CHEMICAL ENGINEERING TRANSACTIONS

VOL. 75, 2019

A publication of AIDIC

The Italian Association of Chemical Engineering
Online at www.cetjournal.it

Guest Editors: Sauro Pierucci, Laura Piazza
Copyright © 2019, AIDIC Servizi S.r.l.
ISBN 978-88-95608-72-3; **ISSN** 2283-9216

DOI: 10.3303/CET1975017

Spoilage Growth and Benzene Evaluation of Non-carbonated Soft Drinks

Marselle M. N. Silva[a,*], Rafael S. Silva[b], Thayana A. Cruz[a], Marcos B. Pinho[b], Karen S. Pereira[b], Maria Alice Z. Coelho[b]

[a]Instituto de Química, UFRJ. Av. Athos da Silveira Ramos, 149, bloco A - Cidade Universitária, Rio de Janeiro - RJ, Brasil, 21044-020

[b]Escola de Química, UFRJ. Av. Athos da Silveira Ramos, 149, bloco E - Cidade Universitária, Rio de Janeiro - RJ, Brasil, 21044-020

marselle@eq.ufrj.br

Benzoic and ascorbic acids are common additives of many non-carbonated drinks but despite the presence of those preservatives, inappropriate production processes may allow the growth of spoilage. Furthermore, over time these acids tend to react, producing benzene, a known carcinogen. This study analysed the regular and low-calorie versions of three brands of commercial non-carbonated soft drinks, assessing their microbiological and chemical quality. Preservative resistant yeast analysis was performed in TGY medium supplemented with glacial acetic acid (TGYA) and incubating at 30 °C/72 h and lactic acid bacteria analysis was done in MRS medium incubating at 35 °C for 5 days. Isolated strains were identified by partial amplification of 16S rDNA. Benzoic acid and benzene quantification were carried out using, respectively, HPLC and HS-SPME-GC. Some soft drinks samples showed evidence of growth of spoilage bacteria, identified as members of the *Acetobacteraceae* family. Spoilage growth can change the sensorial standard of the products and make consumers discredit the product generating great damage to a brand's reputation. In some samples, benzoic acid and benzene concentrations were above the thresholds established by regulatory agencies. Benzene's risk arises from chronic exposure, which is usually the problem for soft drink consumers, and corrective measures must and can be taken.
Keywords: soft drinks, acetic bacteria, benzene, benzoic acid, solid-phase microextraction (SPME)

1. Introduction

Many non-carbonated drinks include additives to prolong their shelf life, as benzoic and ascorbic acids. However, over time these compounds tend to react, producing benzene, a known carcinogen (Gardner and Lawrence, 1993). Additionally, inappropriate production processes may allow the growth of spoilage, despite the presence of preservatives (Stratford et al., 2013; Rojo et al., 2015). The acid environment in soft drinks inhibits the growth of non-acidophilic bacteria, including most of the pathogens, however, other microorganisms such as acid bacteria, yeasts, and filamentous fungi require the use of preservatives. Current regulations worldwide do not call for testing spoilage microorganisms (Brazil, 2001; CFR, 2017).

Interactions involving sodium benzoate and ascorbic acid have recently been the target of research, as it results in benzene formation, a substance which chronic exposure can cause from vomiting to leukaemia (Weisel, 2010; Smith et al., 2011). This reaction, due to benzoate ion decarboxylation by hydroxyl radicals formed by the reduction of oxygen in the presence of ascorbic acid, catalysed by traces of metal ion, is accelerated with exposure to heat, such as during the transportation and storage of beverages (Gardner and Lawrence, 1993). This reaction must be considered by beverages industries to discuss changes in shelf life time or in conservation method, and yet no regulation about benzene in finished products exists worldwide.

Considering many instances of benzene in non-alcoholic beverages reported in the literature (Fabietti et al., 2001; Sanchez et al., 2012), studies about quantification of the preservative precursor and identification of benzene in food matrices are of general concern for public health. In 2016 the worldwide soft drinks industry

Paper Received: 13 April 2018; Revised: 12 July 2018; Accepted: 20 October 2018

Please cite this article as: Silva M., Silva R., Cruz T., Pinho M., Pereira K., Coelho M., 2019, Spoilage Growth and Benzene Evaluation of Non-carbonated Soft Drinks, Chemical Engineering Transactions, 75, 97-102 DOI:10.3303/CET1975017

sold 726 million litres and earned 777 billion dollars, with annual sales growth of 3% in volume (Euromonitor International, 2017), stating the relevance of this market.
This study analysed the regular and low-calorie versions of three brands of commercial non-carbonated guarana soft drinks, used as a proof of concept, which account for a significant share of the beverage market in Brazil (ABIR, 2017), assessing their microbiological and chemical quality.

2. Materials and methods

Regular and low-calorie versions of three brands (A, B and C) of non-carbonated commercial guarana soft drinks were analysed. For each soft drink, four different batches were tested, three samples for batch. All analyses were performed with the drinks before the expiration date, totalizing 72 sample units analysed. The sample units were polypropylene (PP) cups heat- and pressure-sealed with aluminium foil with capacity for 290 mL.

2.1 pH evaluation

The pH analysis was performed using a countertop pH meter (Tecnopon, Brazil) previously calibrated on pH 4 and 7. Each sample was analysed in separate, resulting in triplicate for each batch.

2.2 Microbiological analysis

The analysis of preservative resistant yeast was performed by pour plate method inoculating 1 mL of sample in TGY medium (tryptone, glucose, yeast extract) supplemented with 0.5% (v/v) of glacial acetic acid (TGYA) and incubating at 30 °C for 72 hours (Pitt and Hocking, 2009). The analysis of lactic acid bacteria was also done by pour plate method with overlay inoculating 1 mL of the beverage in DeMan, Rogosa and Sharpe (MRS) medium and incubating at 35 °C for 5 days (Hall et al., 2001).
The isolated colonies were submitted to Gram stain (Hucker, 1921), motility, catalase and oxidase tests to characterize them (MacFaddin, 2004).

2.3 Statistical analysis

The growth frequency of microorganisms in each version of the beverage was determined statistically at a 5% significance level using the Z test for proportions. Statistical analyses were performed with the software STATISTICA 13.0, from StatSoft, Inc.

2.4 Microorganisms' molecular identification

Partial amplification of 16S rDNA was performed using the primer pair 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1512R (5´-ACGGCTACCTTGTTACGACT-3´) (Rebouças et al., 2016), Platinum PCR Supermix (Thermo Fisher Scientific) in a 96 wells thermal cycler (Applied Biosystems). Sequences of identities were determined using the BLASTn algorithm in the GenBank database (NCBI, 2018).

2.5 Benzoic acid determination and quantification

The benzoic acid analysis was performed using high-performance liquid chromatography (HPLC) according to the methodology proposed by Mota et al. (2003). Reverse phase column Hypersil ODS (4.6 X 250 mm, 5 µm) was used. The mobile phase consisted of 0.005 M acetate buffer (pH = 4.4) and methanol (65:35) with a flow rate of 0.8 mL/min and UV detection at 235 nm. The standards were a benzoic acid solution in HPLC grade methanol at concentrations ranging from 50 to 1000 µg/mL.
Preceding the analysis, samples of guarana soft drink were centrifuged for 20 min at 3000 rpm and purified with C18 cartridge previously activated. The activation was performed by passing 2 mL of methanol followed by 4 mL of water, and for purification, 1 mL of the supernatant obtained after centrifugation of the drink was passed through the cartridge, cleaned with 4 mL of n-hexane and then eluted with 3 mL of methanol. The resulting methanol extract was filtered through a 0.45 µm membrane.

2.6 Benzene determination and quantification

The determination of benzene was carried out by gas chromatography coupled to solid phase micro extraction headspace (HS-SPME-GC) following the methodology proposed by Sanchez et al. (2012) with modifications. The chromatograph used was GC-2010 Plus (Shimadzu), associated with FID and MS detectors. The mass spectrometer (MS) was used to identify and later the ionization detector (FID) was used for the quantification of benzene.
The CAR-PDMS (carboxen-polydimethylsiloxane) fibre was used for the adsorption of benzene in the matrix and subsequent desorption in GC injector at 300 °C for 5 minutes. The standards were solutions with concentrations ranging from 0.5 to 15 µg/L of benzene in water prepared from benzene in methanol ampoule

(200 µg/mL). A volume of 10 mL of both standards and samples was added to 3 g of sodium chloride (NaCl) and placed in vials of 20 mL. The vials were submitted to an ice bath at 0 °C for 15 minutes while the fibre was exposed to vial's vapour phase, rich in benzene.
The injector temperature was maintained at 300 °C, the capillary column used was Restek RTX-1 (30 m X 0.25 mm X 0.25 µm) with helium gas flow rate of 5.5 mL/min. The temperature was raised from 40 °C to 100 °C at a rate of 10 °C/min, followed by 15 °C/min until 180 °C. These conditions were maintained in the analysis using MS and in the analysis using FID.

3. Results and discussion

3.1 Some soft drink samples showed evidence of growth of spoilage bacteria

Microbial growth occurred in both regular and low-calorie versions of brand A when tested in lactic acid bacteria-selective medium, although the fact that the pH in all samples was below 4.0 (data not shown) precludes the possibility of most pathogenic bacterial growth (Lawlor et al., 2009). As the current legislation worldwide only determines limit to pathogenic microorganisms (Brazil, 2001; CFR, 2017), the growth of bacteria in samples A and Adiet (diet version of brand A) before the expiration indicates that bacterial inhibition that should occur by the addition of benzoic acid may not be enough as a barrier to decay. Although spoilage growth does not represent a health security problem, it changes the sensory standard and makes the consumer discredit the product; great damage to the brand's representation.
We also observed bacteria, but not yeast, in preservative-resistant yeast-selective medium, as evidenced by microscopic evaluation of gram-stained slides (Figure 1). This may have been because this media was acidified, as specified by the protocol (Pitt and Hocking, 2009), recreating the pH 3 of the drinks, propitious for acid bacteria growth. A similar situation was observed by Chuayana et al. (2003) and Creencia et al. (2014), as they isolated other bacteria, and suggests that this protocol presents important limitations for the analysis of preservative resistant yeasts.

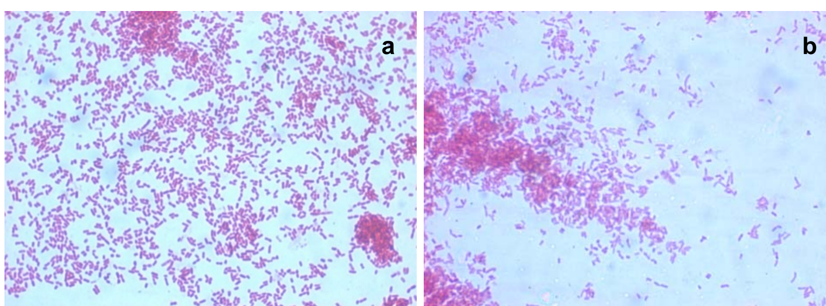


Figure 1: Gram-negative short rods bacteria isolated from TGYA (a) and MRS (b) media

Growth frequency showed no significant difference (at 5% significance through Z test for two proportions) between regular and low-calorie versions of the brand A drink, which contrasts our previous theory pointing to greater microbiological contamination in the regular version, which could be attributed to the fact that there is no metabolizable sugar in low-calorie beverages.

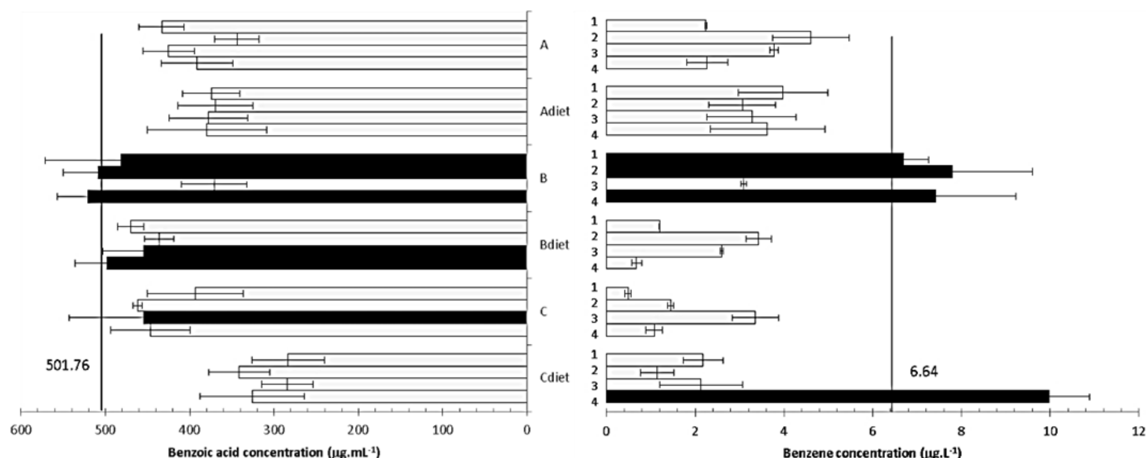
3.2 Bacteria molecular identification

As shown in Figure 1, colonies growing in both culture media were short gram-negative rods. These colonies showed motility, were positive for catalase and negative for oxidase (data not shown), indicating that the strains isolated in both culture media belong to the *Acetobacteraceae* family. This was confirmed by partial sequencing of the 16S rDNA. *Komagataeibacter* (25,5% incidence), *Gluconacetobacter* (23,5%), *Acetobacter* (19,6%), Asaia (15,7%), *Nguyenibacter* (1,96%), *Endobacter* (1,96%), *Kozakia* (1,96%), *Acidomonas* (1,96%), *Neoasaia* (1,96%), *Ameyamaea* (1,96%), *Tanticharoenia* (1,96%) and *Swaminathania* (1,96%) were identified based on homology identity (98-100%) searches at GenBank database, all genera of *Acetobacteraceae* family. Recently, genus *Gluconacetobacter* has been subdivided into the genera *Nguyenibacter*, *Komagataeibacter* and *Gluconacetobacter* (Yamada et al., 2012) and databases have not been fully updated to the reclassification, so it is possible that incidence assigned to *Gluconacetobacter* may belong to the genera *Nguyenibacter* or *Komagataeibacter*. The presence of this family is unusual in non-alcoholic beverages, where the most common isolates are lactic acid bacteria. These two families are very similar to each other in their capacity to grow in acid matrices, but acetic acid bacteria are mostly aerobic, whereas lactic acid bacteria grow better in an anaerobic environment (Kregiel, 2015). However, oxygen contact with the beverage may

occur due to the packaging material of these soft drinks, which consists of polypropylene cups (significantly O2-permeable, Mirzadeh and Kokabi, 2007), and a heat- and pressure-sealed aluminium foil, which may be subject to failures in the seal.
Acetic acid bacteria are biofilm producers (Bartowsky and Henschke, 2008) and this leads us to consider the presence of bacterial biofilm in the mixture tank in the soft drink manufacturer's plant. Cross-contamination between seeds or extracts and manufacturing areas, as well as inappropriate sanitation of the production facility, may be the cause of propagation of these bacteria among different batches.
There is no legislation which includes spoilage analysis in juices, soft drinks and other non-alcoholic non-milk-based beverages, however, it is of vital interest of both the consumer and the industry, to implement more effective control of contamination. HACCP (Hazard and Point Analysis Critical Control) program can be an alternative to prevent the final product quality from being compromised by the lack of appropriate action steps.

3.3 Some soft drink brands showed benzoic acid and benzene concentration above the limit

Figure 2 shows the concentrations of both benzoic acid and benzene for three brands of guarana based soft drinks in regular and low-calorie versions. Benzoic acid limit allowed in soft drinks is 500 µg/mL (Brazil, 2007) and from analytical decision limit calculation (CCα = MRL + 1.64sreproLMR) (ISO, 2000) all values above of 501.76 µg/L can be considered over the maximum allowed. Benzene limit in water is 5 µg/L (Brazil, 2011), and after the calculation of analytical decision limit, all values above 6.64 µg/L can be considered over the limit.



Brands A, B and C – regular versions
Brands Adiet, Bdiet and Cdiet– low-calorie versions
Numbers 1, 2, 3 and 4 on y-axis refer to batches

Figure 2: Benzoic acid and benzene concentrations for guarana soft drinks samples and their limits

In six batches, the benzoic acid concentration was above Brazilian regulatory limits (Brazil, 2007). The fact that industries use benzoic acid in excess demonstrates the lack of control in weighing the supplies, which reflects directly on microbial growth and, probably also in the formation of benzene. Weighing of components, including preservatives, is usually performed manually in this industry and should, therefore, be considered a critical point in the process as it depends on the precision of the operator.
In four batches of guarana soft drinks, benzene concentrations were above the levels allowed for drinking water by regulations of both the Brazilian Ministry of Health and the Food and Drug Administration (Brazil, 2011; CFR, 2017), European Union legislation is even more stringent and only allowed 1 ppb, so almost every sample analysed in our study would be above this limit (Council Directive, 1998). Three of those batches also had benzoic acid levels above regulatory limits, which is significant as benzene is formed from benzoic acid decarboxylation. This reaction is favoured at higher temperatures, suggesting that the presence of benzene above the threshold in some batches may have been due both to problems in production (excess benzoic acid) and in storage or commercialization (storage at too high temperatures) (Nyman et al., 2008). Neither the United States nor Brazil have specific legislation for benzene content in non-alcoholic beverages other than water. Our results suggest that this may be insufficient, as both countries allow the use of benzoic acid as a preservative, generating a potential risk that benzene may reach dangerous levels in the final product if optimal production and storage conditions are not met. Although the concentrations observed here were not much higher than the allowed limit, the risk from benzene arises from chronic exposure, which is usually the case for soft drink consumers. Thus, industry regulators should take corrective measures. For instance, the

formation of benzene can be inhibited by adding chelating agents, such as ethylenediaminetetraacetic acid (EDTA) and sodium hexametaphosphate (SHMP), which form complexes with Cu^{+2} and Fe^{+3} ions, catalysts of the benzoic acid decarboxylation reaction. Alternatively, the International Council of Beverages Associations suggests the removal, reduction or replacement of benzoate by sorbate or another additive or changing the storage system to reduce the need for preservatives (ICBA, 2006).
Benzene migration from packaging material is another potential source of chronic contamination in soft drinks. This issue, together with a review of the control standards for benzene in the water used for the production of soft drinks, should be further investigated if we are to prevent benzene contamination in soft drinks from becoming a public health issue.

4. Conclusions

Neither preservative resistant yeasts nor lactic acid bacteria were found on beverage analysed. Acetic acid bacteria were isolated in both versions commercialized, regular and low-calorie, in one of the brands analysed. The presence of this family is unusual in non-alcoholic beverages, where the most common isolates are lactic acid bacteria.
By the methodology applied, considering the limits of decision (CCα) inherent to analytical procedures and sample standard deviations, seven batches, representing 29.17% of total batches, presented benzoic acid concentration above 500 µg/mL, which is the maximum allowed by law. Regarding benzene, four samples showed a concentration value above 5 µg/L, maximum established for drinking water.
Although this work focused in a non-alcoholic soft drink, used merely as proof of concept, results reflect a lack of regulation that can be extrapolated to all industrialized fruit-based beverages, even for the ones that do not use any preservative. According to this paper, microbiological and chemical quality of this kind of matrix needs to be evaluated to protect both consumers' health and industries' economic interests

Acknowledgements

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

ABIR, Dados de Mercado, 2017, Associação Brasileira das Indústrias de Refrigerantes e de Bebidas Não Alcoólicas. <www.abir.gov.br> accessed 18.03.2017.
Bartowsky, E. J., Henschke, P. A., 2008, Acetic acid bacteria spoilage of bottled red wine—a review, International Journal of Food Microbiology, 125, 60-70.
Brazil, Agência Nacional de Vigilância Sanitária, 2007, Atribuição de Aditivos e seus Limites Máximos para a Categoria de Alimentos: Bebidas Não Alcoólicas, Subcategoria: Bebidas Não Alcoólicas Gaseificadas e Não Gaseificadas. Diário Oficial [da] República Federativa do Brasil, Brasília.
Brazil, Ministério da Saúde. Agência Nacional de Vigilância Sanitária, 2001, Regulamento técnico sobre padrões microbiológicos para alimentos. Diário Oficial [da] República Federativa do Brasil, Brasília.
Brazil, Ministério da Saúde, 2011, Procedimentos de controle e de vigilância da qualidade da água para consumo humano e seu padrão de potabilidade. Diário Oficial [da] República Federativa do Brasil, Brasília.
Chuayana, E. L., Jr., Ponce, C. V., Rivera, M. R. B., Cabrera, E. C., 2003, Antimicrobial activity of probiotics from milk products, Philippine Journal of Microbiology and Infectious Diseases, 32, 71-74.
Code of Federal Regulations (CFR), 2017, Requirements for Specific Standardized Beverages. Washington, DC: U.S. Government Printing Office. <www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=165.110&SearchTerm=bottled%20water> accessed 11.05.2018.
Council Directive 98/83/EC, 1998, Council Directive 98/83/EC of 3 November 1998 relating to the quality of water intended for human consumption, Official Journal of the European Communities, 330, 32–54.
Creencia, A. R., Mendoza, B. C., Migo, V. P., Monsalud, R. G., 2014, Degradation of residual jatropha oil by a promising lipase-producing bacterial consortium, The Philippine Journal of Science, 143, 73-78.
Euromonitor International, 2017, Soft Drinks in 2017: new insights and system refresher, London: Euromonitor International.
Fabietti, F., Delise, M., Bocca, A. P., 2001, Investigation into the benzene and toluene content of soft drink. Food Control, 12, 505–509.
Gardner, L., Lawrence, G., 1993, Benzene production from decarboxylation of benzoic acid in the presence of ascorbic acid and a transition metal catalyst, Journal of Agricultural and Food Chemistry, 41, 693–695.

Hall, P. A., Ledenbach, L., Flowers, R. S., 2001, Acid-Producing Microorganisms. In: Salfinger, Y., Tortorello, M. L. (Eds.), Compendium of Methods for the Microbiological Examination of Foods, 4th ed., Washington, D.C., (Chapter 19).

Hucker, G. J., 1921. A New Modification and Application of the Gram Stain, Journal of Bacteriology, 6, 395-397.

ICBA, International Council of Beverages Associations, 2006, Guidance document to migrate the potential for benzene formation in beverages <www.icba-net.org/ICBA-Benzen e-Guidance.pdf> accessed 18.03.2018.

ISO (International Standards Organization). ISO 11843-2, 2000. Capability of detection - Part 2: Methodology in the linear calibration case. Geneva: ISO, 24 p.

Kregiel, D., 2015, Review Article: Health Safety of Soft Drinks: Contents, Containers, and Microorganisms. BioMed Research International, 2015, 1-15.

Lawlor, K., Schuman, J., Simpson, P., Taormina, J., 2009, Microbiological spoilage of beverages. In: Sperber, W. H., Doyle, M. P. (Eds.), Compendium of the Microbiological Spoilage of Foods and Beverages, Springer: New York, NY, USA. (Chapter 9).

MacFaddin, J. F., 2004. Pruebas bioquímicas para la identificación de bacterias de importancia clínica, 3rd ed., Buenos Aires: Médica Panamericana.

Mirzadeh, A., Kokabi, M., 2007. The effect of composition and draw-down ratio on morphology and oxygen permeability of polypropylene nanocomposite blown films, European Polymer Journal, 43, 3757-3765.

Mota, F. J. M., Ferreira, I. M. P. L. V. O., Cunha, S. C., Oliveira, M. B. P. P., 2003, Optimization of extraction procedures for analysis of benzoic and sorbic acids in food stuff, Food Chemistry, 82, 469-473.

NCBI Resource Coordinators, 2018, Database resources at the National Center for Biotechnology Information, GenBank database <blast.ncbi.nlm.nih.gov/> accessed 30.06.2017.

Nyman, P. J, Diachenko, G. W, Perfetti, G.A, McNeal, T. P, Hiatt, M. H., Morehouse, K. M., 2008, Survey Results of Benzene in Soft Drinks and Other Beverages by Headspace Gas Chromatography/Mass Spectrometry, Journal of Agricultural and Food Chemistry, 56, 571-576.

Pitt, J. I.., Hocking, A. D., 2009, Methods for Isolation, Enumeration and Identification. In: Pitt, J. I., Hocking, A. D. (Eds.), Fungi and Food Spoilage, 3rd ed., New York: Springer Science and Business Media, (Chapter 4).

Rebouças, K. H., Gomes, L. P., Leite, A. M. O., Uekane, T. M., Rezende, C. M., Inês, M. B. T., Almeida, E. L., Aguila, E. M. D., Paschoalin, V. M. F., 2016, Evaluating Physicochemical and Rheological Characteristics and Microbial Community Dynamics during the Natural Fermentation of Cassava Starch, Journal of Food Processing & Technology, 7, 1-9.

Rojo, M. C., López, F. N. A., Lerena, M. C., Mercado, L., Torres, A., Combina, M., 2015, Evaluation of different chemical preservatives to control Zygosaccharomyces rouxii growth in high sugar culture media, Food Control, 50, 349-355.

Sanchez, A. B., Budziak, D., Martendal, E., Carasek, E., 2012, Determination of benzene in beverages by solid-phase microextraction and gas chromatography, Scientia Chromatographica, 4, 209-216.

Smith, M. T., Zhang L., McHale C. M., Skibola C. F., Rappaport S. M., 2011, Benzene, the exposome and future investigations of leukemia etiology, Chemico-Biological Interactions, 192, 155-159.

Stratford, M., Steels, H., Nebe-Von-Caron, G., Novodvorska, M., Hayer, K., Archer, D. B., 2013, Extreme resistance to weak-acid preservatives in the spoilage yeast Zygosaccharomyces bailii, International Journal of Food Microbiology, 166, 126-134.

Weisel, C. P., 2010, Benzene exposure: An overview of monitoring methods and their findings, Chemico-Biological Interactions, 184, 58-66.

Yamada, Y., Yukphan, P., Vu, H.L., Muramatsu, Y., Ochaikul, D., Tanasupawat, S., Nakagawa, Y., 2012. Description of Komagataeibacter gen. nov., with proposals of new combinations (Acetobacteraceae), The Journal of General and Applied Microbiology, 58, 397-404.