

Electrospray Ionization Mass Spectrometry Fingerprinting of Brazilian Artisan Cachaça Aged in Different Wood Casks

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We have investigated the capability of direct infusion electrospray ionization mass spectrometry in the negative ion mode, ESI(–)-MS, to differentiate representative samples of artisan cachaça, a Brazilian sugar cane distillate of large production, aged in four different types of wood casks: amburana (*Amburana cearensis*), jequitibá (*Cariniana estrellensis*), balm (*Myroxylon peruiferum*), and oak (*Quercus rubra*). The ESI(–)-MS were found to be very characteristic, showing sets of diagnostic ions for each of the four types of samples: amburana (m/z 271, 313, 377), jequitibá (m/z 143, 171, 255), balm (m/z 137, 269, 283, 297), and oak (m/z 197, 301, 307). Furthermore, principal component (PCA) and hierarchical cluster analysis (HCA), applied to the ESI(–)-MS data, divided these samples into four definite categories. The influence of the aging time on the ESI(–)-MS fingerprints of the cachaça samples stored in oak casks was also established. An inversion in the relative intensity of the diagnostic ions of m/z 307 and 301 is detected in the ESI(–)-MS as the aging time increased from 1 to 2 years. The chemical structures of the major cachaça components were proposed on the basis of the following: (a) the comparison of the ESI(–)-MS/MS of the diagnostic anions with those of the authentic anions or (b) the interpretation of the fragmentation patterns of the previously unknown diagnostic anions. Hence, direct infusion ESI(–)-MS allows not only a rapid, simple, and accurate way to distinguish among cachaça samples stored in different wood casks but also monitoring changes in their chemical composition according to the aging time.

KEYWORDS: Electrospray ionization; mass spectrometry; fingerprinting; aged cachaça samples; wood casks; principal component analysis; hierarchical cluster analysis

INTRODUCTION

Cachaça, the most typical Brazilian alcoholic beverage, is produced from the fermentation of sugar cane (*Saccharum officinarum*) must. The production of the highest-quality artisan cachaça, aged during at least 1.5 years in wood casks previously to its commercialization, is now close to 180 million liters per year (1). Due to well-known and valuable characteristics, oak casks has been extensively and preferentially employed for the aging process (2–5). In Brazil, however, since no sufficient oak is available, the alternative is to use native woods, such as amburana (*Amburana Cearensis*), (6, 7) jequitibá (*Cariniana estrellensis*), and balm (*Myroxylon peruiferum*) (6). The use of a variety of wood casks causes a predictable variation in the sensorial properties of cachaça, with the production leaning for a local preference. But the lack of a reliable quality control

facilitates adulteration. For instance, to forge cachaça samples aged in oak casks, the better-quality and the most expensive product, Brazilian producers have usually made use of amburana, jequitibá, and balm casks, an illicit practice.

During the aging process, a variety of compounds can be continuously extracted from the wood cask or, alternatively, be slowly formed upon contact with the cask inner surface. The nature and concentration of such compounds in cachaça and other spirits have been systematically related with the type of wood cask used in the aging process and the aging time, respectively (1). For instance, Madrera and co-workers (8), by using high-performance liquid chromatography (HPLC), determined the presence of numerous phenolic compounds at different concentration in several samples of cider brandy aged in casks of both American and French oaks. In a similar study, Gomis and co-workers (9) also verified that the content of monosaccharides in cider samples was noticeably related with the type of wood casks, i.e., of American and French oaks, used during the aging process. Sanza and co-workers (10) observed

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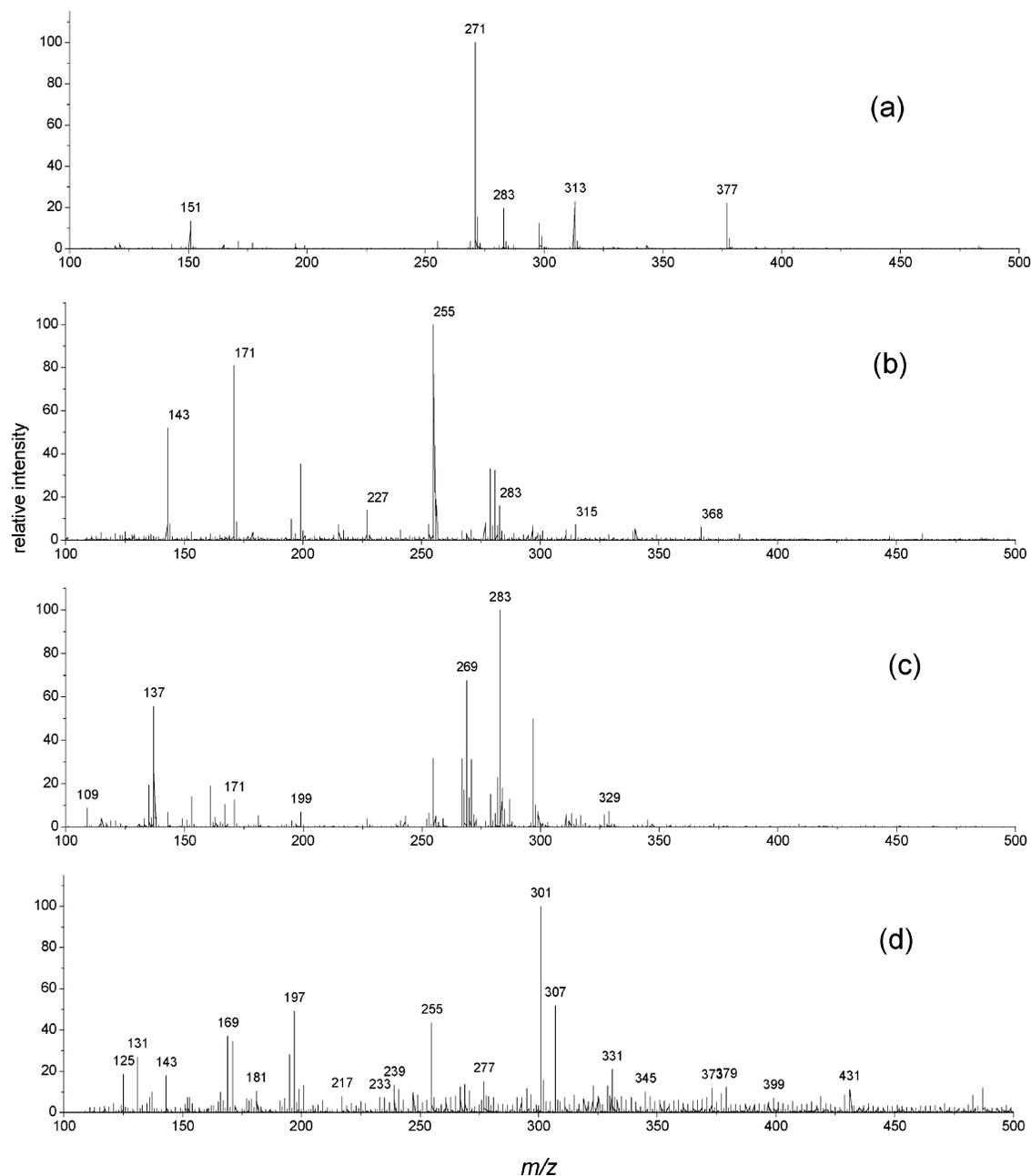


Figure 1. ESI(-)-MS of typical cachaça samples stored in casks of (a) amburana (a1); (b) jequitibá (j1); (c) balm (b1); and (d) oak (o3). All these samples were aged for a period of 2 years.

Table 1. Major ESI(-)-MS Fingerprint Diagnostic Ions Assigned to Each Group of Cachaça Samples, That Is, Those Aged in Casks of Amburana, Balm, Jequitibá, and Oak

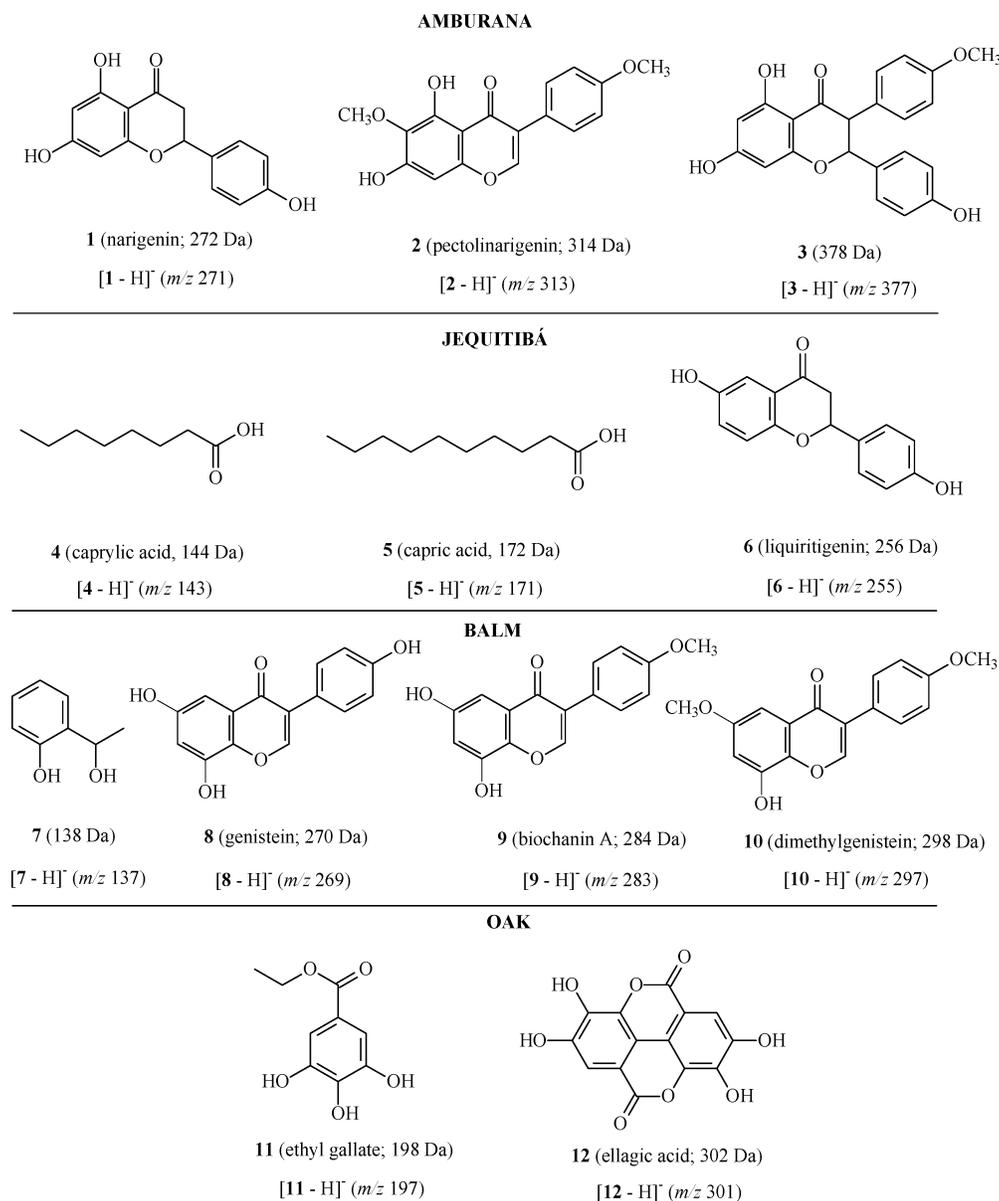
wood cask	diagnostic ions	figure ^a
amburana	271, 313, 377	1a
jequitibá	143, 171, 255	1b
balm	137, 269, 283, 297	1c
oak	197, 301, 307	1d

^a Typical ESI(-)-MS of the samples aged in each wood cask.

that the concentration of anthocyanins, determined by using HPLC, in samples of aged wine was dependent on the type of wood, that is, American, French, and Hungarian oaks, used to fabricate the casks. Fernández de Simón and co-workers (11) also verified, for samples of aged wines, that casks made of

four different oak species (*Quercus robur*, *Quercus petraea*, *Quercus pyrenaica*, and *Quercus faginea*) directly affected the distribution and content of assorted volatile organic compounds (VOC), which were determined by using gas chromatography–mass spectrometry (GC-MS).

Analogous studies have been reported for cachaça samples. For instance, Campos and co-workers (6) evaluated the effect of thermal treatment on casks of amburana and balm (Brazilian native woods) designed for cachaça aging. The authors observed a significant increase in the phenolic aldehydes content in samples aged in casks submitted to such treatment. Faria and co-workers (12) compared cachaça samples aged in several casks of Brazilian native woods and oak by evaluating their sensorial properties and determining their UV absorption spectra, total phenolic compounds content, and color intensity. These parameters, adequately handled by the PCA method, allowed the

Scheme 1. Chemical Structures Proposed for the Major Compounds Detected in the Cachaça Samples Aged in the Different Wood Casks^a

^a The *m/z* of the diagnostic anions (the deprotonated form of the main compounds 1–13 detected by ESI(–)MS) are quoted under the structures).

differentiation among these samples depending on the wood cask used during the aging process.

Direct infusion electrospray ionization mass spectrometry (ESI-MS), (13, 14) due to its highly convenient features, has been consistently used to accomplish fast fingerprints of numerous samples such as beer (15), wine (16, 17), propolis (18), whisky (19), crude petroleum oils (20), vegetable oils (21, 22), and plant extracts (23, 24). Therefore, we describe herein the use of direct infusion electrospray ionization in the negative ion mode, ESI(–)MS, to discriminate among samples of Brazilian artisan cachaça aged in casks of amburana (*Amburana cearensis*), jequitibá (*Cariniana estrellensis*), balm (*Myroxylon peruiferum*), and oak (*Quercus rubra*). The wood casks of balm, amburana, and jequitibá are the most used by the Brazilian producers, whereas oak is usually employed to produce the finest product. The ESI(–)MS data are handled by the PCA and HCA statistical methods to verify if these samples can be split into distinct clusters. The influence of aging on the samples fingerprint is also investigated.

MATERIALS AND METHODS

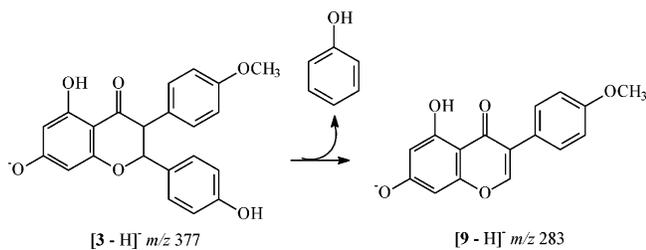
Samples Description. The 34 samples of Brazilian artisan cachaça, produced by using copper distillers and aged in different wood casks, were supplied directly by several qualified producers. The following wood casks were used: amburana (samples a1–a9), balm (b1–b7), jequitibá (j1–j9), and oak (o1–o9). Aging time for the samples stored in casks of amburana, balm, and jequitibá varied from 1.5 to 2 years. To investigate the influence of aging time on the ESI(–)MS fingerprints, the following samples, stored in oak casks, were collected: o1 and o2 (1 year); o3 and o4 (2 years); o5 (3 years); o6 (4 years); and o7–o9 (5 years).

Mass Spectrometry Procedure. Mass spectra were acquired by using a quadrupole/time-of-flight (Q-TOF) mass spectrometer (Micro-mass, Manchester, U.K.). General conditions were as follows: source temperature of 80 °C, capillary voltage of 2.1 kV, and cone voltage of 40 V. Prior to the ESI-MS analysis, 250 μ L of an aqueous solution of 0.1% ammonium hydroxide (v/v) was added to 1.00 mL of each sample and the mixture vigorously stirred for 15 s. Sample introduction was performed by using a syringe pump (Harvard Apparatus, Pump 11) at

Table 2. Main Fragments Arising from the Dissociation of the Diagnostic Anions Detected in the ESI(-)-MS of the Cachaça Samples

wood cask	diagnostic anion (m/z) ^a	main fragment ions ^b	ref ^c
amburana	[1 - H] ⁻ (271)	177, 165, 151, 119, 107, 93	42
	[2 - H] ⁻ (313)	298, 283	43
	[3 - H] ⁻ (377)	283	
jequitibá	[4 - H] ⁻ (143)	99	44
	[5 - H] ⁻ (171)	127	44
	[6 - H] ⁻ (255)	149, 135, 119, 91	45
balm	[7 - H] ⁻ (137)	135, 109, 108, 93, 91	
	[8 - H] ⁻ (269)	241, 225, 213, 135, 133, 91	46
	[9 - H] ⁻ (283)	268, 267, 240, 239, 224, 211	47, 48
	[10 - H] ⁻ (297)	282, 281, 269, 267	49
oak	[11 - H] ⁻ (197)	182, 169, 125, 124	33
	[12 - H] ⁻ (301)	300, 284, 229, 201, 173, 145	50, 51
	unidentified (307)	263, 261, 235, 233, 217, 215	

^a The chemical structures proposed for the corresponding neutral compounds (1–12) are shown in Scheme 1. ^b Obtained upon mass selection and fragmentation of the respective diagnostic anions. ^c Only references showing the ESI(-)-MS/MS of the authentic anions with a fragmentation pattern very similar to that observed for the respective diagnostic anions are indicated in this column.

Scheme 2. Proposed Fragmentation Pathways for the Anion of m/z 377 Attributed to Be [3 - H]⁻

a flow rate of 10.0 $\mu\text{L}\cdot\text{min}^{-1}$ and pumped through an uncoated fused-silica capillary. Each analysis required about 60 s.

Mass spectra were acquired by scanning over the 50–500 m/z range. ESI tandem mass spectra were obtained by mass selection of a specific ion in Q1, by using a unitary m/z window, which was then submitted to collision-induced dissociation (CID) with argon in the collision chamber at energies of 15–20 eV. The product-ion MS analysis was accomplished with the orthogonal ToF (time-of-flight) analyzer.

ESI-MS Data Handling and Statistical Treatment. All mass spectra were accumulated over 60 s, centered, aligned, and handled using MassLynx 3.5 software (Waters, Manchester, U.K.). To discard noise signals, only the ions with a relative abundance higher than 5% were included in the final data matrix. Multivariate analyses by principal component analysis (PCA) and hierarchical cluster analysis (HCA) were performed by running the software Matlab, version 6.0. The experi-

mental data were compiled to generate a final matrix of 34 objects (samples) and 143 variables (m/z ratios and relative intensities of detected ions). These data were previously mean-centered and scaled to variance 1, aiming at assuring that all variables contribute equally to the model, independent of the scale in which they were measured. PCA and HCA were used to evaluate the similarity among the cachaça samples by the formation of different groups according to the types of wood casks used during the aging process. Complete linkage method and Euclidean distance were used to generate the dendrogram in the HCA method.

RESULTS AND DISCUSSION

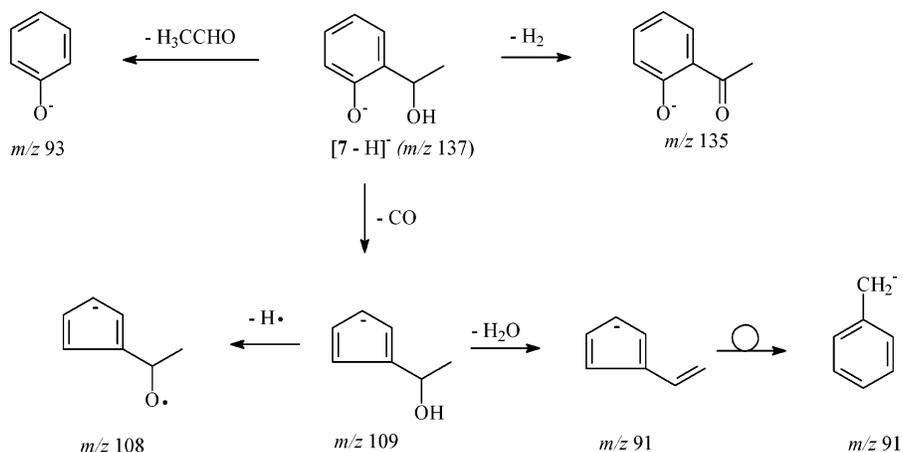
All the cachaça samples were analyzed by ESI-MS in both the positive and negative modes. However, since much cleaner data were obtained from the ESI(-)-MS fingerprints, discussion is restricted to these data.

ESI(-)-MS Fingerprints of Cachaça Samples Aged in Different Wood Casks and the Chemical Structures Proposed for the Major Components. Figure 1 shows the ESI(-)-MS fingerprints of four typical samples of cachaça aged in casks of amburana (Figure 1a), jequitibá (Figure 1b), balm (Figure 1c), and oak (Figure 1d). Cachaça samples from the same group (see Materials and Methods for more details) yielded fingerprints quite similar to those displayed in Figure 1, and Table 1 shows the major diagnostic anions assigned to each group (the selection of the most important anions via the loadings of the principal components will be discussed later in this paper).

The diagnostic anions detected in the ESI(-)-MS (Table 1), most likely deprotonated forms of acidic components, such as carboxylic acids, aliphatic alcohols (25), and phenols, (26–28), characterize each set of samples, which clearly portray diverse chemical composition. These assorted compounds were probably extracted from the wood casks or continuously formed by later and slow reactions during the aging process.

The structural elucidation of all the major components, although not needed for sample classification, was achieved by the interpretation of the ESI(-)-MS/MS of the diagnostic anions and mainly by the comparison with ESI(-)-MS/MS of the authentic anions previously reported in literature. Note that these tandem mass spectra, which normally show a set of product ions arising mainly from losses of small molecules such as H_2O , CO , and CO_2 (Table 2), could be eventually acquired as an additional data dimension for even more selective fingerprint characterization or confirmation.

Thus, the anions of m/z 197 and 301, detected in the ESI(-)-MS of the samples aged in oak casks, were ascribed to

Scheme 3. Proposed Fragmentation Pathways for the Anion of m/z 137 Attributed to Be [7 - H]⁻

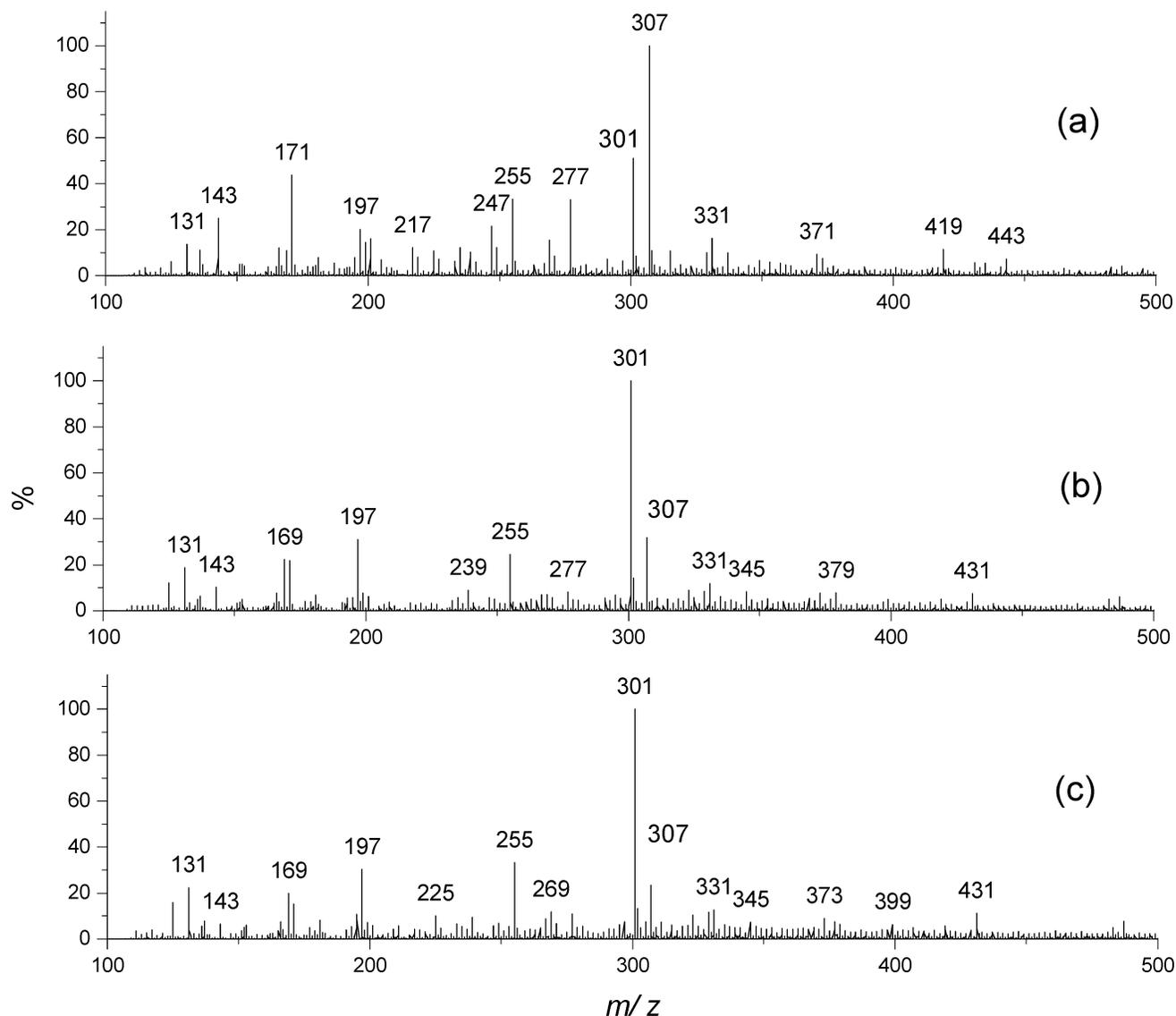


Figure 2. ESI(-)-MS of cachaça samples aged in an oak cask: (a) o1 (1 year); (b) o4 (2 years); (c) o5 (3 years).

be the deprotonated forms of ethyl gallate (**11**) and ellagic acid (**12**), respectively (Scheme 1), which have been usually detected in distillates aged in oak barrels (19, 29–33). This attribution was confirmed by the high similarity between the ESI(-)-MS/MS of both “experimental” anions with those of the corresponding authentic species (Table 2). Furthermore, the high similarity between the tandem mass spectra of the diagnostic anions of m/z 271, 313, 143, 171, 255, 269, 283, and 297 with those of the authentic anions [**1** - H]⁻, [**2** - H]⁻, [**4** - H]⁻, [**5** - H]⁻, [**6** - H]⁻, [**8** - H]⁻, [**9** - H]⁻, [**10** - H]⁻, respectively (Table 2), was thus used to attribute the chemical structures for the flavonoids **1** (narigenin), **2** (pectolarigenin), **6** (liquiritigenin), **8** (genistein), **9** (biochanin A), and **10** (dimethylgenistein) and the carboxylic acids **4** (caprylic acid) and **5** (capric acid) (Scheme 1). Furthermore, the fragmentation behavior of the remaining anions, i.e., m/z 377 and 137, was meticulously analyzed to suggest reliable chemical structures for the corresponding neutral compounds (Scheme 1). For instance, flavonoid **3** (378 Da) was suggested to be formed via a net insertion of a phenol molecule toward the C=C double bond of the benzopyrone moiety of **9** (biochanin A). In fact, the major loss of phenol (94 Da) upon the dissociation of the [**3** - H]⁻ anion (m/z 377) to yield [**9** - H]⁻ of m/z 283 is fully

consistent with the proposed structure for **3**, as indicated in Scheme 2. Similarly, the set of ions arising from the dissociation of the anion [**7** - H]⁻ (m/z 137), as shown in Table 2 and Scheme 3, corroborates the proposed structure for the phenolic compound **7** (Scheme 1). The diagnostic anion of m/z 307 was detected exclusively in the ESI(-)-MS of samples aged in oak casks (Table 1). However, to the best of our knowledge, there are no reports in literature about acidic compounds with a nominal mass of 308 Da detected in alcoholic beverages aged in oak casks or even in oak alcoholic extracts. The dissociation of the ion of m/z 307 (Table 2) yielded mainly the following fragments, formed via consecutive losses of small neutral molecules: 263 (CO₂), 261 (CO₂ + H₂), 235 (CO₂ + CO), 233 (CO₂ + CO + H₂), 217 (CO₂ + CO + H₂O), and 215 (CO₂ + CO + H₂O + H₂). Therefore, based exclusively on this incomplete and vague set of information, a reliable chemical structure for such a compound was not proposed herein. Finally, it must be said that slightly different positional isomers can also be proposed for the chemical structures of compounds **1**–**12**.

Flavonoids, a class of plant secondary metabolites with remarkable antioxidant, anticancer, and anti-inflammatory properties, have been usually detected (including several of the flavonoids proposed to be present in the cachaça samples

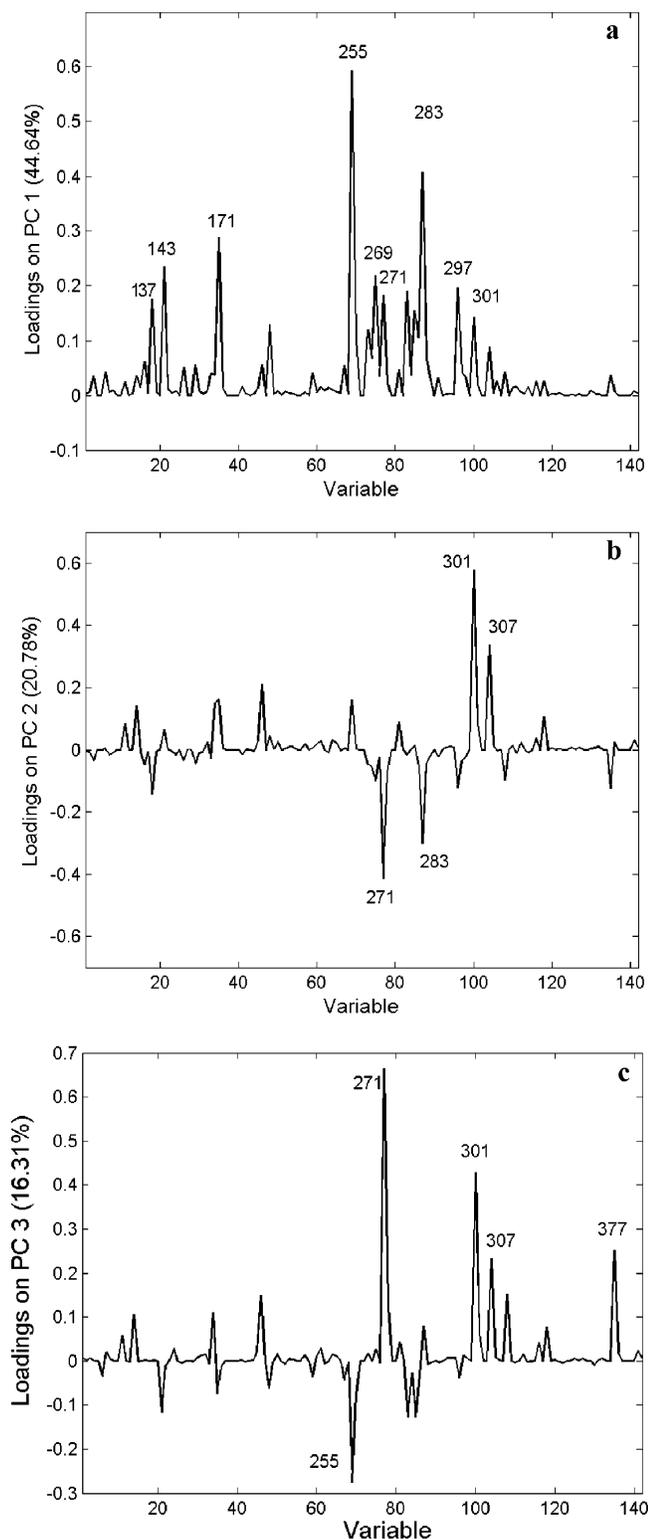


Figure 3. Loadings plots showing the importance of the main variables (m/z of the ions) in the first three PCs: (a) PC1; (b) PC2; (c) PC3. The m/z values and their respective relative intensities were obtained from the ESI(-)-MS of the cachaça samples.

investigated herein) in a number of distillates and their formation have been postulated to be resultant from slow processes that arise during the maturation of the alcoholic beverages in assorted wood barrels (34, 35). Besides flavonoids, other compounds, such as caprylic and capric acids, have also been detected in cachaça samples (28). On the other hand, it is well-known among Brazilian producers that the sensorial properties of

cachaça samples are not significantly altered after a maturation time in jequitibá casks, thus indicating that this wood probably does not significantly modify the chemical composition of the spirit. In fact, the mass spectra of cachaça samples (not shown) recently prepared by using copper distillers (as explained in Materials and Methods) are similar to those of samples aged in jequitibá casks.

Influence of Time on the ESI(-)-MS Fingerprints of Samples Aged in Oak Casks. Figure 2 shows the ESI(-)-MS of three samples stored in the same oak cask and collected after aging times of 1 year (o1), 2 years (o2), and 3 years (o3). Note that as aging time increases the abundance ratio of the ions of m/z 301 (the deprotonated form of ellagic acid 12) and m/z 307 changes drastically. That is, for the young samples this ratio is lower than 1, and for the older samples this ratio becomes higher than 1, whereas the abundances of other diagnostic ions remains approximately the same. The ratio variation from 3 to 5 years of aging is however quite slight. It is interesting to mention that Farias and co-workers (12), during the evaluation of the sensorial quality of cachaça samples aged in oak casks, verified that the product quality improves significantly during the first 2 years but stays with nearly the same quality afterward. This alteration in the sensorial properties of the beverage is probably caused by processes that take place at the interface between the liquid sample and the wood surface, such as the continuous extraction of compounds (including ellagic acid) from the oak wood.

Multivariate Analysis. To test the statistical relevance of ESI(-)-MS fingerprinting to differentiate cachaça samples aged in different wood casks, the data were analyzed first by the PCA method. Examination of the loadings graphics (Figure 3) shows that no more than 13 variables (the diagnostic ions of m/z of 137, 143, 171, 197, 255, 269, 271, 283, 297, 301, 307, 313, and 377) are the most important in explaining the entire set of data consisting of 143 ions (see Materials and Methods). Only the variables with loading values higher than ± 0.15 in at least one of the first three principal components were selected. It was observed that PC1 is mainly correlated with the ions of m/z 255 and 283 (diagnostic for jequitibá and balm casks, respectively), whereas PC2 and PC3 are mostly associated with the ions of m/z 271 and 301 (characteristic of amburana and oak casks, respectively). The scores plot was then built (Figure 4) by using a matrix comprising these 13 most important variables. In this plot, PC1, PC2, and PC3 accounts for 45, 21, and 16% of the total variance, respectively. Four well-defined groups were formed, each one comprising samples aged in casks of amburana, balm, jequitibá, and oak casks.

As the dendrogram of Figure 5 shows, the HCA method when applied to the ESI(-)-MS data results again in four distinct clusters, comprising the samples aged in the different wood casks. In the "oak group", the 1 year aged samples (o1 and o2), likely due to their special characteristics as previously mentioned, seem to be split from the others, hence forming a distinct subgroup. Other subgroups formed by the 2 years (o3 and o4) and 5 years (o7–o9) aged samples are also clearly observed. This typical behavior in the oak group is also observed in the scores plot as the sample o1 (1 year aged) appears to be separated from the main group (Figure 4). Multivariate analysis has usually been applied with success to discriminate among beverage samples submitted to different aging times (9, 36–40). For instance, Piggot and co-workers were succeeded in classifying samples of cognac aged in oak casks by using sensorial analysis data as well as the nonvolatile compounds content (41).

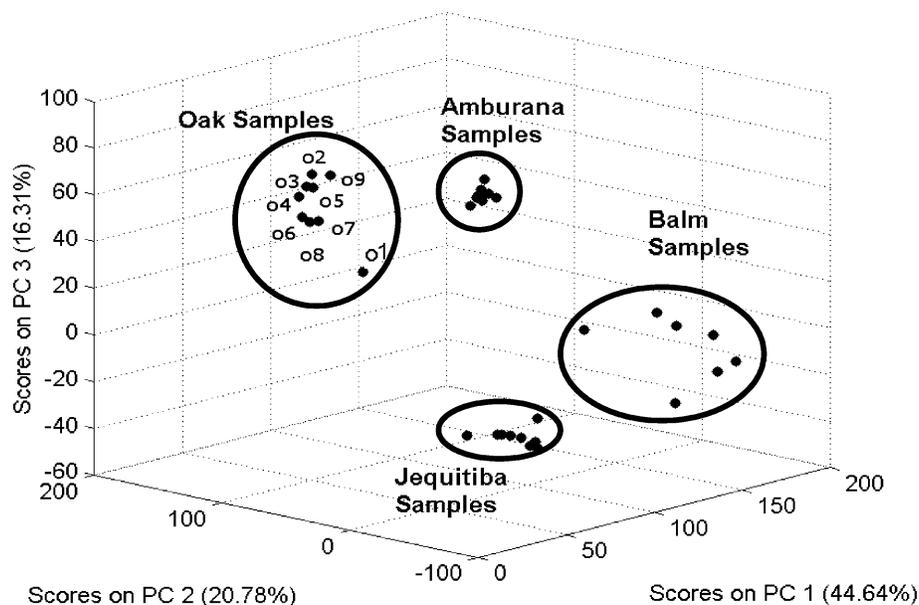


Figure 4. Scores plot for the first three PC of cachaça samples obtained from the data of ESI(-)-MS fingerprints.

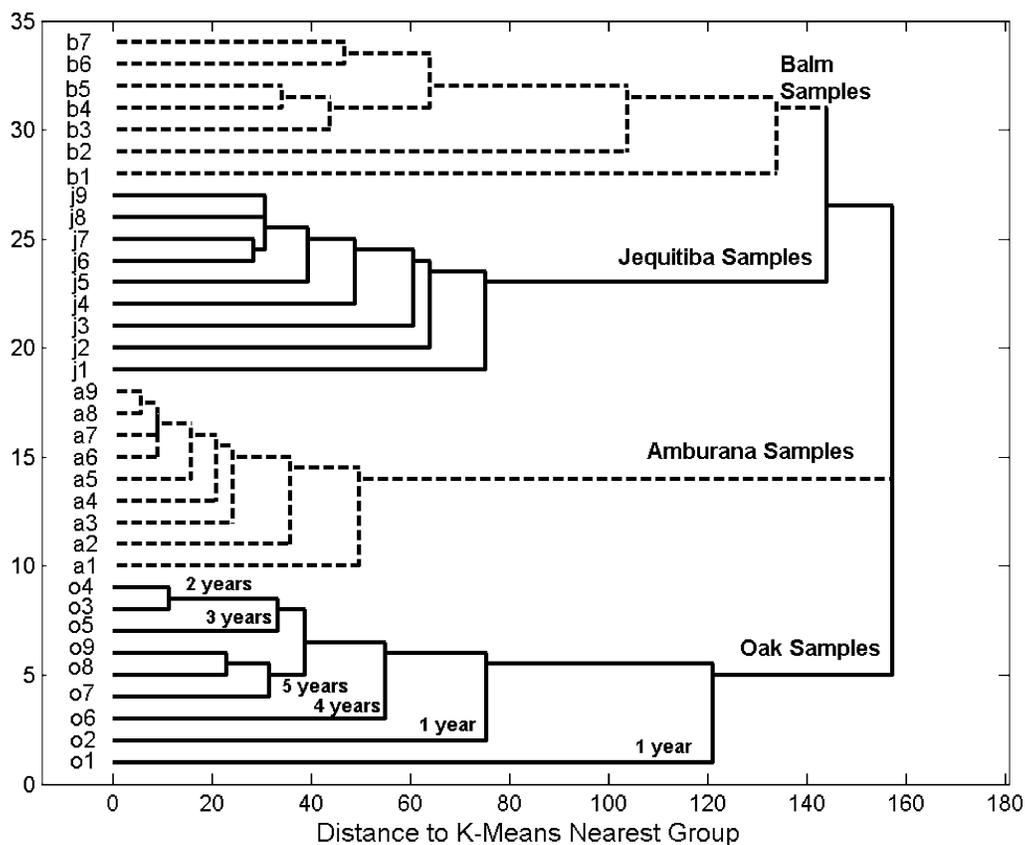


Figure 5. Dendrogram for the cachaça samples obtained from the ESI(-)-MS and built by using unscaled data.

In conclusion, direct infusion ESI(-)-MS fingerprinting, in conjunction with the exploratory statistical methods PCA and HCA, have been shown to provide a simple, rapid, and accurate technique able to differentiate Brazilian artisan cachaça aged in four major different wood casks: amburana, balm, jequitibá, and oak. The effective technique can therefore be used with confidence for quality control and forensic investigations, and the present results also point to its successful use to determine aging. As preliminary results have also pointed out, this technique can detect cachaça adulteration by the addition of

caramel and other substances, a common practice in Brazil. These interesting results are underway in our laboratory.

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