Fatty acids quantifications

The method used for the quantification of four fatty acids (decanoic, dodecanoic, tetradecanoic and hexadecanoic) in the seven alcoholic extracts of woods was described by Serafim *et al.*.[5] It involved a derivatization procedure with MSTFA, followed by gas chromatography with flame ionization detector (GC-FID) analyses.

Derivatization of samples

Aliquots of 20 mL of samples of *cachaça* and alcoholic wood extracts were evaporated to dryness and the subsequent derivatization reaction was performed by adding 200 μL of the derivatizing solution, which was composed of 100 μL of MSTFA and 100 μL of nonanoic acid (internal standard, 100 mg L^{-1}) in acetonitrile solution. The silylated derivatives were then analyzed by gas chromatography.

GC-FID analyses

The fatty acids analyses were done by injecting 1 μL of the samples in a Hewlett-Packard 5890 model gas chromatograph equipped with a flame ionization detector using a capillary column DB-5 (5%-phenyl-methylpolysiloxane) with dimensions of 50 m × 0.20 mm × 0.33 μm. The oven temperature program used was 60 °C (2 min) to 100 °C at a programming rate of 25 °C min^{-1} and raised at 10 °C min^{-1} increments from 100 to 300 °C (5 min), using split mode (1:15). The organic acids were identified by an authentic standard addition method and quantified using standard calibration curves. The limits of detection and quantification (LOD and LOQ, respectively) for the fatty acids were: LOD = 225 μg L^{-1} and LOQ = 750 μg L^{-1} for decanoic acid, LOD = 45 μg L^{-1} and LOQ = 150 μg L^{-1} for dodecanoic acid, LOD = 45 μg L^{-1} and LOQ = 150 μg L^{-1} for tetradecanoic acid and LOD = 45 μg L^{-1} and LOQ = 150 μg L^{-1} for hexadecanoic acid.[5]

Results and Discussion

Chromatographic analyses of glycerol and fatty acids in sugarcane spirits

Figure 1 shows typical chromatograms of a blank solution, a glycerol standard and samples of non-aged and aged sugarcane spirits (*cachaças*).

The aged *cachaça* (Figure 1d) exhibited a more complex chromatogram than the non-aged sample, as expected. Indeed, during aging, the beverage extracts from the wooden casks compounds like furanic, benzoic and cinnamic aldehydes,[8] monosaccharide and polyols[20] that have hydroxyl groups susceptible to derivatization.

In order to evaluate the derivatization reaction with benzoyl chloride, a benzoylated glycerol standard solution (20.0 mg L^{-1}) was analyzed by LC-MS. Figure 2 shows

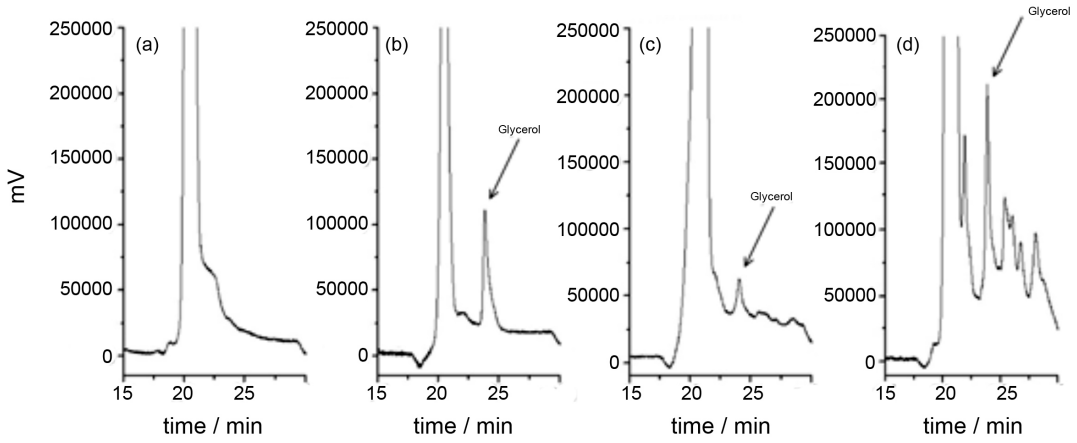


Figure 1. Typical chromatograms of (a) an alcoholic solution (40% v/v); (b) a glycerol standard 11.2 mg L^{-1}; (c) non-aged *cachaça* and (d) aged *cachaça*. The detection wavelength was 230 nm. The arrow points to the glycerol peak.

the extracted ion chromatogram (*m/z* 427.1 ± 0.5). The average mass spectrum of glycerol, which is consistent with the proposed structure for the derivatized molecule, was obtained in 24.5 min, which correspond to the retention time of glycerol derivative. In the insert of Figure 2, the benzoylated glycerol with its fragmentation profile is presented.

The expected *quasi*-molecular ion should be at *m/z* 404.1, although the observed *quasi*-molecular ion was at *m/z* 427.1, as a result of adduct formation with a sodium ion. The other masses observed in the mass spectrum (*m/z* 283.1 and *m/z* 105.0) were attributed to fragments of benzoylated glycerol (see the insert in Figure 2). In order to confirm the presence of glycerol in sugarcane spirits, MS/MS analysis monitoring the *m/z* 427.1 → *m/z* 283.1 transition was performed. The LC-MS/MS analyses of the glycerol standard showed no evidence of other glycerol forms (esterification of only one or two hydroxyl groups

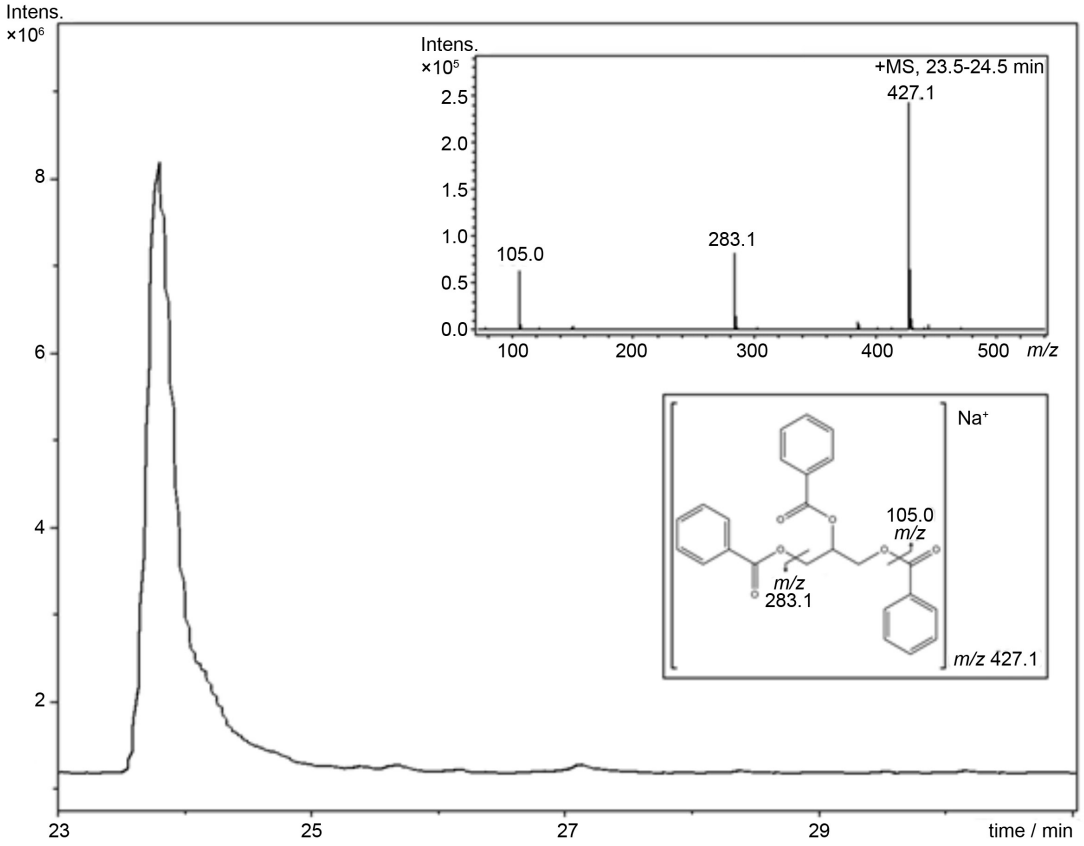


Figure 2. Extracted ion chromatogram (*m/z* 427.1 ± 0.5) of a benzoylated glycerol standard solution (20.0 mg L^{-1}), the average mass spectrum of the glycerol peak and the derivatized glycerol molecule with fragments observed in the mass spectrum.

of glycerol molecule), hence, the reaction between glycerol and benzoyl chloride takes place in the ratio 1:3 and is quantitative. Certainly, glycerol could be quantified using the LC-MS/MS methodology, however due to the practical advantages (more available and less expensive equipment), the HPLC-DAD technique was preferred for such purpose.

Validation of the method

The range of linearity of the DAD (230 nm) response was checked by establishing calibration curves in the range from 0.56 to 112.0 mg L^{-1} of a glycerol standard which had been previously derivatized. The curve exhibited a linear correlation coefficient (r^2) of 0.998. LOD and LOQ of glycerol were 0.25 mg L^{-1} and 0.74 mg L^{-1}, respectively. The average recovery values ranged from 93.9% to 102.3%, whereas the precision and the accuracy for the method was 93.5% and 97.5% respectively.[21]

Quantitative analyses of glycerol and of fatty acids

This glycerol methodology was applied in an aged and non-aged commercial *cachaças* set. Table S1, in the Supplementary Information (SI) section, summarizes the average concentrations of glycerol and fatty acids in the 48 samples of *cachaças*. The contents of glycerol and fatty acids obtained for these samples were quite varied. The fermentation process, probably dominated by different yeasts strains, certainly would influence the production of glycerol before the aging process and, therefore, would account to the differences observed in glycerol contents of the spirits. Table 1 summarizes the concentration profile for glycerol and total fatty acids organized as aged and non-aged *cachaças*.

It was observed that the average glycerol and fatty acids concentrations in the aged sugarcane spirits were respectively about 10 and 4-fold higher than in the non-aged samples (Table 1). It is well known that the aging process accounts for substantial changes in the chemical compositions and the sensory properties of distilled spirits. The concentrations of total aldehydes, organic acids, esters and carbohydrates increase during aging as result of the extraction of components from the wood or due to degradation products of macromolecules of the wood (cellulose, hemicellulose and lignin), as well from reactions between components of the distillate itself and compounds that are originated from the wooden casks. An analogous behavior may well account for the increase of glycerol and fatty acids concentrations in aged samples. The cell walls of woods are composed of diverse compounds such as fats, resins and triglycerides that may be extracted and/or hydrolyzed, leading to formation of glycerol and fatty acids.[22]

Table 1. Minimum, maximum, average and median concentrations of glycerol and total fatty acids (mg L^{-1}), in the aged and non-aged samples of sugarcane spirits (*cachaças*)

	Aged[a]		Non-aged[b]	
	Glycerol	Fatty acids	Glycerol	Fatty acids
Minimum	< LOQ	1.61	< LOD	0.86
Maximum	66	51.3	5.3	7.45
Average	17.5	13.3	1.75	3.63
Median	12.5	10.6	1.4	3.2

[a]34 samples; [b]14 samples.

The samples stored in stainless steel reservoirs, which did not have contact with a wooden cask, presented (Tables 1 and 1S) the lower median concentrations of glycerol (1.4 mg L^{-1}) and fatty acids (3.2 mg L^{-1}). The median concentrations of glycerol (12.5 mg L^{-1}) and total fatty acids (10.6 mg L^{-1}) were higher for the samples of aged *cachaças*.

To get more insight into this subject, glycerol and the fatty acids more abundant in the triglycerides[23] (decanoic, dodecanoic, tetradecanoic and hexadecanoic) were quantified in seven alcoholic extracts of the woods commonly used in the production of casks. The same *cachaça* was used as a blank, to avoid the influence of the process in the glycerol concentration. Table 2 presents the concentrations of glycerol and fatty acids (mg L^{-1}) and the molar ratios between them in the alcoholic extracts of woods.

The quantitative data for glycerol and fatty acids in the *cachaça* wood extracts are consistent with the results obtained for glycerol quantifications in the 48 samples of *cachaças*. The concentration of glycerol and fatty acids expressively increased after the extraction process (Table 2). Taking into account the hydrolysis of triglycerides, for the same sample, the molar ratio between glycerol and fatty acids would, in principle, be 3 to 1. However, such correlation has not been observed neither in the samples nor in the alcoholic extracts of woods. This lack of correlation suggests that fatty acids and glycerol may originate not only from triglycerides hydrolysis, but also from other sources, such as degradation of resins, fats, lignin, cellulose and other carbohydrates present in the woods.[22,24] Furthermore, the high ethanol concentrations in the *cachaças* could promote triglycerides ethanolysis, thereby leading to the respective esters of the free fatty acids,[25] which would account for observed reduction in the molar ratio between fatty acids and glycerol.

Although more and better specifically planned experiments would be necessary for drawing a picture,

Table 2. Average concentrations of glycerol,[a] fatty acids (mg L^{-1}) and the molar ratios between total acids and glycerol in alcoholic wood extracts

Wood	Glycerol[b]	Decanoic acid[c]	Dodecanoic acid[d]	Tetradecanoic acid[e]	Hexadecanoic acid[f]	Molar ratio: acids/ glycerol
Cachaça (blank)	< LOD	< LOD	$0.22 \pm 3.0 \times 10^{-2}$	$0.20 \pm 2.0 \times 10^{-2}$	$0.44 \pm 1.0 \times 10^{-2}$	–
Oak A	15 ± 0.46	6.5 ± 0.17	$0.54 \pm 1.0 \times 10^{-2}$	1.0 ± 0.31	$0.54 \pm 2.0 \times 10^{-2}$	0.34
Oak B	4.2 ± 0.40	$9.5 \pm 5.0 \times 10^{-2}$	$0.53 \pm 7.0 \times 10^{-2}$	$1.2 \pm 2.0 \times 10^{-2}$	$1.1 \pm 2.0 \times 10^{-2}$	1.75
Amburana	42 ± 0.11	$24 \pm 3.0 \times 10^{-2}$	$0.59 \pm 1.0 \times 10^{-2}$	$6.5 \pm 1.0 \times 10^{-2}$	$1.2 \pm 2.0 \times 10^{-2}$	0.40
Jatobá	12 ± 0.33	19 ± 0.13	0.60 ± 0.40	2.4 ± 0.34	1.1 ± 0.15	0.98
Jequitibá Br. A	$3.4 \pm 6.0 \times 10^{-2}$	$1.7 \pm 6.0 \times 10^{-2}$	$0.48 \pm 3.0 \times 10^{-2}$	$0.38 \pm 2.0 \times 10^{-2}$	$0.63 \pm 2.0 \times 10^{-2}$	0.56
Jequitibá Br. B	$7.7 \pm 9.7 \times 10^{-2}$	11 ± 0.15	$0.52 \pm 1.0 \times 10^{-2}$	0.89 ± 0.18	1.5 ± 0.20	0.99
Amendoim	4.0 ± 0.18	7.5 ± 0.41	$0.56 \pm 1.0 \times 10^{-2}$	$0.96 \pm 3.0 \times 10^{-2}$	1.0 ± 0.20	1.43

[a]All data are an average of three independent determinations whose agreement is better than 95%; [b]glycerol: MW = 92.09 g mol^{-1}, LOD = 0.25 mg L^{-1}, LOQ = 0.74 mg L^{-1}; [c]decanoic acid: MW = 172.26 g mol^{-1}, LOD = 225 μg L^{-1}, LOQ = 750 μg L^{-1}; [d]dodecanoic acid: MW = 200.32 g mol^{-1}, LOD = 45 μg L^{-1}, LOQ = 150 μg L^{-1}; [e]tetradecanoic acid: MW = 228.37 g mol^{-1}, LOD = 45 μg L^{-1}, LOQ = 150 μg L^{-1}; [f]hexadecanoic acid: MW = 256.42 g mol^{-1}, LOD = 45 μg L^{-1}, LOQ = 150 μg L^{-1}.

this preliminary collected data would suggest that the concentrations of glycerol in the samples of aged *cachaça* varied according to the kind of wood used in the casks during the spirits storage period.

Conclusions

A methodology based on glycerol derivatization using benzoyl chloride followed by solid-phase extraction and subsequent HPLC-DAD analysis has been successfully applied for the quantification of glycerol in sugarcane spirits (*cachaças*). High sensibility (LOD = 0.25 mg L^{-1}) and absence of matrix interferences, due to the SPE extraction and chromatographic separation were observed in comparison to previously described amperometric and potentiometric methods.[13,17] The proposed methodology as described or alternatively using MS/MS detector, could, on principle, be extended to fuel ethanol, spirits in general and others alcoholic beverages. It has been observed that glycerol might be present in different types of *cachaças*, while its average concentrations are unequivocally higher in aged spirits.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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