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MEASUREMENTS OF DISTRIBUTION COEFFICIENTS AND LIPOPHILICITY VALUES FOR OLEANOLIC ACID AND BETULINIC ACID EXTRACTED FROM INDIGENOUS PLANTS BY HOLLOW FIBRE SUPPORTED LIQUID MEMBRANE

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ABSTRACT. Hollow fibre supported liquid membrane (HFSLM) has been applied in the measurements of distribution constants, K_D and lipophilicity (log P) values for the isomeric triterpenic acids, betulinic acid (BA) and oleanolic acid (OA) isolated from indigenous plants. The results have shown that BA had an optimum pH of 3.5 while the optimum sample pH for OA was ranging from 0.5 to 2.5. The log P values obtained for BA and OA were 6.61 and 6.12, respectively. The K_D value obtained for BA was 0.29 while that for OA 0.16. The method has advantages of simplicity, use of minimal organic solvents and the ability to selectively extract only one isomer (OA) in the mixture with BA by optimizing pH conditions.

KEY WORDS: Betulinic acid, Oleanolic acid, Hollow fibre supported liquid membrane, Distribution coefficient, Lipophilicity

INTRODUCTION

Betulinic acid (BA) and oleanolic acid (OA) are isomeric triterpenoid compounds with medicinal properties that have been isolated from many indigenous plants species. Betulinic acid for example, has been isolated from *Quisqualis fructus* (Fam. Combretaceae), *Coussarea paniculata* (Fam. Rubiaceae), Argentinean legume *Caesalpinia paraguariensis* (Fam. Fabaceae), *Vitex negundo* (Fam. Verbenaceae), *Ilex macropoda* (Fam. Aquifoliaceae), *Anemone raddeana* (Fam. Ranunculaceae), and *Doliocarpus schottianus* (Fam. Dilleniaceae), to mention a few [1]. Oleanolic acid has been isolated from *Rosa woodsii* (Fam. Rosaceae), *Prosopis glandulosa* (Fam, Fabaceae), *Phoradendron juniperium* (Fam. Viscaceae), *Syzygium claviflorum* (Fam. Myrtaceae), *Hyptis captata* (Fam. Labiatae), and *Temstromia gymnanthera* (Fam. Theaceae), amongst others [2]. Betulinic acid has been reported to selectively induce apoptosis in tumor cells through mitochondrial permeability transition pathway [3-5]. Oleanolic acid on the other hand has been used for treatment of lung cancer and has also anti-inflammatory, anti-hyperlipidemic properties and antitumor effects [6, 7]. Both BA and OA are also known to have anti-HIV-1 properties [8, 9].

Due to the medicinal values of BA and OA, it is imperative that the bioactivity properties for these compounds be determined using simple and reliable methods. The bioactivity of medicinal compounds can be determined by their lipophilicity values as well as the distribution coefficient (K_D). Knowledge of the relationships between affinity to lipids and biological (anesthetic) activity of chemical substances was firstly reported by Overton and then by Meyer and Baum [10-12]. It is therefore important to develop simple and rapid procedures for determining lipophilicity parameters and K_D values for bioactive compounds with very small samples.

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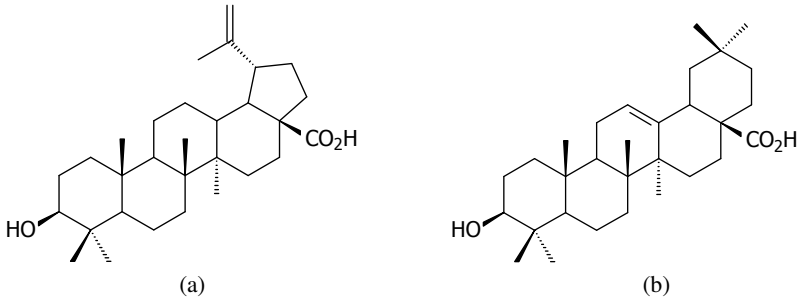
Traditionally, tube method has been predominantly used for the evaluation of lipophilicity values [13]. This method involves mixing a known amount of solute in a known volume of octanol and water, followed by measuring the distribution of the solute in each solvent using UV/Vis spectroscopy or other methods [14]. The method has advantages of high selectivity, broad range of solutes (neutral and charged compounds applicable) and also the fact that the chemical structure does not have to be known beforehand. The limitation of the method is realized from the fact that the method is time consuming.

In this work, we report the use of hollow fibre supported liquid membrane (HFSLM) technique to determine the log P values of BA and OA in addition to its role as extraction and separation means for the two isomers. Reversed phase high performance liquid chromatography (RPHPLC) with UV-DAD detection as well as HPLC coupled to a mass spectrometer was used for separation and detection of the extracts.

HFSLM is a non-porous membrane technique which has been widely used for selective pre-concentration of both organic and inorganic extracts [15-17]. With the HFSLM technique, the analyte is contained in an aqueous sample solution and for it to be extracted it should be in its neutral form. The organic solvent is impregnated in the pores of the hollow fibre which separates the sample solution from stripping (acceptor) solution contained in the lumen of the hollow fibre [18]. The concentration gradient develops between sample solution and acceptor phase which allows the dissolved analytes to be transported through the organic phase (impregnated in the pores of the hollow fibre) to acceptor solution (in the lumen of the hollow fibre) [19].

HFSLM was chosen for the study because of its numerous advantages. HFSLM techniques incorporate low pore sized hollow fibre and therefore more efficient in its selectivity. It involves simple extraction set up, easy sample preparation with less preparation time unlike most sample preparation methods. Moreover, there is no direct contact between the sample solution and acceptor phase and therefore ion transfer occurs through the organic solvent filled hollow fibre pores. The extract is very clean as contaminants do not cross the hydrophobic membrane and is highly selective when parameters affecting extraction are well optimized. HFSLM also lowers the limit of detection because of the high pre-concentration factors obtained which result into enhanced signals [20].

In this study, authentic samples of BA and OA were isolated from *Tectonia grandis*, family Verbenaceae (stem bark) and *Syzygium aromaticum*, family Myrtaceae (cloves), respectively. BA/OA mixture was isolated as a co-mixture from the leaves of *Meleleuca bracteata*, family Myrtaceae, 'Johannesburg Gold' (South African Tea Tree). The structures of BA and OA are shown in Figure 1.



(a) (b)

Figure 1. (a) Betulinic acid (BA). (b) Oleanolic acid (OA).

EXPERIMENTAL

Instrumentation and apparatus: HPLC-UV-DAD and HPLC–ESI-MS experiments

Agilent 1200 series HPLC (Wilmington, DE, USA) equipped with auto sampler, degasser, and a UV-DAD detector as well as a C18, XDB column, 4.6 mm x 50 mm x 1.8 µm) were used. The detection wavelength of 254 nm was used for the compounds. The mobile phase was 75% methanol and 25% water. Injection volume was 5 µL at a flow rate of 0.3 mL/min.

The HPLC-ESI-MS system used for this work consisted of an Agilent Hewlett-Packard 1100 Series HPLC connected to Agilent 1100 Series LC/MSD Trap mass spectrometer (Wilmington, DE, USA). The quadrupole temperature of the MS was 120 ºC and the mass chromatograms were acquired in the selected ion mode (SIM) for the negative mode of both OA and BA (m/z = 455). The temperature of the drying gas, N_2, was 300 ºC and the flow rate of the nebulizing gas, N_2, was 40 mL/min, being maintained at 80 psi.

An analytical Mettler AE240 balance was used for all measured weights. Magnetic stirrer bar and stirrer plate (Bibby HB 502) were used during sample extraction. The polypropylene hollow fibre tubing (50 µm wall thickness, 280 µm inner diameter and 0.1 µm pore size) was used with needle of BD micro-fine syringe (needle of 0.3 mm outer diameter, a length of 8 cm and 100 µL holing volume (SGE, Australia). pH meter – 430 Corning was used for all pH measurements.

Chemicals, reagents and materials

Di-n-hexyl ether (DHE), isooctane, octanol and undecane were purchased from Sigma Aldrich (St. Louis, MO, USA), while sodium hydroxide (analytical grade) and hydrochloric acid (32%) were obtained from Merck (Darmstadt, Germany). The organic solvents used as mobile phase during separation (methanol and acetonitrile) were of HPLC grade and were purchased from BDH (Poole, England). Reagent water used was purified using a Mill-Q® reagent water system (Millipore, Molsheim, France).

Buffer solutions of different pH values were prepared according to the standard procedures [22]. The Q3/2 Accurel polypropylene hollow fibre membranes (200 µm wall-thickness, 600 µm inner diameter, 0.2 µm pore size) were obtained from Membrana GmbH (Wuppertal, Germany). A 500-µL HPLC syringe was used for filling the acceptor solution into the lumen of the hollow fibre for extraction as well as flushing out the acceptor after extraction into a 100 µL conical glass microinsert fitting in standard 2-mL autosampler vials.

Plant materials

The leaves of *Melaleuca bracteata* F. Muell. 'Revolution Gold' or *Melaleuca bracteata* F. Muell. 'Johannesburg Gold' (Myrtaceae family) (common names: Johannesburg gold or South African tea tree) were collected from the Nursery of the Ethekweni Municipality Parks and Gardens, KwaZulu-Natal, Durban and authenticated by Prof. H. Baijnath, School of Biological & Conservation Sciences, University of KwaZulu-Natal (UKZN), Durban Centre, South Africa. A voucher number, Glow OO1 was given to a sample of the leaves and deposited at the Ward Herbarium, UKZN, School of Biological & Conservation Sciences. *Syzygium aromaticum* (cloves) were purchased from a local spice shop in Durban, South Africa. *Tectonia grandis* stem barks were collected along Ondo road, Ile Ife, Nigeria. The plant was identified by Mr. Yakubu of the herbarium section of the Faculty of Pharmacy, Obafemi Awolowo University (OAU), Ile Ife.

Analytical procedures

Preparation of plant materials and Isolation of OA and BA

Oleanolic acid. *Syzygium aromaticum* (cloves) (whole, 100 g) were defatted using hexane followed by extraction with dichloromethane and ethyl acetate, successively. The oily residues obtained after the removal of organic solvents under reduced pressure were combined and triturated/pulverized with hexane to give a precipitate of crude OA. Column chromatography of the crude OA over silica gel 60 (230-400 mesh ASTM) using solvent system hexane-ethyl acetate (7:3) gave pure OA with spectral data identical with the literature values [21].

Betulinic acid. T. grandis stem barks were oven-dried (60 °C) for 2 days and powdered using a laboratory mill. The powdered stem barks (860 g) were macerated in ethyl acetate (3 L) for 24 h, filtered and evaporated to a minimum volume *in vacuo* using a rotary evaporator. The residue was allowed to dry at room temperature to give crude BA. Upon recrystallisation from methanol, pure BA (2.64 g) was obtained.

Betulinic acid/oleanolic acid mixture. Revolution gold (dried leaves, 342 g) were exhaustively extracted with ethyl acetate. The extract was evaporated to complete dryness *in vacuo* using a rotary evaporator, to yield a crude extract (34 g). A portion of the solid crude extract (29 g) obtained was purified by column chromatography over silica gel 60 (230-400 mesh ASTM) with hexane-ethyl acetate (7:3) solvent system as the mobile phase. The obtained BA/OA solid was charcoaled to give colourless mixture of BA/OA.

Preparation of analyte sample. The samples of pure BA and OA and the mixture of the two acids were extracted from *Tectona grandis*, *Syzygium aromaticum*, and *Melaleuca bracteata* 'Revolution gold', respectively. BA and OA mixture (0.1 g) was placed in a glass vial and dissolved with methanol (20 mL). From this solution, 2 mL were transferred into 250 mL volumetric flasks and made up to volume with deionized water. The pH meter was calibrated using pH 4 and pH 7 solutions. From the analyte sample, 10 mL aliquots were transferred into vials and adjusted to pH ranging from 0.5 to 4.5 for optimization experiments.

Preparation of standard solution. Standard solutions were prepared from pure BA and OA by dissolving 0.02 g of each compound in 10 mL methanol followed by transferring 3 mL of resulting solution into 100 mL volumetric flask which was further diluted to give series of standard solutions. This was done to identify the acids according to how they elute from the HPLC column.

Preparation of HFSLM and analyte sample extraction and separation. The hollow fibre tube used in this work was Q3/2 Accurel polypropylene hollow fibre membrane (200 µm wall thickness, 600 µm inner diameter, 0.2 µm pore size) obtained from Membrana GmbH (Wuppertal, Germany). The hollow fibres were cut into 4 cm length pieces, soaked in acetone for 20 minutes for cleaning and one end of each piece was sealed. The lumen of the hollow fibre was filled completely with pH 8 buffer solution (acceptor aqueous phase) using a needle of BD micro-fine syringe, 100 mL. The syringe was attached to the open end of the fibre and the outer membrane of the hollow fibre was impregnated with di-n-hexyl ether (organic phase) for about 5 seconds. The excess organic solvent was then washed with deionized water [19].

The HFSLM was immersed in pH 0.5 analyte sample in a vial with a magnetic stirrer bar on a stirrer plate, and extracted with constant stirring for a period of 30 minutes. Thereafter the accepter solution was flushed into a modified HPLC sample vial consisting of well sized micro pipette tip and the extract was injected into the HPLC. The resulting solution from extraction

was further extracted for 30 min (each sample extracted twice at the same pH) and the pH was increased to 3.5 and the same extraction procedure was followed to exhaustively extract BA component.

Determination of K_D and lipophilicity (log P) experiments. For the determination of distribution coefficient and lipophilicity values for a sample solution with the same concentration levels and at optimal conditions (acceptor pH 8.0, room temperature) were extracted after having been enriched at different length of times. The enrichment time in this case was varied from 5 to 90 min. The amount extracted and the flux rate were then determined which made it possible to evaluate both K_D and log P values for BA and OA.

RESULTS AND DISCUSSION

The choice of organic solvent

Selection of an organic solvent for HFSLM extraction is very crucial and many considerations had to be taken into account to get a suitable solvent. Some of these considerations include that the solvent has to have low volatility to prevent loss due to evaporation during the extraction process, low water solubility and also the analytes must be soluble in it. In this work, four different organic solvents were evaluated for their suitability. The solvents evaluated included DHE, n-undecane, DHE + n-undecane (1:1), toluene and isooctane. Of the various liquid membranes tested, DHE was found to be the best liquid for the selective extraction of OA and BA.

Sample pH optimization for the selective HFSLM extraction and separation of the isomers (BA and OA)

It has been reported that a reduction in sample pH for polar weakly acidic compounds improves their extraction efficiency [23, 24]. The influence of sample pH on the selective extraction of OA and BA was investigated in the pH range of 0.5 to 4.5. Figures 2 and 3 show the results of sample pH optimization for all the compounds analyzed. The results show that the sample pH made it possible to selectively extract one isomer (OA) while leaving the other isomer (BA) in the sample solution. At a very low pH of 0.5-2.5 OA became more extractable than BA and therefore, this made it possible to extract OA exhaustively (at lower pH of 0.5) and then obtain a higher percentage of pure BA component (at slightly higher pH of 3.5).

From Figures 2 and 3, it can be observed that as the sample pH was increased, the peak areas increased to an optimum value. The peak area is proportional to the concentration of the analyte therefore, at pH 2.5 the greatest concentration of the compound OA was extracted and this was the optimum sample pH and the greatest concentration of compound BA was at pH 3.5.

Separation and detection of the extracts using HPLC-UV-DAD and LC-ESI-SIM-MS

Extracts from HFSLM were analyzed using both HPLC-UV-DAD and LC-ESI-SIM-MS. Figure 3 shows the chromatograms of the separated isomers at the optimal sample pH for the two isomers, OA and BA. At pH 0.5 it was possible to selectively extract only OA. When the pH was increased, it was observed that, both OA and BA were extracted. With this method, it is possible to extract exhaustively OA at pH 0.5 and then increase pH of the remaining solution to between 2.5 and 3.5 and extract BA. The results prove the high selectivity of the HFSLM technique which removes the necessity of the HPLC separation step as far as these isomers is concerned.

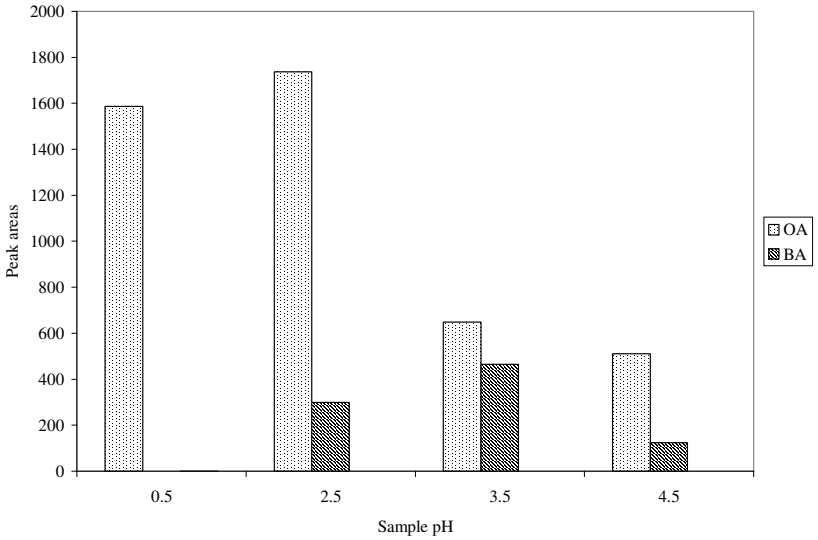


Figure 2. The effect of increasing sample pH for OA and BA.

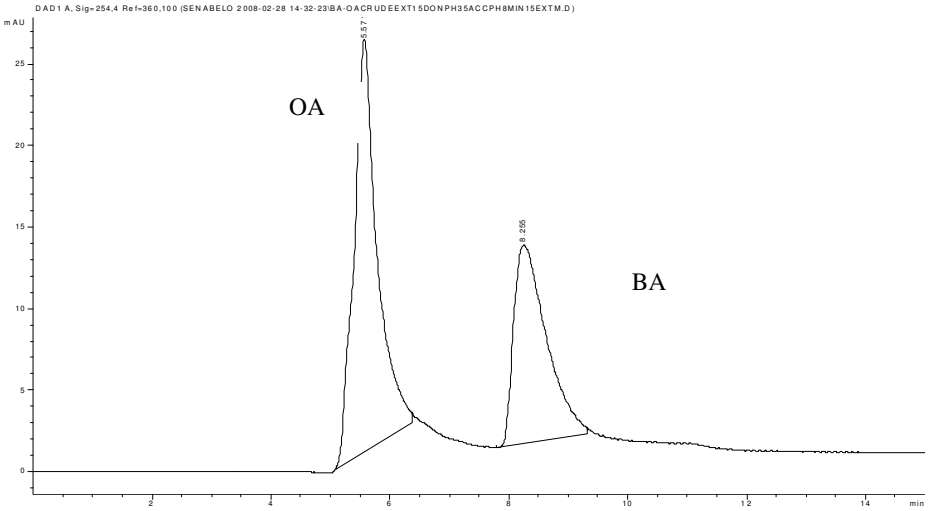
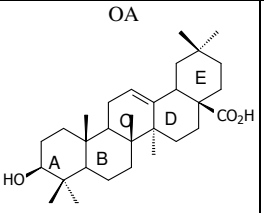
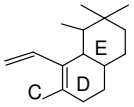
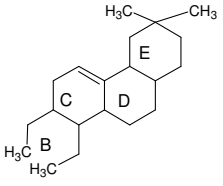
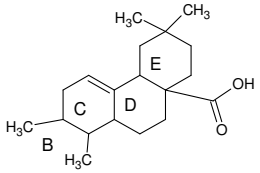
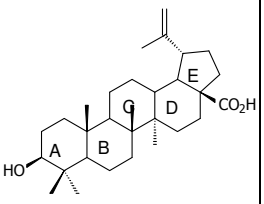
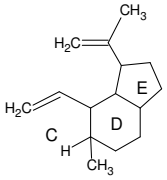
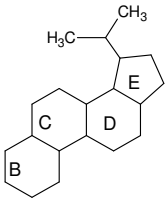
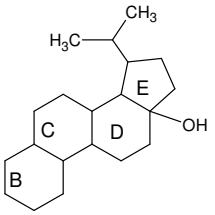


Figure 3. HPLC-UV-DAD chromatograms for sample from HFSLM extraction of crude mixture containing both OA and BA at pH 3.5.

The extraction and separation of the two isomers were further verified using LC-ESI MS (negative mode). With this method, it was possible to monitor a number of ions that were observed which included the deprotonated molecular ion (m/z 455). However, it is assumed that the other ions (apart from the deprotonated molecular ions) were formed in the MS and not during the HFSLM extraction, since in the HPLC chromatograms only two peaks were identified. For this reason, the deprotonated ion was the only ion used for the quantification process in the determination of K_D and log P values for the HFSLM extracts.

The identification of ESI-SIM-MS ion masses from both OA and BA is given in Table 1. All the fragments that were observed can be best described using retro-Diels-Alder mechanism.

Table 1. Identity of LC-ESI-SIM-MS fragments.

Possible structures for OA and BA ions and fragments under ESI-SIM-MS			
Isomer (m/z 456)	m/z 205	m/z 275	m/z 291
OA 			
BA 			

Measurements of K_D and log P values for OA and BA

The distribution coefficient (K_D) for the isomers (BA and OA) in the aqueous samples across the hydrophobic organic solvent made up of di-n-hexyl ether to the aqueous solution inside the lumen of the hollow fibre was calculated as follows (equation 1)

$$K_D = \frac{\text{Concentration of the isomer (OA or BA) in the acceptor aqueous solution (inside the lumen of hollow fibre)}}{\text{Concentration of the isomer (OA or BA) in the aqueous sample solution}} \quad (1)$$

The diffusive flux for each of the isomers (OA and BA) in the sample solution was worked out according to Fick's first law of diffusion. By using Fick's first law**,** the diffusive flux of components in a sample solution can be related to the concentration. According to this law the flux proceeds from regions of high concentration to regions of low concentration, with a magnitude that is proportional to the concentration gradient. Mathematically, Fick's law can be represented as follows:

$$\text{Flux (F) across the hollow fibre} = -D\frac{\Delta C}{\Delta \chi} \quad (2)$$

where, D is the diffusion coefficient with units of length^2 time^{-1}, while $\Delta C/\Delta\chi$ refers to the concentration gradient with units of (concentration of isomer) length^{-2} time^{-1}. NB: χ denotes radius of the hollow fibre with units of [concentration of isomer (per unit volume) length^{-1}].

Also, diffusion flux has units of [(amount of isomer)/(area.time)] = [(amount of isomer).length^{-2}.time^{-1}]. Diffusion coefficient or diffusivity is expressed as [area/time] = [length^2.time^{-1}]. Gradient of C/gradient of x accounts for [concentration/length], which is equal to [(amount of isomer/volume)/length] = [(amount of isomer).length^{-4}].

The flux and concentration relationship is governed by the law of conservation of mass which states that the cumulative amount of solute crossing any medium x in a total amount of time, t should be equal to the total amount of that substance at that medium x at time t (27).

$$V\theta_m \int_0^t C_F(x,t')dt' = \theta_{tot} \int_x^\infty C_{R,tot}(x',t)dx' \quad (3)$$

where C_F is the flux rate, C_{Rtot} is the original concentration and θ_{tot} refers to total porosities.

Rearranging equation 3, making C_F subject we get the following.

$$C_F(x,t) = \frac{1}{\vartheta_V} \int_x^\infty C_{R,tot}(x',t)dx' \quad (4)$$

The amounts of the deprotonated ions for both isomers as observed in the MS results were calculated from the standard calibration curves which were prepared by injecting increasing concentrations of standards of both OA and BA. The intensities for each concentration were recorded and plotted against the corresponding concentrations to get the linear calibration curves. The average K_D values obtained for BA and OA was 0.29 and 0.16, respectively. The calibration curves for a deprotonated molecular ions of BA and OA (m/z = 455) gave acceptable calibration curves with r^2 = 0.9. Extracts from HFSLM process were quantified using these calibration curves.

The concentrations of extracted OA and BA obtained from HFSLM process (Tables 2 for BA isomer, m/z = 455) were used to calculate the flux across the hydrophobic liquid membrane which was made up of di-n-hexyl ether. The flux measurements obtained were used to determine the flux rate for the extractions which were performed at different enrichment times. These data made it possible to calculate both distribution coefficient K_D (according to equation 1) as well as the log P values for the two isomers. Figures 4a and 4b show that the flux was increasing as enrichment time was increased.

The flux for BA seems to be almost twice as much as compared to that of OA for the same enrichment times. This means that BA has a higher rate of crossing the hydrophobic organic membrane than OA when each is extracted at its optimal conditions for the same amount of time. This may be attributed to the differences in their chemical structures. From the measurements of flux and the distribution coefficients, the graphs for the diffusive flux rate versus K_D were plotted which gave the values for log P of the two isomers (Figures 5a and 5b).

Table 2. BA concentrations (m/z = 455) used for the calculations of flux and flux rate.

Time	BA ion count, m/z = 455	BA Concentration, mg/L	Flux rate, mg/min	Initial concentration, mg/L	K_D
5	83	7.699083	1.539817	100	0.076991
30	167	12.73536	0.424512	100	0.127354
60	216	15.67318	0.26122	100	0.156732
90	322	22.02848	0.244761	100	0.220285

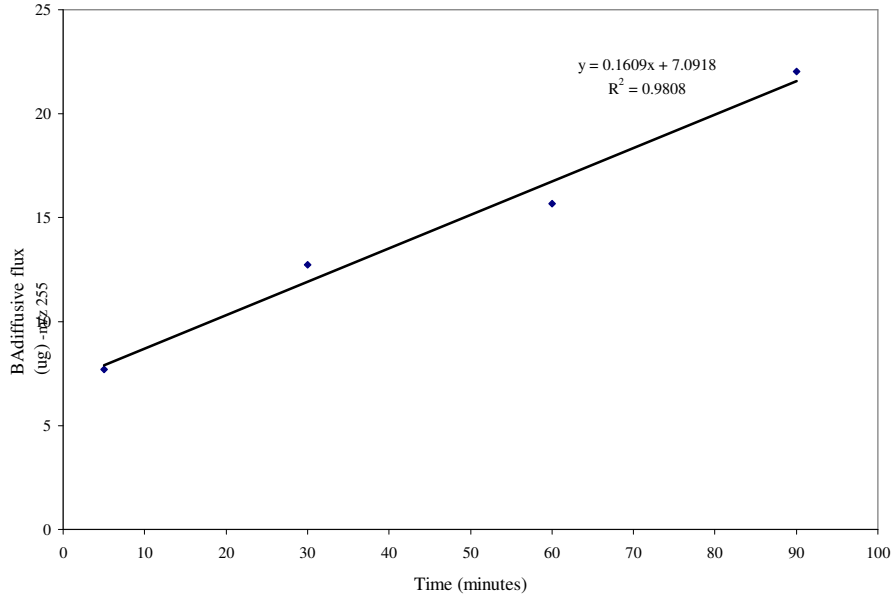


Figure 4a. Diffusive flux for BA (m/z = 455) at different enrichment times.

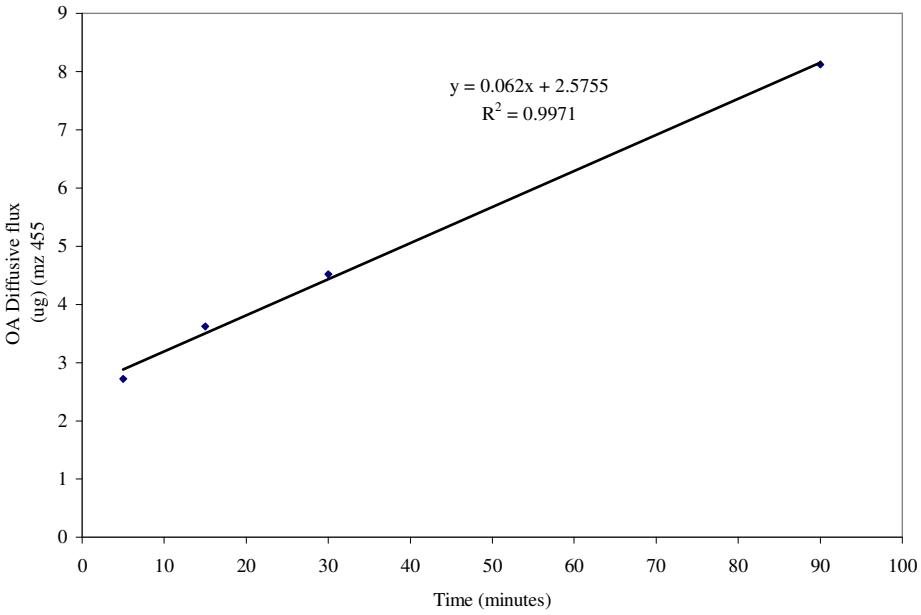


Figure 4b. Diffusive flux for OA (m/z = 455) at different enrichment times.

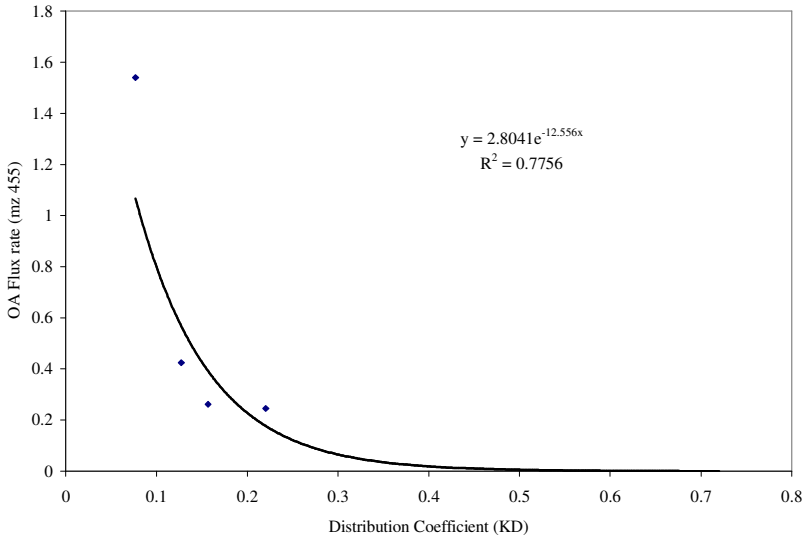


Figure 5a. OA diffusive Flux rate Vs K_D (m/z = 455).

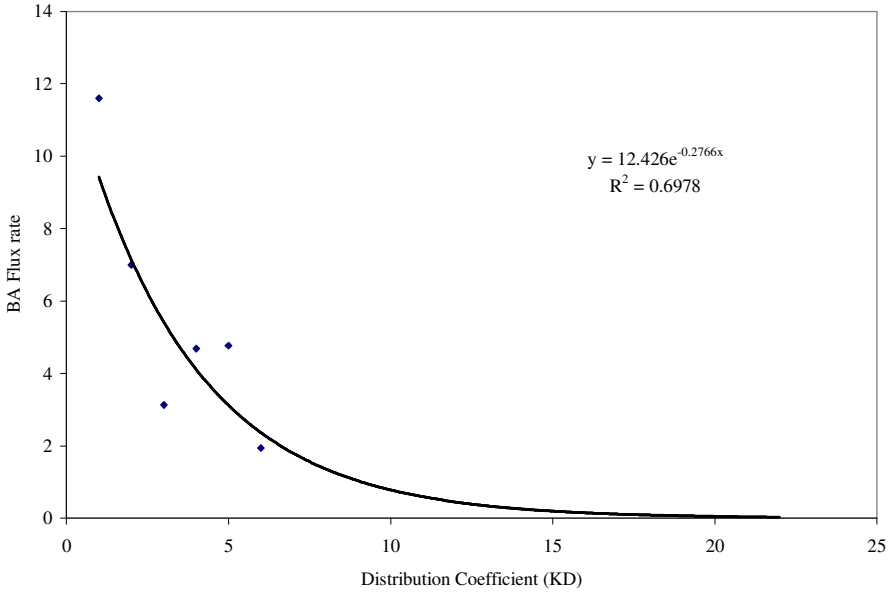


Figure 5b. BA Diffusive flux rate Vs K_D (m/z = 455) BA.

From the exponential equations obtained from the curves, it was then possible to calculate the log P values for both OA and BA. The results showed that BA was slightly more hydrophobic than OA as the data for log P showed that BA had 6.61 while OA had log P value of 6.1. The obtained data were compared to the data reported in the literature using other techniques. The literature has log P values for BA as 6.85 and 6.32 for OA [25]; other

researchers have reported values ranging from 1.24 to 6.72 for OA [26]. Generally, comparisons for the log P values obtained from this work are slightly lower than those reported by Claude and co-workers, and this may be due to the differences in the experimental conditions and set up. Table 3 summarizes the comparison for the log P values for OA and BA.

Table 3. Comparison of log P values obtained from this work and literature values.

Isomer	log P (This work)	log P (Literature)
BA pK_a 5.50	6.61	6.85 [25]
OA pK_a 5.11	6.12	6.72 [25] 6.46-7.47 [26]

CONCLUSIONS

HFSLM has been successfully used for the extraction, separation and for the measurements of K_D and log P values for BA and OA. A comparison with other techniques show that there is a close similarity between the values obtained using other techniques and this affirms the strength of the method developed in this study.

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