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Quantification of ethanol and identification of other chemical constituents in homemade morula beer using gas chromatography-mass spectrometry (GC-MS)

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Morula (*Sclerocarya birrea*) beer is a seasonal homemade alcoholic beverage made from morula fruits pulp. Unlike the commercial morula alcoholic beverage, the alcohol content of homemade morula beer is not known. The major challenges with homemade alcoholic beverages arise from batch-to-batch differences in product quality and safety due to the variability of raw materials and lack of quality control. Consequently, the alcohol content of alcoholic beverages made by the same individual may differ. In this study, a validated liquid-liquid extraction gas chromatography-mass spectrometry (GC-MS) method is used for the quantification of ethanol and identification of volatiles in homemade morula alcoholic beverage. The analysed samples contained ethanol with concentrations ranging from 0.5 to 5.8% v/v depending on the number of days the sample was fermented. Other volatile compounds that were identified were acetic acid, propanol, propanal, butanal, 3-methyl-1-butanol; 2-methyl-1-butanol, butanoic acid, iso-amyl acetate, butanoic acid ethyl ester, benzyl alcohol, phenyl ethyl alcohol, lactic acid, humulene, decanoic acid ethyl ester and 2-methyl-1-hexadecanoic acid ethyl ester. The volatile compounds were found to decrease over time. These results provides a validated method for the characterisation of homemade alcoholic beverages and information on the quality of morula beer.

Key words: Morula beer, home-made alcoholic beverage, gas chromatography-mass spectrometry (GC-MS), standardisation.

INTRODUCTION

Many wild plant food resources are often eaten across Africa (Kamanula et al., 2022). They are a source of

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vitamins, minerals, amino acids, and trace metals (Mdziniso et al., 2016; Bonić et al., 2013). One such type of plant whose fruits have been part of a supplemental diet in many Southern African countries is Morula (*Sclerocarya birrea*). Morula fruits have been known and consumed for a very long time (Sinthumule and Mzamani, 2019). It occurs naturally in various types of woodland, on sandy soil or occasionally sandy loam though some plant it in their homesteads and farms worldwide (Jinga et al., 2022). The tree itself is used for firewood as well as to make housing/fencing poles and wooden utensils (Tapiwa, 2019). Fruits are used for food, whereas leaves, branches, bark, and roots are used for traditional medicine (Sinthumule and Mzamani, 2019; Mojeremane and Tshwenyego, 2004). The fruit is processed to make jams, candies, and juices, and the kernel is extracted for the oil which is used in the manufacturing of a range of cosmetics (Manyeula et al., 2021; Hilou et al., 2017). Morula fruit juice has higher ascorbic acid than orange juice and the oil extracted is rich in antioxidants and oleic acid which prevent diseases such as cancer and heart disease (Murye et al., 2018; Galvão et al., 2020; Mashau et al., 2022).

A widely known commercial morula-based liqueur, known as 'Amarula cream' with an ethanol content of 17% v/v is produced, bottled, and marketed across the world by Cape Distell Pty Ltd. in Stellenbosch (South Africa) (Sinthumule and Mzamani, 2019). Furthermore, homemade fruit 'beer' called morula beer is common among rural communities. Unlike the commercial type, the alcohol content of the home-made beer' is not known. Morula beer is consumed between December and April every year as this is the time when the fruit ripens. The recipe for making morula beer is adopted from the traditional fermentation recipe of making wines but without the aging process. To make morula beer, the ripe fruits are collected, and then the skins are removed. The pulp, pips, and juice are then mixed with water and mashed thoroughly until the liquid thickens. The stones and then removed from the juice. The juice is then left to ferment for a few days (Shackleton et al., 2012). When fermentation is complete, the mixture is filtered, and the alcoholic beverage is ready for consumption. This recipe is passed from generation to generation and has remained the same.

The production and selling of homemade alcoholic beverages are not regulated in Botswana. The fermentation process in traditional home brews is not regulated and as such the amount of alcohol in these drinks may continue to rise even after filtration and during storage (Tulashie et al., 2017). Traditionally fermented alcoholic beverages contain a lot of volatile organic compounds arising from various chemical reactions taking place during the fermentation process or from raw materials used with ethanol as the main psychoactive component (Mashau et al*., 2022)*. While morula beer has been consumed for a very long time in Botswana, research on the safety, quantification of ethanol and identification of other chemical constituents of morula beer has never been done. Previous studies have been done on the fermentation process and alcohol content of khadi, a local homemade alcoholic brew (Mapitse et al., 2015; Motlhanka et al., 2020). It is worth noting that in these reports the methods were not validated, and some were costly (Isaac-Lam, 2016). Therefore, this research reports a validated method for the quantification of ethanol and identification of other volatile components in morula alcoholic beverages using GC-MS.

MATERIALS AND METHOD

A validated Liquid–Liquid Extraction method for the quantitative analysis of ethanol in the different types of homebrewed alcoholic beverages of Botswana using the Gas Chromatography Flame Ionization Detector method was adopted and used for the purpose of this study (Tsenang et al., 2022).

Chemicals and reagents

Ethanol (99.9%) and Ethyl acetate (99.9%) were purchased from Merck Chemicals (Pty) Ltd (Germiston, South Africa). Sodium chloride (AR) was purchased from Rochelle Chemicals (Johannesburg, South Africa).

Sampling

500 ml of each fermented morula beer samples (1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B, 5A, and 5B), were collected from local brewers in five different villages in Central District near Palapye. The collected samples were collected from five different drinking sites. Figure 1 shows the villages where samples were collected. The samples were collected in glass bottles and transported in cooler boxes containing ice. Upon arrival at the laboratory, they were extracted and kept at 4°C for an average of 3 days.

Preparation of calibration standards

Nine ethanol standard solutions were prepared by mixing 0.25, 0.5, 0.75, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 ml of ethanol with the required volumes of ethyl acetate in separate volumetric flasks to achieve a final solution volume of 10 ml. The resultant concentration of the prepared solutions was 2.5, 5.0, 7.5, 10, 20, 30, 40, 50 and 60% v/v. Three replicate injections of each solution were performed, and their response was recorded.

Liquid-liquid extraction of samples

The samples were extracted by dissolving 1.5 ml of homemade morula beer sample in 0.5 ml of each ethyl acetate. NaCl (0.5 g) was added to each solution to enhance phase separation. Following this, the solutions were vortexed for 1 min and centrifuged for 10 mins at 10000 rpm. The organic layer was removed and transferred into GC vials.

Gas chromatography conditions

The ethanol content in the samples was determined using an

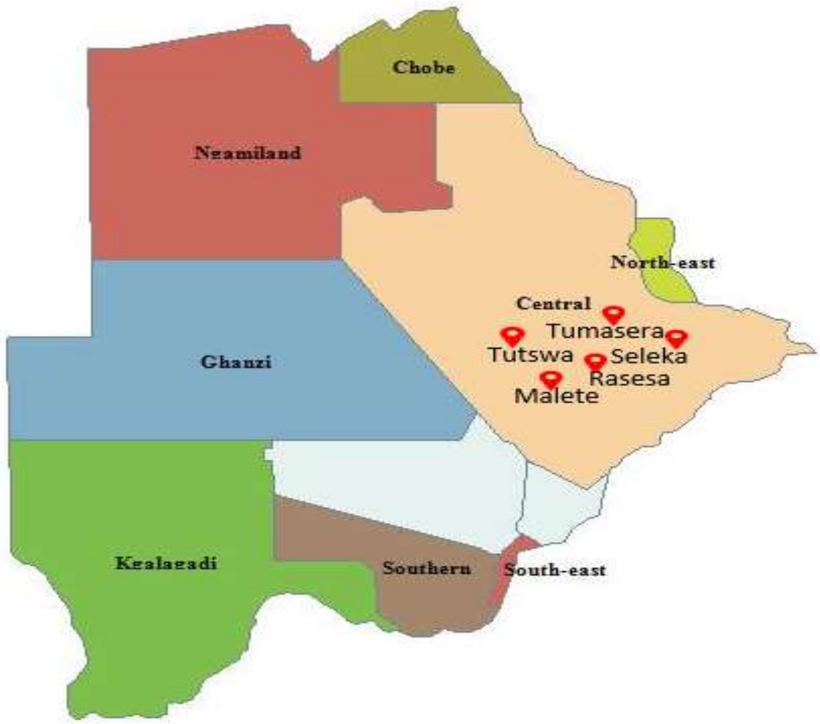

Figure 1. Map of Botswana showing the different sampling sites in the central district.
Source: Tsenang et al., 2022

Agilent 6890N capillary gas chromatograph connected to an Agilent G5977 mass spectrometer and a PAL 3 auto-sampler. The NIST library was used for the identification of compounds (NIST, Mass-spectrometry Data Center, 2017). Separation was achieved on a standard mid-polar DBALC1 capillary column, (30 m length, 0.32 mm i.d, and 0.25 µm film thickness). Sample injections were made in a split mode using a general-purpose split/split-less liner packed with glass wool. The GC oven temperature program was started at 35°C and held for 2.5 min, then increased to 90°C at a rate of 10°C min^{-1} and held for 4 min, and then ramped to 220°C at a rate of 10°C resulting in a total run time of 23 min. Extracted sample volumes of 1 µL were injected into the instrument at a split ratio of 50:1 using helium as a carrier gas. The flow rate of the helium was set at a constant flow of 0.5 ml min -1. The injector and Mass transfer line temperature settings were 220°C and 280°C, respectively.

RESULTS AND DISCUSSION

The results obtained (Figure 2), revealed that the ethanol concentration of samples ranged between 0.5 and 5.8% v/v. The results are comparable to those obtained by Motlhanka and coworkers during the analysis of microbes of Khadi (a homemade alcoholic beverages made from *Grewia flava* fruits) who found ethanol concentrations to range from 4-8% v/v (Motlhanka et al., 2020). The concentration of ethanol increased with an increase in the number of fermentation days. The lowest concentration was found in sample 3A which was collected during the day it was prepared while the highest ethanol content was observed in sample 1B which was collected 2 days after it was prepared. The difference in ethanol concentration in samples 2B and 3B which had fermented for the same number of days (1 day) before sampling may be due to different conditions and utensils used. While some just fermented the fruit juice, others added a previously fermented morula beer to the freshly prepared beer (back slopping) leading to the difference in the amount of ethanol. In general, the ethanol content of the fermented homemade morula beer (2.5-5.8% v/v) was similar to those found in most commercial alcoholic beers (2.92-15.66 % v/v) (Destanoğlu and Ateş, 2019; Galvão et al., 2020; Sawadogo-Lingani et al., 2021). After a successful quantification of ethanol in the samples, they were analysed for the presence of other volatile components. Apart from ethanol and carbon dioxide, the fermentation process also produces a broad range of secondary metabolites (Pinho et al., 2006). While these substances are only produced at very low concentrations, they are responsible for the complex aromas and taste of fermented beverages (Leão et al., 2018; Bettenhausen et al., 2018). The major higher alcohols found in morula beer were n-propanol, 2-methy-l-propan-1-ol, 3-methylbutan-1-ol, and the aromatic alcohols β-phenyl-ethanol and benzyl alcohol (Figure 3). Most of these compounds are common to those previously reported for other alcoholic beverages

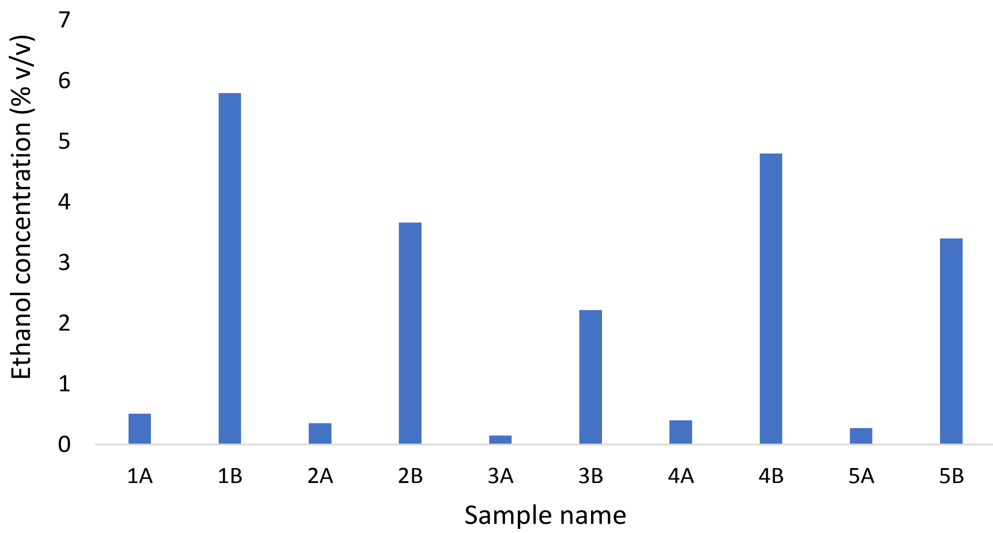

Figure 2. Concentration of ethanol in morula beer samples [1,2, 3,4,5: Sampling sites, A: 1-day old samples, B: 2-day old sample].
Source: Authors

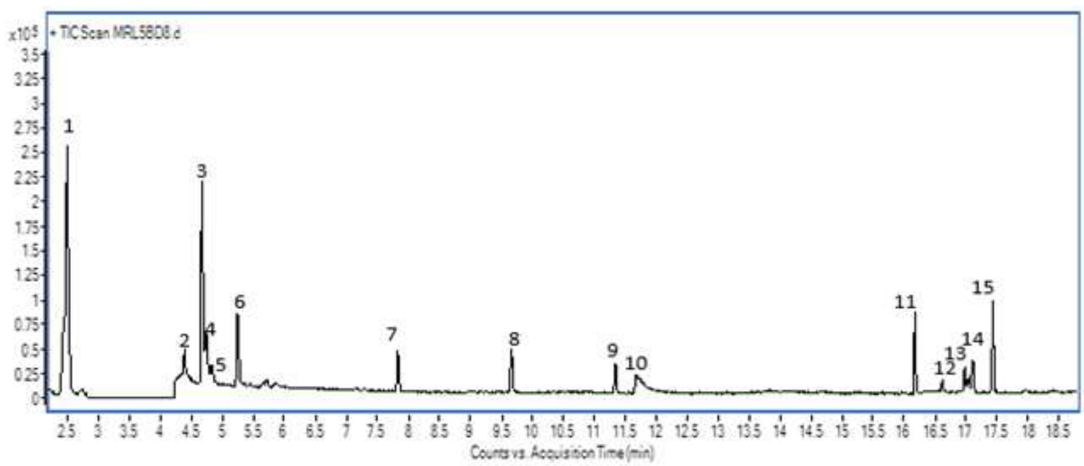

Figure 3. GCMS chromatogram of a 2-day-old morula beer. 1: ethanol, 2: acetic acid, 3: propanol, 4: propanal, 5: butanal, 6:3-methyl-1-butanol; 2-methyl-1-butanol,7: butanoic acid,8: isoamyl acetate,9: butanoic acid ethyl ester,10: benzyl alcohol, 11: phenyl ethyl alcohol, 12: lactic acid, 13: humulene, 14: decanoic acid ethyl ester, 15: 2-methyl-1-hexadecanoic acid ethyl ester.
Source: NIST, Mass-spectrometry Data Center, 2017.

(Mapitse et al., 2015, Destanoğlu and Ateş, 2019; Bettenhausen et al., 2018). The chemical composition of these homemade alcoholic beverages have different impacts on the aroma and flavor as high concentrations of higher alcohols can lead to a strong, pungent smell and taste, whereas reduced amounts impart desirable characteristics (Lu et al., 2020). During yeast metabolism, higher alcohols are formed as by-products of amino acid synthesis from pyruvate through the anabolic pathway or they could be produced through amino acid catabolism (Zhu et al., 2021). Two aldehydes (propanal and butanal) were found in morula beer. Aldehydes are formed from the oxidation of primary alcohols (Sinthumule and Mzamani, 2019). Esters present in morula beer were iso-amyl acetate, butanoic acid ethyl ester, decanoic acid ethyl ester, and 2-methyl-1-hexadecanoic acid ethyl ester. During alcoholic fermentation, several esters can be produced due to yeast metabolism (Torres-Guardado et al., 2022). Esters are some of the compounds found in beer and therefore impact greatly on its aroma. In optimal quantities, they can give a pleasant, full-bodied character to beer aroma, but in large amounts, they give beer aroma an overly fruity quality, which is disliked

Table 1. Difference in the relative abundances of volatile compounds based on the number of fermentation days.

Volatile compounds	Relative abundances of volatile components (peak area)				
	Day 0 (Fresh)	Day 1	Day 2	Day 3	Day 4
ethanol	201	9545	20598	15125	11021
acetic acid	5003	4523	2548	1547	514
1-propanol	ND	1578	2485	2045	1457
3- methyl butanol	ND	2514	6654	4214	2141
propanal	ND	1986	2801	3651	2958
butanal	ND	2541	ND	3024	2541
butanoic acid	ND	912	3510	4510	4501
iso-amyl acetate	ND	1484	3307	2599	2841
butanoic acid ethyl ester	ND	1042	6585	5874	5584
benzyl ethanol	ND	4521	4856	1457	ND
phenyl ethyl alcohol	2156	5865	10254	9847	5140
lactic acid	7548	5142	4215	2145	658
humulene	3584	3216	3201	3120	2914
decanoic acid ethyl ester	ND	1040	5247	5547	5915
2-methyl-1-hexadecanoic acid ethyl ester	ND	4244	9855	10240	10954

ND: Not detected.
Source: Authors

by most consumers (Mapitse et al., 2015). Acetic acid and lactic acid were also found in morula beer. Organic acids are formed from the oxidation of alcohols and acidic hydrolysis of esters while fatty acids form from the oxidation of aldehydes (Bettenhausen et al., 2018). Humulene was also found in morula beer. It is a biogenic volatile compound present in some plants, and it is a naturally occurring monocyclic sesquiterpene (Hilou et al., 2017). Humulene and its reaction products in the brewing process of beer give many beers their hoppy aroma (Galvão et al., 2020). It is an anti-bacterial, anti-inflammatory, and appetite suppressant and is also believed to kill cancer cells (Manyeula et al., 2021). Most of the volatile compounds detected in morula beer have also been reported by Destanoğlu and Ateş (2019) in homemade and commercial beers.

Different compounds identified in the morula beer sample on Day 0 (fresh) and how they varied over the 4 days are shown in Table 1. The volatile compounds in this study were not quantified, therefore the discussion was made in terms of the peak area (which is directly proportional to concentration) of volatile compounds that are present in morula beer relative to the peak areas obtained at Day 0. It was observed that the most important volatile compounds increased in amount up to Day 2 and eventually decreased gradually. In the fresh sample, only ethanol, acetic acid, lactic acid, and humulene were detected. Ethanol amount increased up to day 2 of fermentation but eventually decreased in a 3- and 4-day-old sample. The same trend was observed in other alcohols (n-propanol, 2-methy-l-propan-1-ol, 3-methylbutan-1-ol, phenyl ethyl alcohol, and benzyl alcohol) from day 1 to day 2. Acetic and lactic acid concentrations also decreased. The decrease in the amounts may be a result of the oxidation of alcohols and organic acids to form esters and aldehydes whose concentration increased from day 1 to day 3. The concentration of aldehydes decreased after day 3 and this might be due to the formation of fatty acids (for example, butanoic acid) whose concentration increased up to day 4 of fermentation. The amount of humulene dropped from day 1 but at a lower rate compared to other volatiles. The high amounts of aldehydes on days 3 and 4 could lead to off-flavour (soapy/cardboard) reported by consumers as the beer ages (Tulashie et al., 2017). It can be concluded that day 2 is an important day during morula beer shelf life because, after day 2, the amounts of important compounds like alcohols started to drop while unwanted compounds like aldehydes increase.

Conclusions

A simple, rapid, cheap, and highly sensitive method that requires the use of a minimal amount of solvent was used for the analysis of homemade morula alcoholic beverages. This method can be used for routine analysis of alcohol and other volatile substances in homemade alcoholic beverages to provide reliable quantitative and qualitative data. The results show that the quality of homemade alcoholic beverages is variable and unpredictable, both quantitatively and qualitatively due to the lack of specifications and the absence of routine scientific quality control. Ethanol was quantified in morula beer and ranged from 0.5 to 5.8% v/v in all samples, which is lower than that of the commercial Amarula cream liquor (17% v/v). This work revealed the presence of fifteen other volatile compounds which included alcohols, aldehydes, and esters. The results revealed a general decrease in the amount of volatile compounds over the fermentation period. The amount of some compounds (especially alcohols and esters) that contribute to important beer flavors was maximum around Day 2 of brewing before dropping significantly. No harmful substances were detected in Morula beer.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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