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Extraction of Cashew Nutshell Liquid from Cashew Nutshells Using the Polyol Induced Extraction (PIE) Method

Ibrahim L. Adewole

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By

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Governors State University
University Park, Illinois 60484

2023

WE, THE UNDERSIGNED MEMBERS OF THE COMMITTEE

HAVE APPROVED THIS CAPSTONE REPORT

Extraction of Cashew Nutshell Liquid from Cashew Nutshells Using the Polyol Induced Extraction (PIE) Method

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Table of Contents

Tables and Figures

List of Tables

List of Figures

ABSTRACT

Polyol-induced extraction (PIE) is applied to the extraction of cashew nutshell liquid (CNSL) from cashew nutshells using glycerol as a mass-separating agent. In this process, a 1:1 mixture of acetonitrile (ACN) and water (H_2O) exists as a completely miscible homogeneous phase. Cashew nutshells are soaked in this mixture at room temperature. After soaking the nutshells in acetonitrile/water, a polyol, glycerol, is added to the mixture, and a phase separation is observed. Upon cooling to –20 ˚C, the phase separation increases with an acetonitrile-rich top layer containing analytes that are soluble in acetonitrile.

Initially, the PIE process was studied with pure caffeine as a test molecule. Using a 1:1 mixture of acetonitrile and water, 20 % (w/v) of glycerol and cooling to -21 ˚C, an acetonitrile-rich upper layer formed in 30 % of the total volume. The partition coefficient for caffeine (K_{PC}) was 1.0.

Extraction of 2.06 g of cashew nutshells produced 0.33 g of CNSL in 16.0 % yield. By comparison, extraction of 2.05 g of cashew nutshells with cyclohexane produced 0.38 g of CNSL in 18.5 % yield. While extraction of 12.01 g of cashew nutshells by the Soxhlet method using acetonitrile as a solvent produced 2.45 g CNSL in 20.4 % yield. The extracts were analyzed by HPLC, GC-MS, and NMR instruments. The HPLC peak area analysis revealed that the saturated form of anacardic acid was only a minor component (about 2 %). Moreover, ^{1}H NMR analysis indicates that the major component of the extract is a decarboxylated derivate of anacardic acid (**Figure 1)** which is cardanol (**Figure 2)**. This decarboxylation reaction occurred in the GC-MS due to the loss of the carboxylic acid group from the anacardic acid.

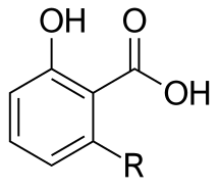

Figure 1. General anacardic acid structure.

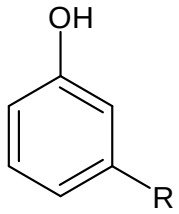

Figure 2. General cardanol structure.

Keywords

Anacardic acid (2-hydroxy-6-alkylbenzoic acid), Cardanol, PIE (Polyol-induced extraction) method, High-Performance Liquid Chromatography (HPLC), Cashew nutshell liquid (CNSL), Gas chromatography-mass spectrometry(GC-MS), Nuclear magnetic resonance (NMR).

Introduction

The cashew tree (*Anacardium occidentale*) is native to eastern Brazil and spread by the Portuguese in the 16th and 17th centuries to other tropical regions, including India, Africa, Indonesia, and Southeast Asia.[1] "*It is a tiny evergreen tree that can reach a height of 10 to 12 m*."[2] Its trunk is usually short and irregularly curved (**Figure 3)**. The tree then starts to grow flowers indicating that the fruit is about to be produced **(Figure 4)**. The nut, a kidney-shaped object about 2 to 3 centimeters in length, is the actual fruit of the cashew tree. The end of a fleshy pulp known as the cashew apple has a nut connected to it (**Figure 5)**. The cultivation of this tree is not too difficult. The fruits known as cashew apples are used to make juices, jellies, jams, wine, and syrups. Fruit waste has also been suggested as an ingredient in high-fructose or protein-rich animal feeds.[3] The cashew tree has been the source of traditional remedies for malaria and toothache due to the chemicals in its leaves and bark.[4] Around 570,000 acres of Indonesian land are used for cashew tree farming, and 142,000 metric tons of cashew nuts are produced yearly.

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[1] Wikipedia contributors. (2022, December 1). Cashew. In *Wikipedia, The Free Encyclopedia*. Retrieved 20:49, January 4, 2023, from https://en.wikipedia.org/w/index.php?title=Cashew&oldid=1124964648

[2] Atteya, A. K. G., El-Serafy, R. S., El-Zabalawy, K. M., Elhakem, A., & Genaidy, E. A. E. (2022). Exogenously Supplemented Proline and Phenylalanine Improve Growth, Productivity, and Oil Composition of Salted Moringa by Up-Regulating Osmoprotectants and Stimulating Antioxidant Machinery. *Plants*, *11*(12), 1553. https://doi.org/10.3390/plants11121553

[3] Arinzechukwu, C., & Nkama, I. (2019). Production and Quality Evaluation of Fruit Bars from Banana (Musa sapientum) and Cashew (Anacardium occidentale) Apple Fruit Blends. *Asian Food Science Journal*, *10*(2), 1-16. https://doi.org/10.9734/afsj/2019/v10i230032

[4] Rossetti, A. G., Vidal, F. das C., & Barros, L. de M.. (2019). Sampling of cashew nuts as an aid to research for the genetic improvement of cashew tree. *Pesquisa Agropecuária Brasileira*, *54*(Pesq. agropec. bras., 2019 54). https://doi.org/10.1590/S1678-3921.pab2019.v54.00962

Figure 3. Cashew tree.[5a]

Figure 4. Cashew flower.[5b]

Figure 5. Cashew fruit.[5c]

The nuts are shelled either manually **(Figure 8)** or mechanically as an initial preparative procedure to extract their constituents. They are spread out on a flat stone and smashed with a wooden hammer during the hand-shelling process. However, in a larger industry where the production of cashew nut kernel is commercialized, a faster and more effective way is employed. Oltremare, is an Italian company specializing in the design and manufacture of plants and machinery for the cashew nut processing industry.[6]

Despite the nutritious value of the kernel, the shell of cashew nut has traditionally been used as a manufacturing byproduct. Since 50 % of the cashew kernel is composed of a shell, this by-product could cause environmental issues if not managed properly. Furthermore, a study suggests that the shell of the cashew kernel produces a heavy liquid with a strong odor known as cashew nutshell liquid (CNSL).[7] Because of the high

[5]Cashew. (2022, September 5). In *Wikipedia*. https://en.wikipedia.org/wiki/Cashew; a) "Cashew Tree" by MJEHermann - Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=64082857; b) "Flower of Cashew Tree "by Kateelkshetra - Own work, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=30569277; c) "Ripe Cashew Apples" by Abhishek Jacob - Own work, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=12698315.

[6] Strocchi, A., & Lercker, G. (1979). Cardanol in germ and seed oils extracted from cashew nuts obtained by the oltremare process. *Journal of the American Oil Chemists' Society, 56*, 616-619.

[7] Tamiello-Rosa, C. S., Cantu-Jungles, T. M., Iacomini, M., & Cordeiro, L. M. (2019). Pectins from cashew apple fruit (Anacardium occidentale): Extraction and chemical characterization. *Carbohydrate Research*, *483*, 107752. https://doi.org/10.1016/j.carres.2019.107752

concentration of unsaturated long-chain phenols, including anacardic acid, cardanol, cardol (**Figure 6**), and their isomers, CNSL is considered to be a valuable commodity. CNSL comprises three major components: anacardic acid, cardol, and cardanol. Each of these compounds can be found in four forms depending on the presence of C=C bonds in the alkyl chain: saturated, monoene, diene, and triene (**Figure 6**).

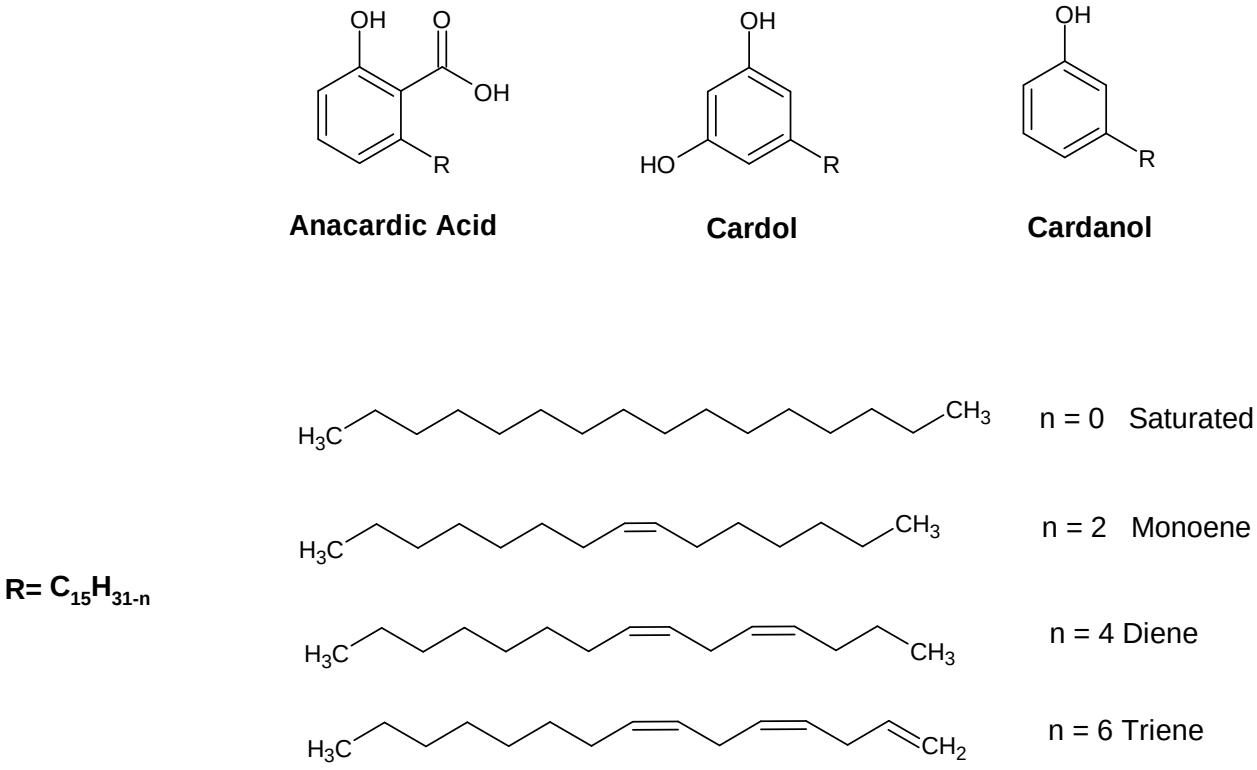

Figure 6. Chemical structures and compositions of major components of CNSL.

Anacardic acid and cardol both show anticancer, antibacterial, urease inhibitory, and lipoxygenase properties.[8] Anacardic acid is thermally unstable and may quickly convert to cardanol by decarboxylation at high temperatures. On the other hand, the remarkable

[8] Borges, I., de Araújo, J., & de Sousa, F. (2020). Bactericidal and antibiofilm activity of anacardic acid loaded-zein nanoparticles against Enterococcus faecalis Ex Vivo. *Journal of Computational and Theoretical Nanoscience*, *17*(7), 2918-2925. https://doi.org/10.1166/jctn.2020.9270

resistance to the softening effects of mineral oil and the great resistance to acids, alkalis, microbes, termites, and insects of cardanol make it a popular substance in the coating and resin industries.[9]

Figure 7. Cashew nutshell.[10]

Figure 8. Crushed cashew nutshell.[10]

Figure 9. Cashew nutshell liquid.[10]

CNSL is a viscous liquid with a bitter flavor that is dark brown in color and found in the honeycomb structure of the cashew nutshell (**Figure 9**).[11] The percentages of the components of natural CNSL have been reported variously by several writers. According to most estimates, it comprises between 70 and 80 percent by weight of anacardic acid and derivatives.[12] As previously mentioned, anacardic acid quickly decarboxylates when heated at 92.5 - 93 ˚C and transforms into the respective cardanol derivatives (**Figure 10**).[13]

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[9] Ngwira, K., Kühlborn, J., Mgani, Q., de Koning, C., & Opatz, T. (2019). Valorisation of cashew nut shell liquid phenolics in the synthesis of UV absorbers. *European Journal of Organic Chemistry*, *2019*(30), 4778-4790. https://doi.org/10.1002/ejoc.201900743

[10] Images in Figures 12 – 14 were created by the author of this work, Ibrahim Adewole.

[11] Ma, Y., Gong, X., Xie, B., Geng, X., & Jia, P. (2019). Synthesis and characterization of DOPO-g-CNSL and its effect on the properties of phenolic foams. *Journal of Renewable Materials*, *7*(10), 1037-1046. https://doi.org/10.32604/jrm.2019.07454

[12] Erick, H. R., Rosa, N., & Juliá S. (2018). Recovery of Anacardic Acids from Cashew Nut Shell Liquid with Ion-Exchange Resins. *Industrial & Engineering Chemistry Research,* 57 (49), 16903-16908. https://doi.org/10.1021/acs.iecr.8b04192

[13] David, W., & Charles R. D. (1948). Cashew Nut Shell Liquid. III. The Cardol Component of Indian Cashew Nut Shell Liquid with Reference to the Liquid's Vesicant Activity. *Journal of the American Chemical Society, 70* (11), 3675-3679. https://doi.org/10.1021/ja01191a041

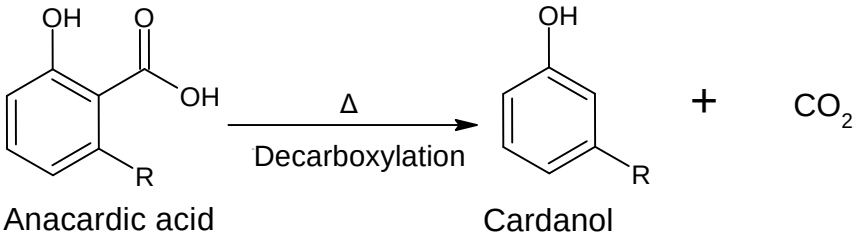

Figure 10. Decarboxylation reaction of anacardic acid to cardanol.

Cardol (**Figure 6**) makes up 8 to 10 percent of CNSL, and, is primarily in charge of the substance's vesicant function (i.e., potential to cause local tissue injury and irritation).[14] The methodological process, inclusive of the type and duration of heat, is used to create CNSL and has an impact on the substance's real composition, color, and stability.[15] The production of cardanol-based resins and varnishes, cardanol, foundry resins, brake linings, clutch facing, acid-resistant paints, insecticides and fungicides, rubber compounding resins, lacquers, and enamels all employ cashew nutshell liquid (CNSL).[16]

As shown in **Figure 10**, anacardic acid is heated to produce cardanol. Since the side chain's composition varies depending on the level of unsaturation, each of the cardanol fractions contains four different chemicals. Cardanol's distinctive molecular structure, particularly the unsaturation of its lengthy hydrocarbon side chain, makes it

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[14] Makwana, K., Ichake, A., Valodkar, V., Padmanaban, G., Badiger, M., & Wadgaonkar, P. (2022). Cardol: Cashew nut shell liquid (CNSL) - derived starting material for the preparation of partially bio-based epoxy resins. *European Polymer Journal*, *166*, 111029. https://doi.org/10.1016/j.eurpolymj.2022.111029

[15] Suwannahong, K., Thongkao, K., Thongmuang, P., Kreetachat, T., & Sudjaroen, Y. (2020). Larvicidal activity of Aedes Aegypti from a simple preparation of cashew (Anacardium occidentale L.) Nut shell extract for community level use. *Indian Journal of Forensic Medicine & Toxicology, 14*(4), 3306–3311. https://doi.org/10.37506/ijfmt.v14i4.12135

[16] Ifa, L. (2021). Techno economic study of liquid smoke from cashew nut shell waste. *Journal of Industrial Engineering Management*, *6*(1), 26-37. https://doi.org/10.33536/jiem.v6i1.879

simple to cross-link during polymerization.[17] To create specialized, high-value products, structural alterations can be done at the hydroxyl group, on the aromatic ring, and on the side chain. Cardanol is flexible and hydrophobic. Resin made from CNSL has an exceptional level of strong resilience to acids and alkalis.[18] Numerous items made from CNSL are employed in petroleum products, such as antioxidants, stabilizers, and demulsifiers.[19] The resistance of lubricating oils to oxidation and the production of sludge is enhanced by using soluble metal derivatives of CNSL.

Several researchers have employed different methods of extracting CNSL, which include solvent extraction,[20] supercritical CO_2 extraction,[21] pyrolysis,[22] and screw

[17] Barbosa, L., Souza, D., Queiroz, L., Neto, A., de Lima, D., & Beatriz, A. et al. (2019). Unequivocal structural assignments of three cardanol derivatives: An experimental and theoretical approach. *Journal of Molecular Structure*, *1175*, 357-366. https://doi.org/10.1016/j.molstruc.2018.07.112

[18] Wahyuningsih, S., Ramelan, A. H., Rahmawati, P., Tamtama, P. B. N., et al. (2017). Development of Refined Natural Resin based Cashew Nut Shell Oil Liquid (CNSL) for Brake Pads Composite. IOP Conf. Ser.: Mater. Sci. Eng., 176, 012051. https://doi.org/10.1088/1757-899X/176/1/012051

[19] Raji, V., & Menon, A. R. R. (2019). Phosphorylated cashew nutshell liquid prepolymer modified kaolin as a reinforcing filler for rubber vulcanizates—comparison with srso modified kaolin and cloisite. *Journal Of Materials Science and Engineering B*, *9*(2), 66-84. https://doi.org/10.17265/2161-6221/2019.3-4.004

[20] Senthil K. P., Arun K. N., Sivakumar, R., Kaushik, C. (2009). Experimentation on solvent extraction of polyphenols from natural waste. *J. Mater. Sci.*, *44*, 5894–5899, https://doi.org/10.1007/s10853-009-3834-8.

[21] Patel, R.N., Bandyopadhyay, S., Ganesh, A. (2006). Extraction of cashew (*Anacardium occidentale*) nutshell liquid using supercritical carbon dioxide. *Bioresour. Technol.*, *97*, 847–853, https://doi.org/10.1016/j.biortech.2005.04.009.

[22] Ábrego, J., Plaza, D., Luño, F., Atienza-Martínez, M., Gea, G. (2018). Pyrolysis of cashew nutshells: Characterization of products and energy balance. *Energy, 158*, 72–80, https://doi.org/10.1016/j.energy.2018.06.011.

pressing [23] with (w/w) yields ranging between 15 and 30 %. This research is focused on a new method of extraction of cashew nutshell liquid using the PIE method.[24]

The PIE method stands for polyol-induced extraction and is a process by which the addition of a polyol to a homogeneous mixture of an organic solvent and water results in phase separation and by which the level of phase separation increases as the temperature is decreased. The hypothesis of this research is that, when the PIE process is performed on cashew nutshells, the hydrophobic cashew nutshell liquid will separate with the upper organic phase. To explore this hypothesis, acetonitrile/water was chosen as the solvent mixture, glycerol as the polyol and -21 ˚C as the temperature for the phase separation.

To become familiar with the PIE process, an initial PIE extraction was performed using pure caffeine as an analyte. Two test extractions were done with caffeine dissolved in a 1:1 mixture of acetonitrile and water. Glycerol was used as the polyol and samples were equilibrated at -21 ˚C. This investigation allowed the partition coefficient for the PIE extraction of caffeine to be determined, which is the relative concentration of caffeine in the upper organic phase vs. the lower aqueous phase. In addition, this investigation helped inform the design of the cashew nutshell PIE extractions.

$$K_{PC} = [\text{caffeine}_{(org)}]/[\text{caffeine}_{(aq)}] \quad (1)$$

[23] Chaudhari, A.P., Thakor, N.J. (2015). Extraction of CNSL using screw pressing. *Asian J. Biol. Life Sci.*, *4*, 71–75.

[24] DelMastro, T., Snow, N., Murphy, R. W., & Sowa, J. R. (2017). Polyol Induced Partitioning of Essential Oils in Water/Acetonitrile Solvent Mixtures. *Journal of Liquid Chromatography & Related Technologies*. 0:0, pages 1-11 https://doi.org/10.1080/10826076.2017.1308379.

To compare the effectiveness of the PIE process, two other procedures were performed, which were traditional soaking of cashew nutshells in cyclohexane and Soxhlet extraction. The extracts obtained by these methods were analyzed by HPLC, and a comparison of the results was carried out.

The key objectives of the study are:

- To determine the yield and partition coefficient for the PIE extraction of pure caffeine;
- To extract cashew nutshell liquid from cashew nutshells using the PIE method, determine the yield, and compare it with other methods;
- To analyze the constituent components of cashew nutshell liquid (CNSL) using HPLC, GC-MS, and NMR instrumentation.

Materials and Methods

Anacardic acid (**Figure 10**) standard was purchased from Enzo Life Sciences (via VWR International, Inc.) as a 5 mg sample in 95 % purity. The GC-MS (gas chromatography coupled with mass spectrometry) analysis of the sample revealed the saturated form of anacardic acid (**Figure 10**) as the major component, with 5 % of the monoene, diene, and triene derivatives.

HPLC instrument and method

The HPLC (high-performance liquid chromatography) instrument was an Agilent 1260 Infinity HPLC System with Diode Array Detectors. The instrument was operated with the Agilent OpenLab CDS ChemStation Edition for LC and LC-MS Systems software Rev. C.01.05 [35], copyright 2001-2013.

The HPLC analysis of caffeine was performed using the “caffeine baldrich Sowa” method with a C18 column. The flow rate was set to 2 mL/min using a mixture of 78 % of 0.1 % formic acid in water and 22 % acetonitrile. The column was a Grace Platinum C18-EPS column (3 µm, 53 mm, ID 7 mm) with lot # 61/010, part # 50573, and serial # 615060461. The anacardic acid (**Figure 1)** standard and cashew nutshell liquids were analyzed with the “Anacardicacid.M” method with the following parameters: 0.5 mL/min flow rate, 80 % ACN, 20 % H_2O, and 1 % acetic acid, injection volume 1 µL, DAD 254 nm, and a column temperature of 25 ˚C.

GC-FID instrument and method

The GC-FID instrument was an Agilent 7890B GC System with an FID detector and a G4513A autosampler. The instrument was operated with the Agilent OpenLab CDS ChemStation Edition for GC Systems software Rev. C.01.05 [35], copyright 2001-2013.

The GC analysis was performed on an HP-5 capillary column (30 m, 0.320 mm id, 0.25 µm) using the "Anacardic acid" method with the following parameters: N_2 (g) constant flow, 2.66 mL/min; inlet temperature, 250 ˚C; injection volume, 1 µL (split 5:1, inlet pressure, 6.93 psi, total inlet flow, 6.19 mL/min, inlet septum purge, 3 mL/min); initial oven temperature, 40 ˚C, held for 3 min, then a 30 ˚C/min ramp to 250 ˚C and held for 7 min. The FID temperature was set at 325 ˚C with an H_2 (g) flow rate of 30.0 mL/min, an airflow rate of 400 mL/min, and a makeup N_2 flow of 10 mL/min.

Caffeine PIE extraction method

Two 15 mL graduated conical polypropylene tubes (Eppendorf) were obtained with screw caps. Precisely weighted (7.0 mg) of caffeine was placed in the first tube labeled as Sample 1; 10.0 mL of a 1:1 mixture of acetonitrile/water was added and the contents were mixed until the caffeine completely dissolved. In the second tube, 2.0 g of glycerol was added. The contents of the first tube were quantitatively transferred to the second tube containing glycerol. The solution was thoroughly mixed to allow all the glycerol to dissolve, and then the tube was allowed to stand for about two minutes to observe phase separation. The tube was placed in the freezer to cool to -21 ˚C. The temperature was monitored by using a temperature monitoring tube consisting of a 15 mL conical polypropylene tube containing a well-mixed solution of 1:1 acetonitrile/water (10.0 mL) and glycerol (2.0 g) and a thermocouple probe (Kitchen Assistant, ETHMEAS Digital Refrigerator/Freezer/Fridge Thermometer, 50 – 70 ˚C). When the probe in the temperature monitoring tube read -21 ˚C, the sample tube was removed from the freezer and the volumes of the top and bottom layers were recorded. Using an automatic pipet, 1.00 mL of the top and bottom layers were withdrawn and placed in sample vials (from

VWR stores) for analysis by HPLC. The procedure was repeated for Sample 2 with precisely weighted (8.0 mg) caffeine.

To facilitate the HPLC analysis, a calibration curve was prepared as follows. A stock solution of 1.5 mg/mL of caffeine standard and four other concentrations were prepared by dilution from this stock solution with the following concentrations of caffeine: 0.75 mg/mL, 0.50 mg/mL, 0.25 mg/mL, and 0.10 mg/mL. These were analyzed by HPLC; the peak areas (**Table 1**) were obtained, and a calibration curve was plotted (see **Figure 12** in **Results and Discussion**).

Table 1. Calibration data of caffeine.

Concentration (mg/mL)	**Area (mAu*s)**
1.50	2166.50
0.75	1184.00
0.50	753.80
0.25	312.00
0.10	160.40

PIE extraction of cashew nutshell liquid

Whole cashew nutshells for this study were ordered from Esty (T.M.D Nadeesha stores located at 38 mile's post, Maha bulankulama, Anuradhapura, Sri Lanka). The package was stored at room temperature upon receipt. And upon opening, (which is done in a fume hood), a large-sized cashew nut of about 45-50 mm and dark brown in color, as shown in **Figure 7**, was observed. The whole shells were removed and crushed with a hammer to break them apart and processed with a knife to remove the nut from the shell. The shell was further cut into several small pieces. Approximately 15 g of raw cashew nutshell was obtained.

Two 15 mL graduated conical polypropylene tubes (Eppendorf) were obtained with caps. The cashew nutshell was precisely weighted at 2.0580 g and placed in the first tube labeled as Sample 3. Then, 10.0 mL of a 1:1 mixture of acetonitrile/water was added. In the second tube, 2.0 g of glycerol was added. The contents of the first tube were transferred carefully to the second tube, which contained glycerol. The solution was thoroughly mixed to allow all the glycerol to dissolve and then allowed to stand for about two minutes to observe phase separation. The mixture was then placed in the freezer, and the temperature was monitored using a temperature monitoring tube described above. When the probe read -21 ˚C, Sample 3 was removed from the freezer and the volumes of the top and bottom layers were recorded. Using a 1 mL automatic pipet, 1.00 mL of the top and bottom layers were withdrawn and placed in sample vials for analysis by HPLC, GC-FID, and GC-MS.

Gravimetric analysis of the cashew nutshell PIE extract

Using Sample 3 obtained from the above “PIE extraction of cashew nutshell liquid,” 2.00 mL was removed from the top layer. This was processed as follows to remove the water and glycerol present in it. The 2.00 mL aliquot was mixed in 10 mL of cyclohexane. Then, 5 mL of saturated sodium chloride (brine) was added, and the mixture was transferred into a 60 mL separatory funnel. The saturated sodium chloride formed two layers, leaving the organic layer at the top and the aqueous at the bottom. Because glycerol is water soluble, the bottom layer was dispensed from the separatory funnel as water and glycerol. The top layer was dried with anhydrous sodium sulfate. The dried organic layer was decanted, followed by a 5 mL rinse of the sodium sulfate residue. The cyclohexane was evaporated on a rotary evaporator to give 0.33 g (16.0 %) of cashew nutshell liquid.

Other extraction methods

Another sample labeled Sample 1 was prepared using a Soxhlet extractor. Precisely weighted 12.0108 g of cashew nutshell (Sample 1) was wrapped in filter paper and placed in the Soxhlet extractor reservoir. Then, 200 mL of acetonitrile was added to the Soxhlet extractor pot, and the pot was heated on a hot plate until the acetonitrile came to a boil that was vigorous enough to condense into the Soxhlet extractor reservoir. After about two and a half hours, the heating was stopped, and the extracted oil with the solvent was transferred to the rotary evaporator to evaporate the acetonitrile present. This gives 2.45 g (20.4 %) of CNSL.

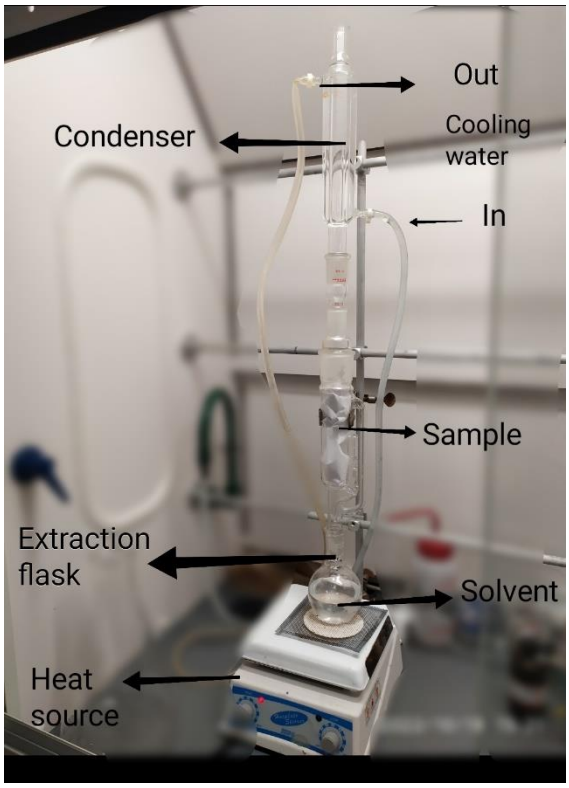

Figure 11. Soxhlet extractor apparatus.

The last sample was labeled Sample 2, which was to be extracted in cyclohexane. Precisely weighted (2.0518 g) of cashew nutshell (Sample 2) was placed in a 10 mL volume of cyclohexane and allowed to extract at room temperature for 24 hours. After this

time, the mixture was filtered, and the cyclohexane was evaporated on a rotary evaporator. 0.38 g was the extracted CNSL which resulted in an 18.5 % yield.

Results and Discussion

Caffeine PIE extraction method

The organic/water partitioning of caffeine using the PIE process was introduced to every member of the Sowa research group starting a research project on the PIE extraction method. The aim was to provide adequate experience with the PIE process of extraction with a procedure that has already been used and proved effective.

For HPLC analysis, a calibration curve was prepared using five caffeine standards ranging from 0.10 to 1.50 mg/mL. As shown in **Figure 12**, the relationship between absorbance and caffeine concentration is linear with a correlation coefficient (R^2) of 0.9956.[25]

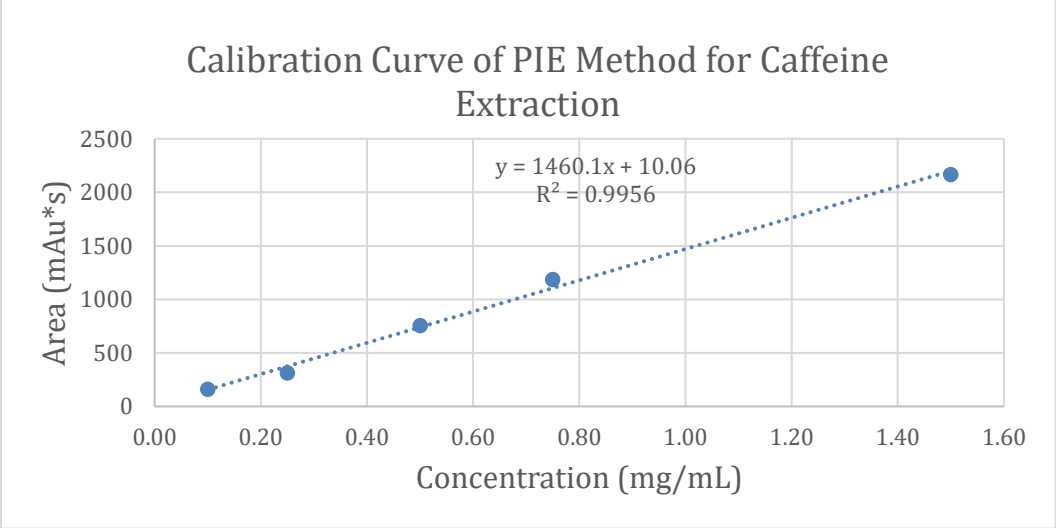

Figure 12. Caffeine calibration curve.

Linear regression analysis gives the equation shown in eq 2.

$$y = 1460.1x + 10.06 \qquad (2)$$

[25] At this stage of the analysis, the values for the linear equation and correlation coefficient are not corrected for significant figures. However, the final concentration results in **Table 2** reflect the correct number (two) of significant figures.

$$A = \varepsilon l c \qquad (3)$$

The value of the slope (1460.1 mg/mL) is the εl (eq 3). The y-intercept (10.06) is less than 1 % of the value of the slope and is negligible. The concentrations (c, mg/mL) of caffeine in the top and bottom layers were calculated by dividing the absorbance of caffeine by the Beer's Law constant and are given in **Table 2**.

Table 2. Caffeine concentration in Sample 1 and Sample 2.

Sample name	**Concentration (mg/mL)**	**Area (mAu*s)**
Top 1	0.51	754.00
Bottom 1	0.50	741.50
Top 2	0.57	846.50
Bottom 2	0.57	838.40

In addition to the caffeine concentrations for Samples 1 and 2, other important parameters for the phase separation process are reported in **Table 3**. For example, for the 7.0 mg of caffeine in Sample 1, it was determined that the combined recovery of caffeine in the upper and lower phases is 6.3 mg (% recovery of 90 %). The % recovery for Sample 2 is 89 %. The extent of the phase separation is calculated as the **% volume of the upper phase** (eq 4) and the **phase ratio** (eq 5).

% Volume of upper phase = volume of upper phase / total volume *100 % (4)

phase ratio (Rv) = volume of upper phase / volume of lower phase (5)

If the acetonitrile were to have completely separated, the **% volume of the upper phase** would be 43.1 %. However, on average, the volume was observed to be 30.0 %. Thus,

even at -21 ˚C, 1.25 mL of acetonitrile remains in the lower layer. Using the volumes of the major components of this system at room temperature (acetonitrile = 5.00 mL, water = 5.00 mL, glycerol = 1.59 mL), the maximum possible **phase ratio** was estimated to be 0.759 for this system and the **phase ratio** was observed to be 0.438. Again, this also demonstrates that even at -21 ˚C, 1.25 mL of acetonitrile remains in the lower aqueous layer.

Table 3. Caffeine table of data.

Caffeine Extraction Data	**Sample 1**	**Sample 2**
Volume of upper phase (mL)	3.70	3.80
Volume of lower phase (mL)	8.80	8.70
% Volume of organic phase	**29.60**	**30.40**
Phase ratio (Rv)	**0.42**	**0.44**
Caffeine concentration in upper layer (mg/mL)	0.51	0.57
Caffeine concentration in lower layer (mg/mL)	0.50	0.57
Partition coefficient (K_{PC})	**1.00**	**1.00**
mg of caffeine in the upper layer	1.89	2.17
mg of caffeine in lower layer	4.40	4.96
mg of Caffeine in both upper and lower layers	6.30	7.10
Original mg of caffeine used	7.00	8.00
% Recovery	**90**	**89**

The partition coefficient, K_{PC}, (eq 1) is an important equilibrium parameter that indicates how analytes are distributed between the upper and lower phases in a two-phase mixture.

For example, the octanol/water partition coefficient for caffeine (K_{OW})[26] is 26 at 20 ˚C which indicates that caffeine has a high affinity for the upper octanol phase. As shown in **Table 3**, Samples 1 and 2 each have a partition coefficient of 1.0.[27] This indicates that equal concentrations of caffeine were found in the top and bottom layers at equilibrium at -21 ˚C. Comparing K_{OW} and K_{PC}, it was noted that caffeine has a much greater affinity for the octanol layer than acetonitrile. However, the temperature differences in these two systems are large (20 vs. – 21 ˚C) and there is a need to be careful about making too much of a comparison between the partition coefficients. To make a more reliable comparison, future comparisons of K_{OW} and K_{PC} for caffeine should be done at the same temperature.

Cashew nutshell extractions

This research work is focused on the extraction of cashew nutshell liquid from cashew nutshells using the PIE method. In addition, the PIE extractions are compared to other extraction methods. Photographic figures of the PIE extraction of cashew nutshells are shown in **Figure 13**. These show an upper acetonitrile-rich layer that is darker in color compared to the lower aqueous layer. The figure also shows that the upper layer is physically separated from the denser cashew nutshells. This indicates an unforeseen benefit of the PIE extraction as it also helps to separate the organic layer from the solid components.

[26] Freire, M. G., Neves, C. M. S. S., Marrucho, I. M., Lopes, J. C., Rebelo, L. P. N., Coutinho, J. (2010). High-performance extraction of alkaloids using aqueous two-phase systems with ionic liquids. *Green Chem.*12, 1715-1718, https://doi.org/10.1039/c0gc00179a.

[27]Recently, Mueller (Josephine Mueller, Extraction of Carvone from Spearmint Using the PIE Method, M.S. Thesis, Governors State University, 2022) measured K_{PC} for caffeine for the PIE process to be 0.94 +/- 0.02. However, this was measurement was based on a 70:30 ratio of acetonitrile and water.

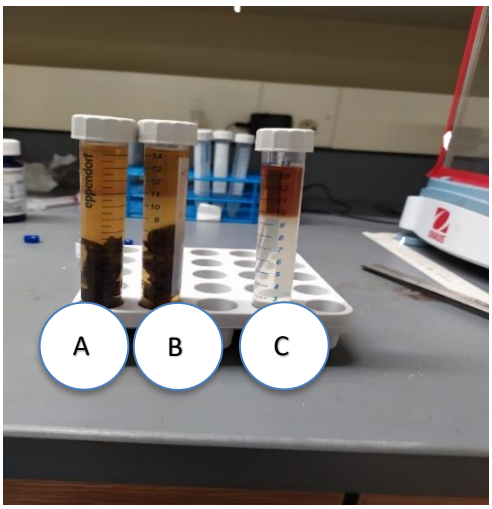

Figure 13. Partitioning phase of the PIE method (**A and B**) and Soxhlet extracted CNSL (**C**).[28]

Starting with 2.06 g of cashew nut shells, 0.42 g of CNSL was obtained. However, this contains trace amounts of water and glycerol. To obtain a more accurate measure of water-free and glycerol-free CNSL, the extract was dissolved in cyclohexane and washed with water to remove glycerol. The cyclohexane solution was then dried with brine and anhydrous sodium sulfate to give 0.33 g of CNSL in 16.0 % yield.

In **Table 4**, a summary of the three extraction methods employed in this study was provided. A higher yield was obtained using cyclohexane, 18.5 %. This is likely due to a longer extraction time of 24 hours compared to a few minutes in the PIE extraction. In addition, a 20.4 % yield was obtained using the Soxhlet extraction method. This extraction used a much larger mass (6 x) of cashew nutshells, a greater volume of solvent relative to the cashew shells (17 mL/g vs. 5 mL/g) and the extraction was performed with boiling acetonitrile (b.p. 82 ˚C). Future experiments should aim to optimize the PIE extraction

[28] The Soxhlet extracted CNSL was a minor experiment carried out using the PIE method to confirm that, indeed, the CNSL from the original PIE method experiment (1st and 2nd from the left) will partition to the upper phase.

using a larger amount of material, longer extraction times and, higher extraction temperatures.

Table 4. Comparison of extraction methods.

Extraction parameters	Soxhlet with acetonitrile	cyclohexane	PIE (Polyol induced extraction) method
Sample name	Sample 1	Sample 2	Sample 3
Mass of CNS	12.01 g	2.05 g	2.06 g
The volume of Solvent used	200 mL, Acetonitrile	10 mL, cyclohexane	10 mL, acetonitrile/water (1:1)
Extraction time	2 h	24 h	< 2 min
Extraction temp	82 ˚C	Room temperature	Room temperature, then, -20 ˚C
Mass of oil (CNSL)	2.45 g	0.38 g	0.17 × 1.95 = 0.33 g[a] (See Note)
% Yield	20.4 %	18.5 %	16.0 %

[a]Note. The volume of the Top layer from the PIE method is 3.9 mL, 2 mL was removed from the 3.9 mL for gravimetry analysis. In order to scale up the result, 3.9 mL / 2 mL = 1.95. Therefore, our dilution factor is 1.95.

Analysis of anacardic acid standard

To assist with the analysis of the chemical composition of the CNSL extracts, an analysis of anacardic acid from a commercial source was performed. This sample was indicated in the product literature to contain 95 % saturated anacardic acid (see **Figure 6** for structures of anacardic acid and the unsaturated derivatives). An HPLC analysis of a 1.0

mg/mL solution of this sample gave the chromatogram shown in **Figure 14** and only the saturated derivative of anacardic acid in this analysis was detected.

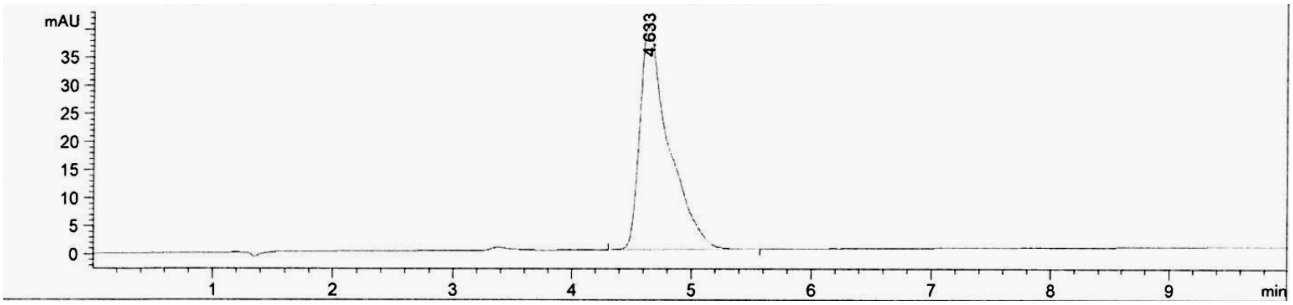

Figure 14. HPLC chromatogram of anacardic acid (saturated) standard (1 mg/mL) monitored at 244 nm.

A GC-MS analysis of the same sample gave the gas chromatogram shown in **Figure 15**. This sample shows a major peak at a retention time of 14.5 min, which is attributed to the saturated derivative of anacardic acid. Prior to this peak are three overlapping peaks attributed to the monoene, triene, and diene derivatives of anacardic acid, respectively. The MS spectra of each of the four derivatives (**Figures 16 – 19**) reveal a molecular ion consistent with a loss of 44 amu corresponding to a loss of carbon dioxide for each derivative.

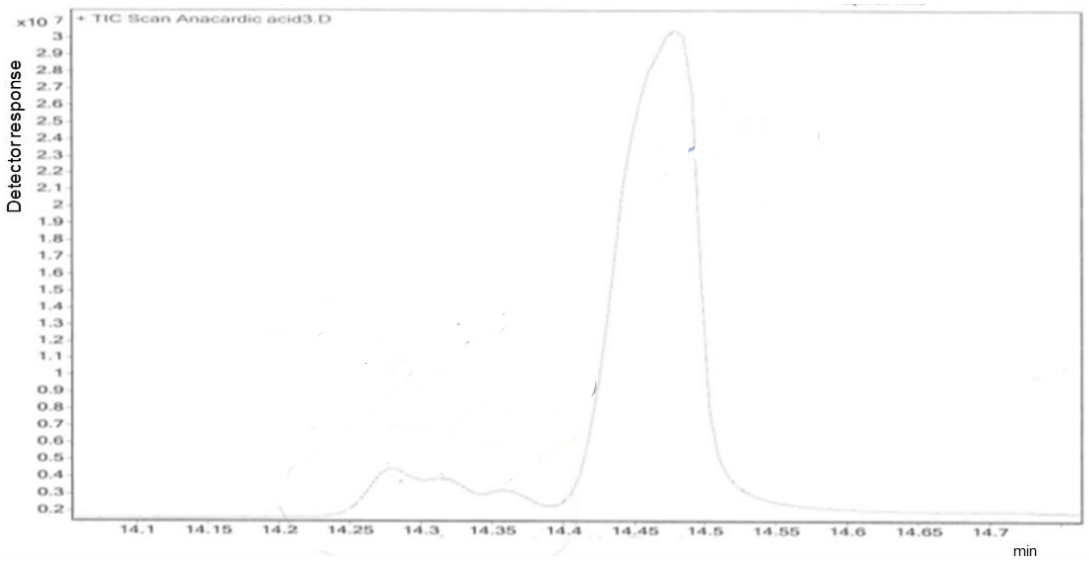

Figure 15. GC-MS Chromatogram of the commercially purchased sample of anacardic acid.

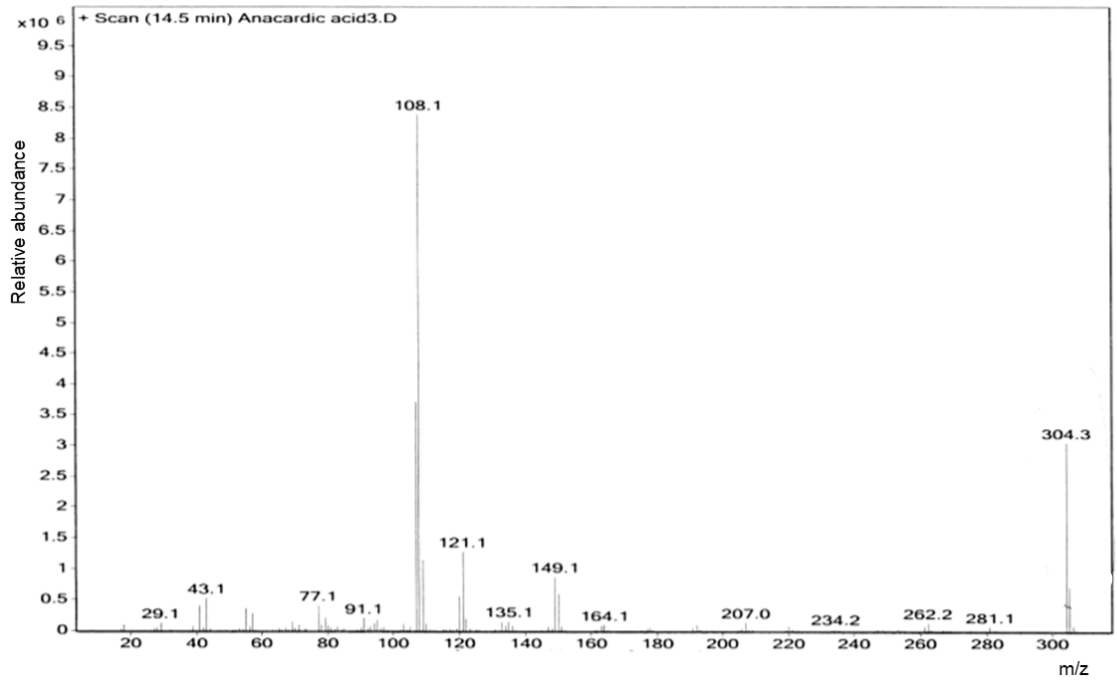

Figure 16. Mass spectrum scan of the GC peak at 14.5 min attributed to saturated anacardic acid showing m/z = 304.3 amu corresponding to M^+ - 44 amu.

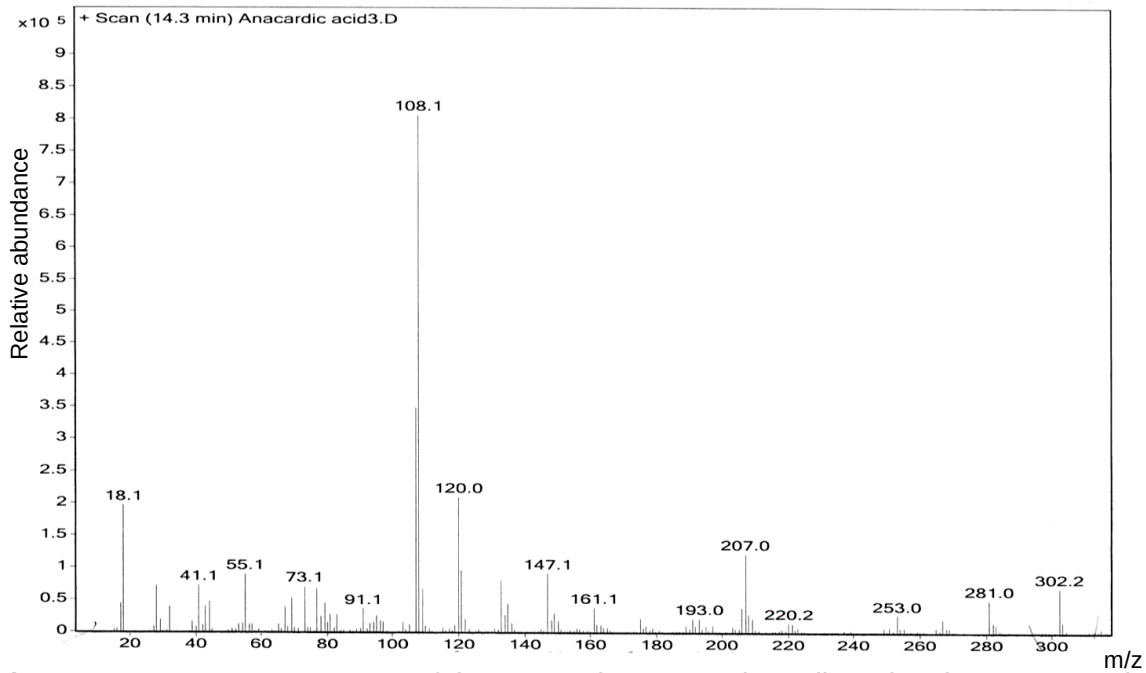

Figure 17. Mass spectrum scan of the GC peak at 14.3 min attributed to the monoene derivative of anacardic acid showing m/z = 302.2 amu corresponding to M^+ - 44 amu.

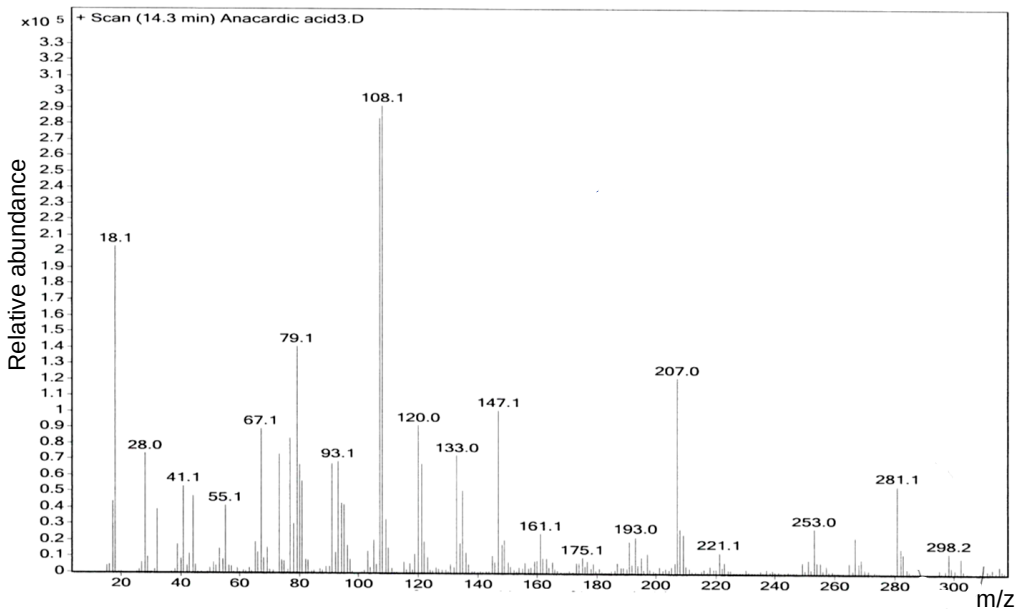

Figure 18. Mass spectrum scan of the GC peak at 14.3 min attributed to the triene derivative of anacardic acid showing m/z = 298.2 amu corresponding to M^+ - 44 amu.

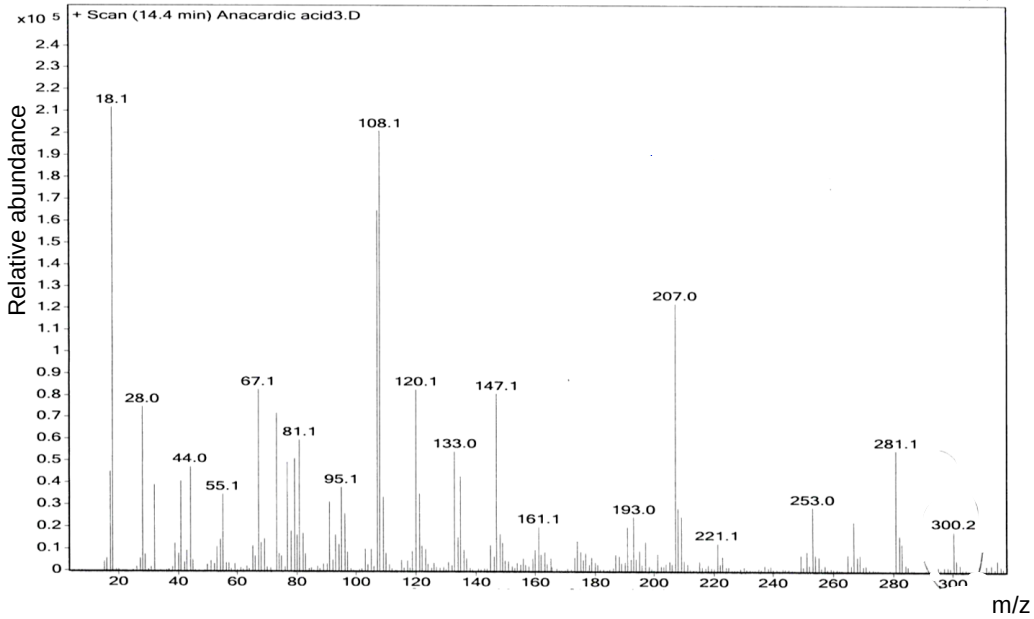

Figure 19. Mass spectrum scan of the GC peak at 14.4 min attributed to the diene derivative of anacardic acid showing m/z = 300.2 amu corresponding to M^+ - 44 amu.

The difference between the observed and expected molecular ion results can be explained by a decarboxylation reaction occurring during the GC-MS experiment. The molecular weight of saturated anacardic acid is 348 amu. After the loss of the carbon

dioxide of 44 amu, the resulting molecular ion is 304 amu (**Figure 16**). At the same time, anacardic acid monoene, diene, and triene each have lower masses by 2, 4, and 6 amu, respectively, corresponding to the of double bonds present in each derivative (**Figures 17 – 19**).

There are two possible places in the GC-MS experiment where decarboxylation is likely to occur. The first place is in the GC inlet, where the temperature is 250 ˚C. The second place is in the mass ionization chamber, where the compound is ionized at 50 eV. Below is the comparison of the GC retention times of the anacardic acid standard and the cardanol derivates found in the extract. This comparison is evidence that the decarboxylation reaction is occurring in the GC inlet. A thermally driven 4n+2 pericyclic mechanism for the decarboxylation was proposed as shown in **Figure 20**.

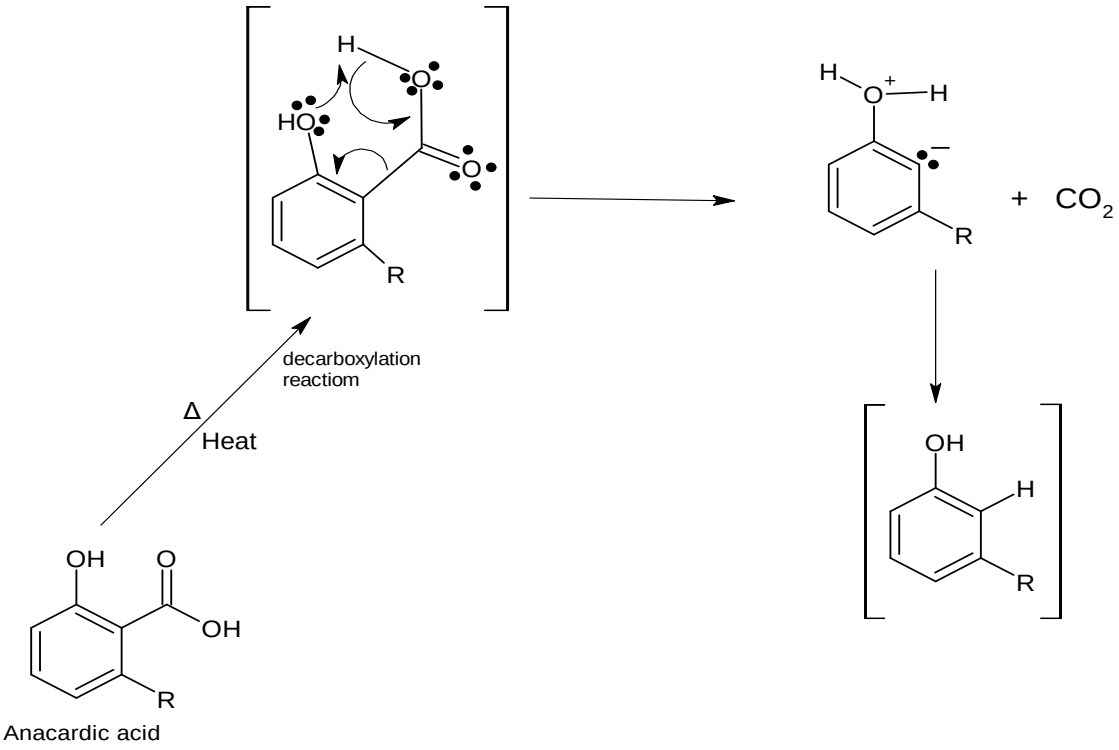

Figure 20. Mechanism of decarboxylation reaction of the molecular ion of anacardic acid.

Analysis of cashew nutshell liquid extracts

After performing a PIE extraction on cashew nutshells, the upper acetonitrile-rich layer was analyzed by HPLC. As shown in **Figure 21,** the upper layer shows three major peaks with retention times of 2.31, 2.65, and 3.28 min. In addition, the cyclohexane (**Figure 22**) and Soxhlet extractions (**Figure 23**) show very similar liquid chromatograms. This indicates that all three of the extraction methods yield products of similar chemical composition.

To see if one of the major peaks corresponds to anacardic acid, a spiked sample of the PIE extract was prepared with the anacardic acid standard and obtained a surprising result shown in **Figure 24**. The chromatograms show that none of the three major peaks correspond to saturated anacardic acid. Instead, a peak at 4.626 min was observed, and an increase in the intensity of a region of the chromatogram containing two minor peaks at 4.561 and 4.913. This experiment clearly indicates that saturated anacardic acid, if present at all, may only be a minor constituent of the sample. So, assuming that one of the minor peaks at 4.561 and 4.913 is the saturated anacardic acid, then, based on the peak area, this represents ~2 % of the sample. The HPLC chromatogram of the samples in the aqueous layer is shown in **Figures 25 – 27**, and this confirms, within limits of detection, that the saturated form of anacardic acid is not present in the bottom layer.

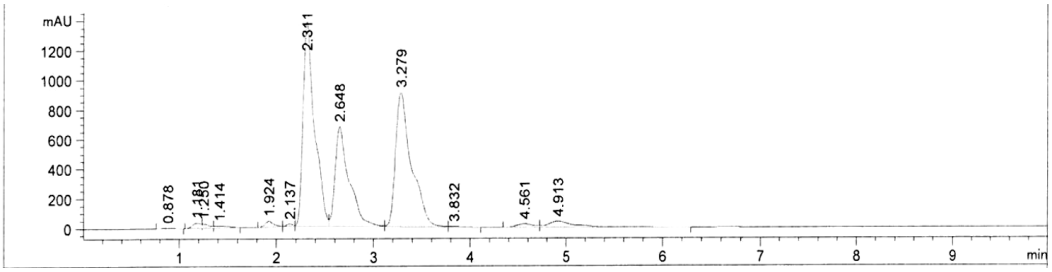

Figure 21. HPLC chromatogram of PIE extracted CNSL monitored at 244 nm.

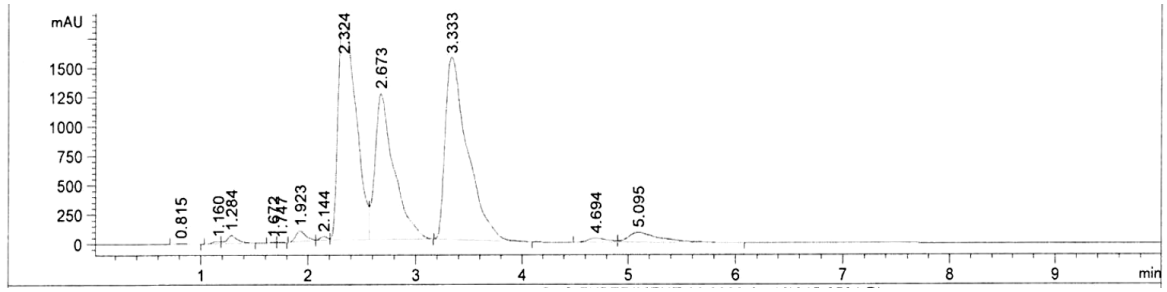

Figure 22. HPLC chromatogram of Cyclohexane extracted CNSL monitored at 244 nm.

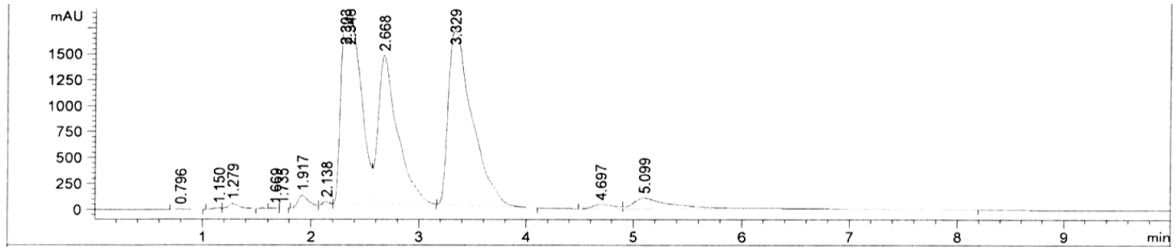

Figure 23. HPLC chromatogram of Soxhlet extracted CNSL monitored at 244 nm.

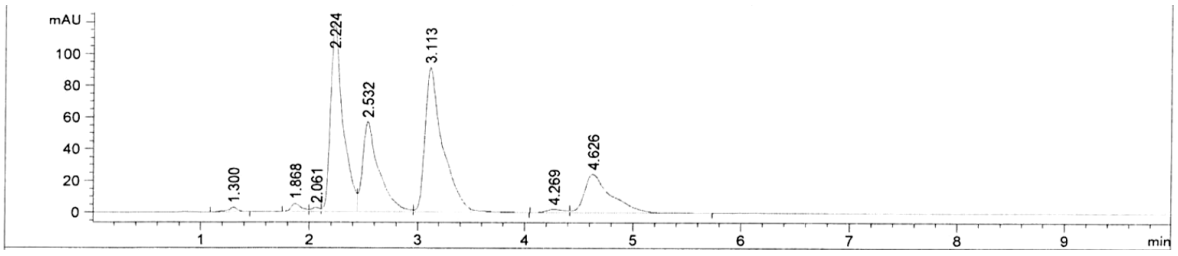

Figure 24. HPLC chromatogram of PIE extracted CNSL with spike monitored at 244 nm.

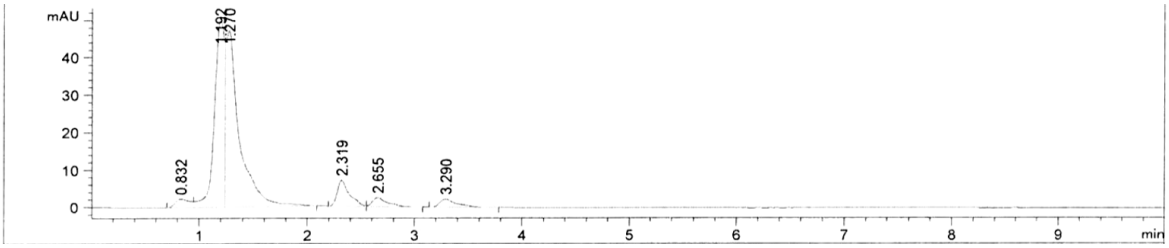

Figure 25. HPLC chromatogram of PIE extracted CNSL (aqueous layer) monitored at 244 nm.

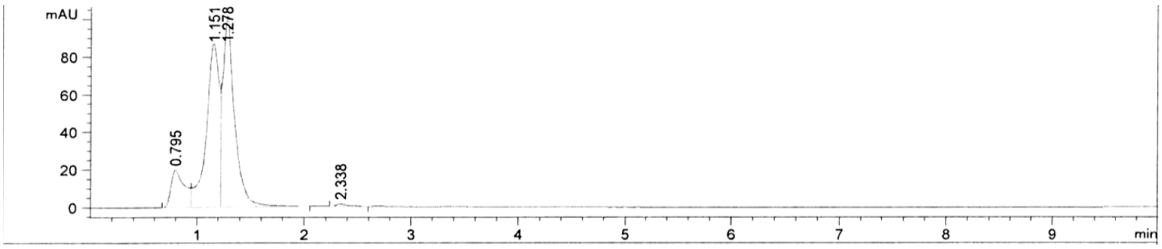

Figure 26. HPLC chromatogram of Cyclohexane extracted CNSL (aqueous layer).

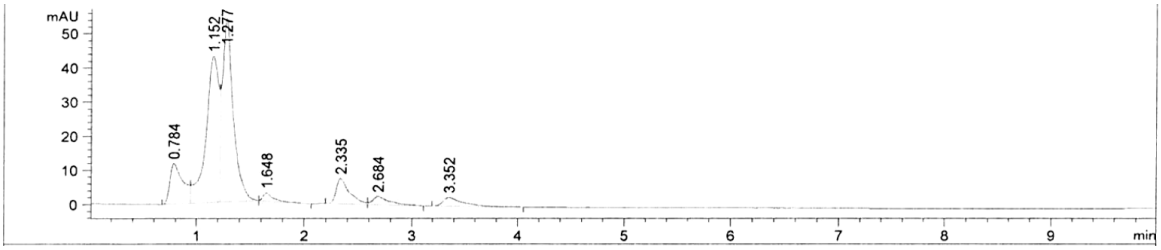

Figure 27. HPLC chromatogram of Soxhlet extracted CNSL (aqueous layer).

GC-MS analysis of Anacardic Acid present in CNSL

A GC-MS analysis of Sample 3 which is the CNSL isolated from the PIE extraction was performed using the same parameters as the anacardic acid standard.

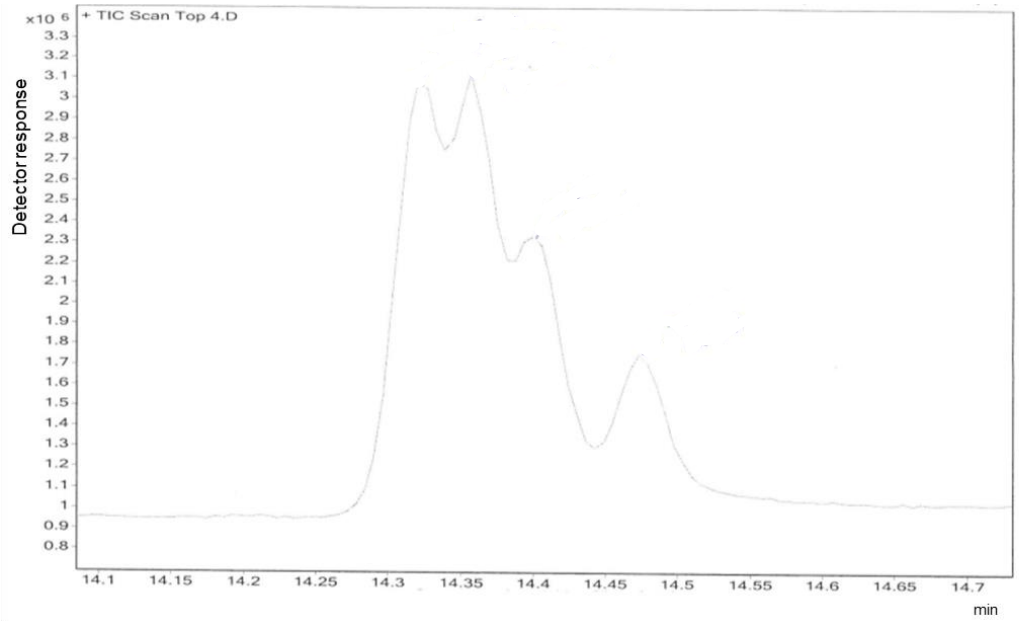

Figure 28. GC-MS chromatogram of CNSL Sample 3.

The chromatogram of Sample 3 is shown in **Figure 28**. It gives a peak at 14.3 min corresponding to the monoene, 14.4 min corresponding to the triene, 14.4 min corresponding to the diene, and 14.5 corresponding to the saturated form. While the same order of elution as the standard was observed, the relative intensities of the peaks corresponding to the monoene, triene and diene are different. In addition, only a trace amount of saturated anacardic acid was detected. As will be discussed below, ^{1}H NMR evidence indicates that the compounds observed in this sample are not anacardic acid derivatives but are cardanol derivatives.

As with the anacardic acid standard, Sample 3 also shows molecular ions corresponding to the loss of carbon dioxide giving m/z's corresponding to the saturated (304.3), monoene (302.2), triene (298.2), and a diene (300.3), as shown in **Figures 29 - 32**. Surprisingly, these show the same retention times as those of the anacardic acid standard. However, as will be discussed below, a ^{1}H NMR study indicates these signals are due to cardanol derivatives.

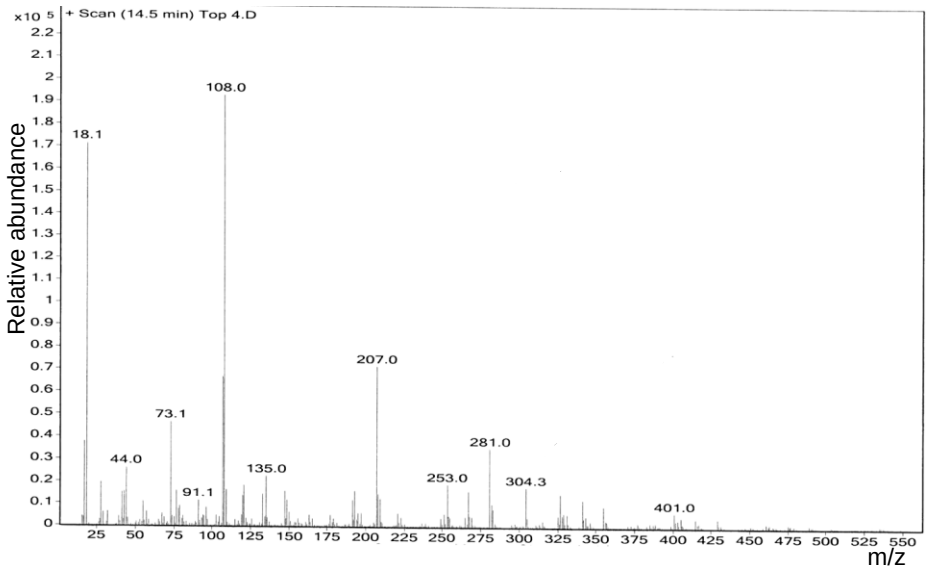

Figure 29. MS scan at 14.5 min of Sample 3 corresponding to the saturated cardanol.

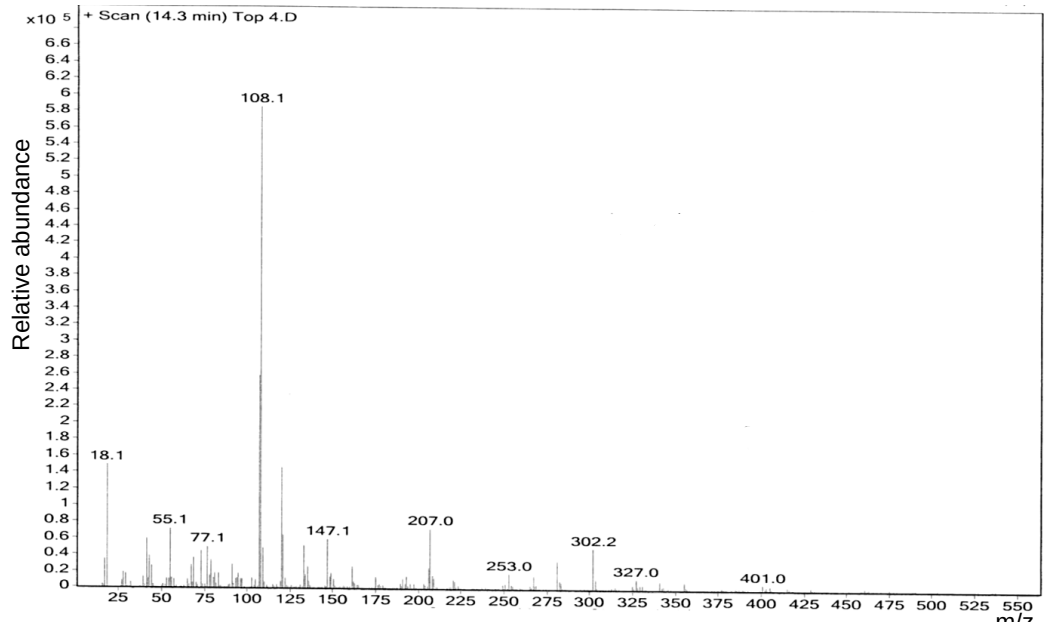

Figure 30. MS scan at 14.3 min of Sample 3 corresponding to the monoene of cardanol.

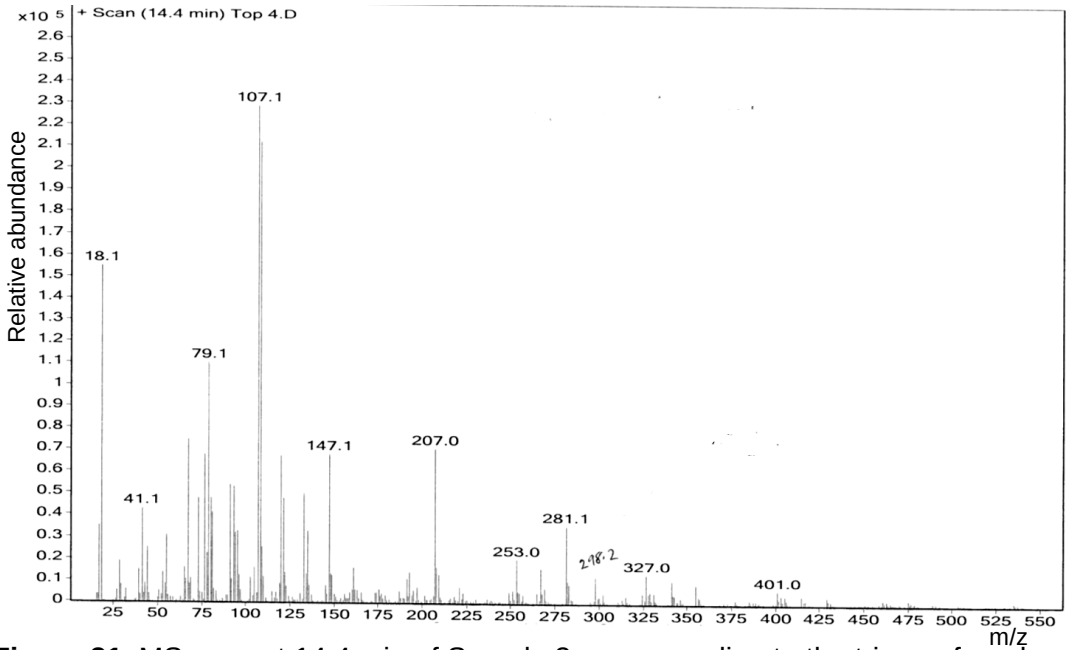

Figure 31. MS scan at 14.4 min of Sample 3 corresponding to the triene of cardanol.

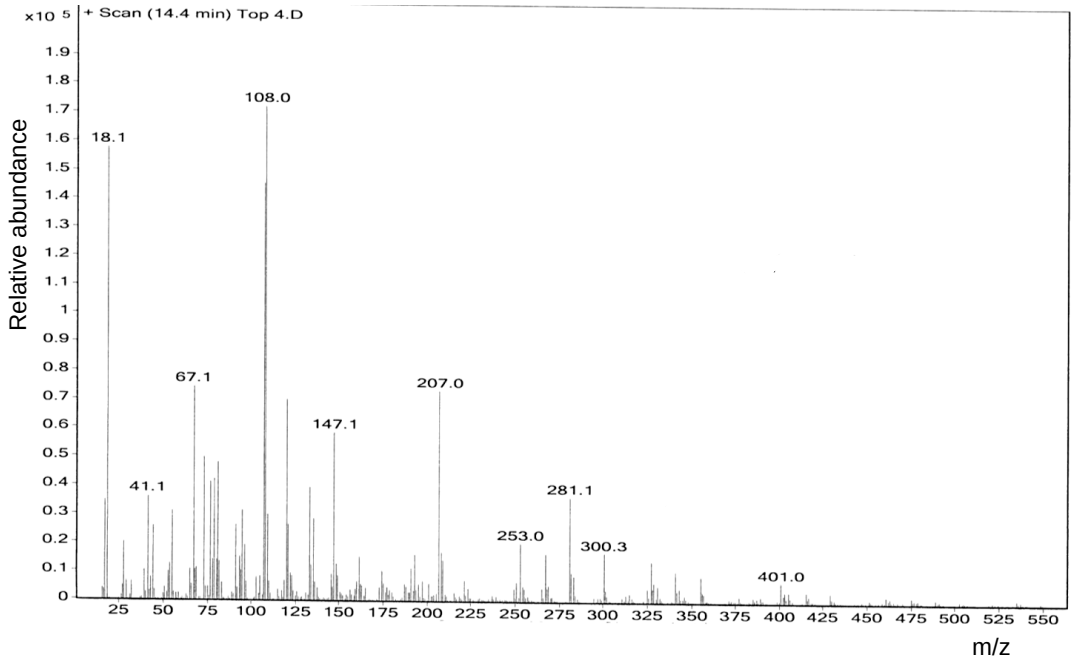

Figure 32. MS scan at 14.4 min of Sample 3 corresponding to the diene of cardanol.

^{1}H NMR study of CNSL extract

The ^{1}H NMR spectrum of a Soxhlet-extracted CNSL is shown in **Figure 33**. This spectrum shows four prominent structural features: 1) a broad phenol OH resonance at 9.2 ppm and expanding the spectrum to 15 ppm does not show a COOH resonance, 2) four signals in the aromatic C-H region (6 – 7.5 ppm), 3) three signals in the vinylic C-H region (4.5 – 6 ppm), and 4) signals from 0.9 to 3.0 ppm corresponding to aliphatic groups.

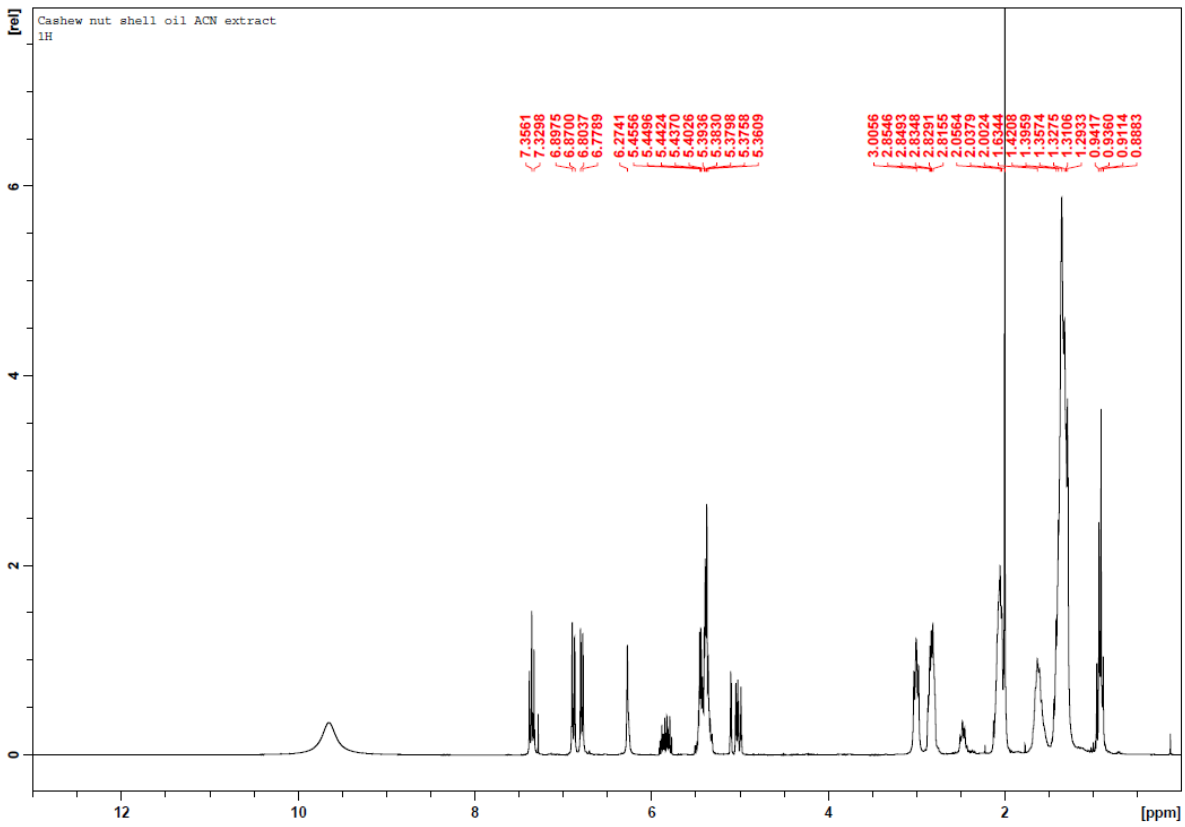

Figure 33. ^{1}H NMR spectrum of a Soxhlet extracted CNSL.

Focusing on the aromatic C-H region, if the extract were to consist of anacardic acid derivatives, three sets of signals would have been observed, with each integrating into one proton due to the three distinct protons in the aromatic ring. This is illustrated in **Figure 34** along with the calculated ^{1}H NMR[29] for the aromatic/vinylic region of the triene derivative of anacardic acid.

[29] Simulate and predict NMR spectra, https://www.nmrdb.org/ (accessed December 20, 2022). See also: Binev, Y., Marques, M.M., Aires-de-Sousa, J. (2007). Prediction of 1H NMR coupling constants with associative neural networks trained for chemical shifts. *J. Chem. Inf. Model. 47*(6), 2089-2097. http://dx.doi.org/10.1021/ci700172n

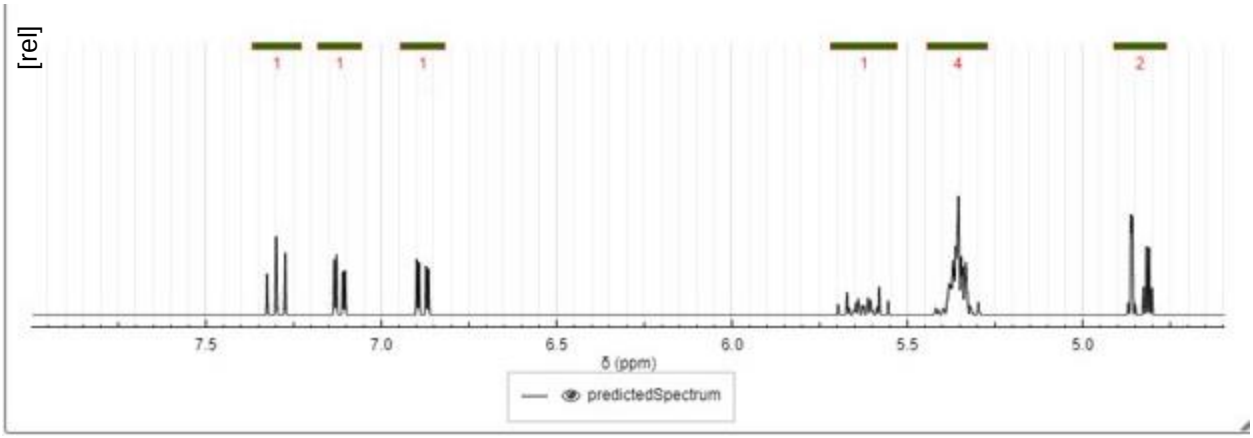

Figure 34. Calculated ^{1}H NMR spectrum[29] of the aromatic/vinyl region triene derivative of anacardic acid.

Instead, four sets of signals were observed which match perfectly well with the calculated ^{1}H NMR of the aromatic/vinylic region of the triene derivative of cardanol. To see this more clearly, the experimental spectrum with the aromatic/vinylic region expanded (**Figure 35**) and the calculated spectrum (**Figure 36**) were shown below. Integrations of the aromatic protons of 1.3 : 1.3 : 1.3 : 1.0 are observed, which are close to the expected values of 1 : 1 : 1: 1.[30]

[30] The discrepancy in the experimental vs. expected values is likely due to a longer relaxation time for the proton at 6.25 ppm. This can be further explored by setting a long relaxation delay in the ^{1}H NMR acquisition parameters.

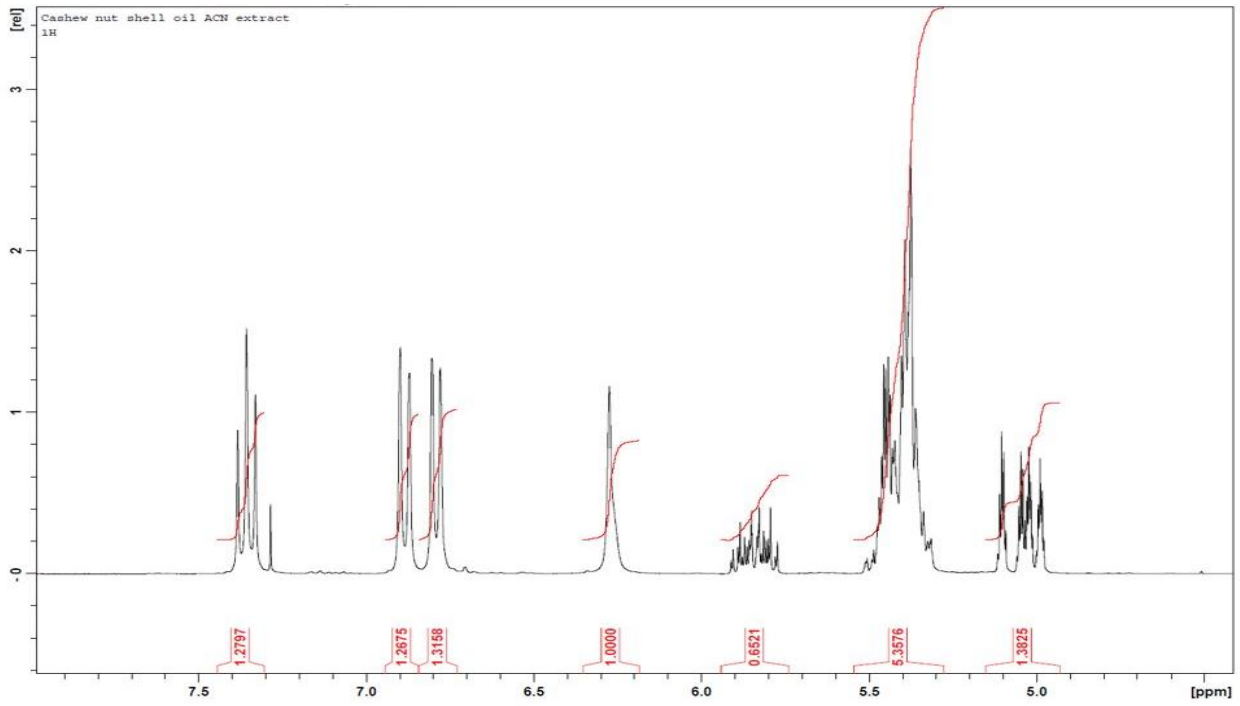

Figure 35. Experimental ^{1}H NMR spectrum of the CNSL extract expanded to show the aromatic/vinylic region.

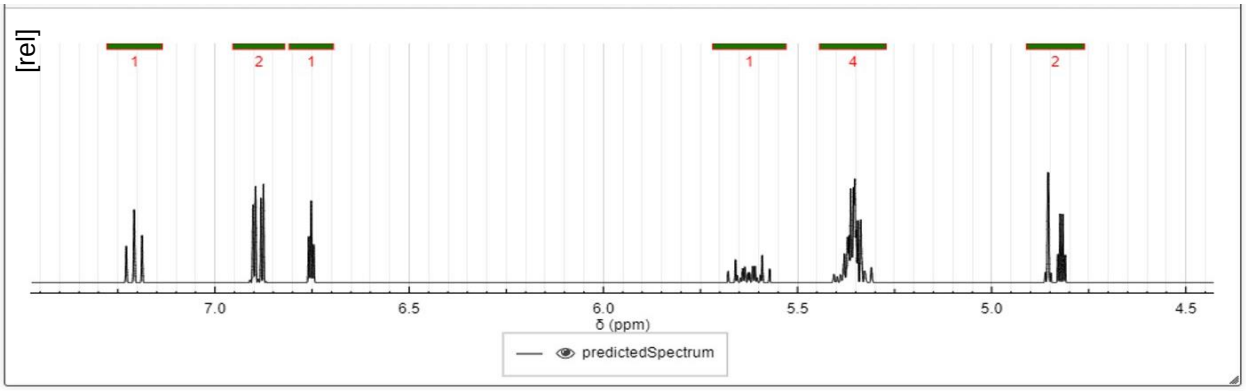

Figure 36. Calculated ^{1}H NMR of the triene derivative of cardanol.[29]

In addition, there is good agreement between the experimental and calculated proton signals in the vinylic region. In this region, the multiplet at 5.25 ppm uniquely corresponds to the triene derivative. The complex multiplet between 5.3 and 5.6 ppm corresponds to overlapping signals of the monoene, diene, and triene. Because of the contributions of the monoene and diene derivatives, this signal has a higher integral intensity (5.36 vs.

2.6) than it would have if it only consisted of the triene. The multiplet between 4.9 and 5.1 also uniquely corresponds to the triene. This ^{1}H NMR study indicates that the extract is mostly composed of cardanol derivatives, and by inspection of the aromatic region, anacardic acid derivatives are not observed.

This leads to several interesting questions about the formation of the cardanol derivatives in the CNSL extract, and also in the anacardic acid standard. First, are the cardanol derivatives in the extracts a result of the extraction procedures, or were they already formed in the cashew nutshells? Here, it is unlikely that mild conditions of the PIE and cyclohexane extractions would have caused the anacardic acids to degrade to cardanol. Since very little about the storage and shipping conditions of the cashew nutshells are known, it is suggested that most likely, any anacardic acids that were present had degraded to cardanol derivatives prior to this analysis. An interesting future experiment would be to test cashew nutshells from a more secure source with known storage and shipping conditions. Second, what is the source of cardanol in the GC-MS analysis of the anacardic acid standard? Here, it is known that the gas chromatograms of the standard and the extracts are similar. There is also good evidence that the chromatograms of the extract are from cardanol derivatives. This leads to the conclusion that the anacardic acid standard has degraded to cardanol early in the GC-MS experiment, so, early, the GC retention times match those of cardanol. This indicates that the anacardic acid standard must have degraded in the GC inlet, which was set at 250 ^{o}C, and, as previously mentioned,[13] the decarboxylation temperature of anacardic acid is 92.5 - 93 $^{\circ}$C. This can be further tested by running a GC-MS at a lower temperature to prevent it from decarboxylating in the inlet.

Conclusion

The PIE extraction method was used in this research to extract anacardic acid from cashew nutshell liquid. Other extraction methods, including the cyclohexane extraction method and Soxhlet method of extraction, were also used, and the data were compared to the PIE method. The percentage yield of anacardic acid in the cashew nutshell liquid from the PIE method, cyclohexane extraction, and Soxhlet method was recorded to be 16.0 %, 18.5 %, and 20.4 %, respectively. This was then analyzed on the HPLC, GC-MS, and NMR instruments. CNSL extracted from CNS was analyzed by HPLC and showed a peak at 4.913 mins and 695.70 mAu*s as the retention time and peak area, respectively. The extracted sample was compared with a purchased anacardic acid standard (saturated), and it showed a retention time of 4.63 min and 668.38 mAU*s as the peak area. The HPLC peak area analysis revealed that the saturated form of anacardic acid was only a minor component (about 2 %). However, the results from the GC-MS indicated that CNSL is mostly comprised of cardanol derivatives due to a decarboxylation reaction. This decarboxylation reaction occurred in the GC-MS due to the loss of the carboxylic acid group, which brings the mass/charge of anacardic acid monoene, diene, triene, and saturated form to 298.2, 300.2, 302.2, and 304.3 respectively. The NMR instrument was finally used to confirm the structure of components present in the CNSL, with the ^{1}H NMR showing signals confirming cardanol's presence in the CNSL.

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