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Full Length Research Paper

Chemometric investigation of the effects of chemical properties and concentrations on the extractability of benzimidazoles with supported liquid membrane

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Principle component analysis (PCA) and a two-way analysis of variance (ANOVA) were employed in the study of factors affecting extractability of benzimidazole anthelmintics using supported liquid membrane (SLM) in liver, kidney, milk and urine at four concentration levels. The SLM extraction process was monitored by liquid chromatography - mass spectrometer (LC-MS). The results showed that the extractability of benzimidazoles is dependent on both the concentration levels and the chemical properties of compounds. Based on chemical properties, extraction of the compounds from the liver matrix showed no significant difference (p = 0.05) for the following pairs; albendazole and oxibendazole, thiabendazole and mebendazole, oxibendazole and fenbendazole, and oxibendazole and mebendazole. At some of the concentration levels, mainly between 1000 and 100, 100 and 10, and 10 and 1 µg/Kg, there was no significant difference. It was also found that, there was significant difference (at p = 0.05) in the extractability in milk between oxibendazole and albendazole, and also oxibendazole and fenbendazole. For milk also, the concentration range from 10 to 100 µg/L, showed no significant difference (p = 0.05). Urine matrix on the other hand, showed significant difference in the recoveries at all concentration levels.

Key words: Benzimidazoles, supported liquid membrane (SLM), liquid chromatography-mass spectrometry (LC-MS), two-way analysis of variance (ANOVA), principle component analysis (PCA).

INTRODUCTION

The use of antibiotics and antibacterial in animal husbandry has been in practice for decades (Gustafson, 1986). Benzimidazole anthelmintics together with a number of other similar compounds are used or abused in animal husbandry mainly as growth promoters, prophylactics and chemotherapeutic agents (Msagati and Nindi, 2001; Msagati and Ngila, 2003; Msagati, 2005; Msagati and Nindi, 2006). The widespread use of these antibiotics has resulted in the potential adverse effects to human health, due to consumption of contaminated foodstuffs of animal origin. Moreover, parent drug molecules or their metabolic degradation products may directly or indirectly find their way to the environment, through wastewater coming from shelters where treated animals reside. This may result in the contamination of waters in streams, as well as in wastewater treatment plants and thus cause adverse effect on the microbial ecosystem responsible for degradation of complex organic materials.

Normally, the drug molecules interact chemically with molecules in the environment where they are being disposed off. The occurrence and significance of the interaction between antibiotic residues and matrix molecules have been of interest to analytical chemists who are responsible for developing chemical methods capable of effective sample clean up from different matrices of either biological or environmental origin (Msagati and Nindi, 2006). Several methods utilizing different strategies to isolate benzimidazole anthelmintic,

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mainly those involving the use of solid phase extraction, have been reported (Nerenberg et al., 1982; Michiels et al., 1982; Bushway et al., 1990). Bradon and coworkers reported a method utilizing a combination of liquid-liquid extraction and SPE for the extraction of benzimidazole compounds (Bradon et al., 1990). Our research group reported successful use of supported liquid membrane (SLM) to extract benzimidazole anthelmintic compounds from a variety of biomatrices (Msagati and Nindi, 2001; Msagati and Ngila, 2003; Msagati, 2005; Msagati and Nindi, 2006). Analytical techniques for detection of these compounds after sample treatment (using SLM, SPE, LLE, etc) include ELISA (Horton, 1990) and immunoassay (Newsome and Collins, 1987; Barker et al., 1986) electrochemical (Msagati and Ngila, 2003; Struck and Elving, 1964; Passet and Tsivina, 1972; Smola and Sontag, 1985; McClean et al., 1994), liquid chromatography (Msagati and Nindi, 2001; Rose, 1999; Hajee and Haagsma, 1996; Steenbaar et al., 1993; Al-Kurdi et al., 1999) and liquid chromatography coupled to mass spectrometry (LC-MS) (Msagati and Nindi, 2006; De Ruyck et al., 2001 and Balizs, 1999).

In spite of good intentions of using established sample pretreatment strategies to achieve effective and efficient extractability, the effect of interactions of variables that impact on the extractability of analytes from biomatrices, cannot be ignored. The practice in the investigation of the interaction between antibiotic residue and matrix molecules is to use a classical approach that involves varying one parameter while keeping all other variables constant (Saltzman and Yaron, 1986). However, this approach has limitations in that, it cannot provide the information on how variables interact in synergistic or antagonistic way.

In this study, we have employed both PCA and a two-way ANOVA which is a statistical technique that can establish the nature of interactions of variables that affect the response signal during the determination of the analytes, in this case, benzimidazole anthelmintics (Figure 1). Using ANOVA, experiments were performed which allow the flexibility of having independent variables modified.

Design for PCA test model

With PCA analysis, the data was obtained by computing the eigenvalues and eigenvectors of the covariance matrix, such that it was possible to get the original variables and the principal component scores, using equations obtained from a number of PCA equations, e.g. principal component 1(Prin1) and principalcomponent 2 (Prin2) which are the new variables or linear combinations and the original mean-corrected variables (x1 and x2). The PCA equations were formulated as follows:

$$\xi 1 = Prin1 = V1x1 + V2x2 \quad (1)$$

$$\xi 2 = Prin2 = -V2x1 + V1x2 \quad (2)$$

Where the sum of squared weights of each component is unity, that is (V1)2 + (V2)2 = 1 and also (-V2)2 + V12 = 1, on the other hand, the sum of the cross products of the weights is zero, that is, [V1 x – (V2) + V2 x V1)] = 0.

Design for a two-way ANOVA test model

A statistical model was designed to provide an optimal method for investigation of the relationship between response (Y) and control variables (i, j, k). If (Yijk) is a response signal related to the recovery in the form of extraction efficiency of the SLM, while αi and βj denotes concentration levels and compounds fortified in matrices respectively. Using a set of control variables in each case represented by i, j and k, we can then propose a functional relationship (mathematical model) which gives Yijk as a function of αi and βj.

$$Y_{ijk} = f\left[\alpha_i + \beta_j + \alpha\beta_{ijk}\right] \quad (3)$$

The overall form of the model being tested in this study is as follows:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + e_{ijk} \quad (4)$$

where; Y_{ijk} is the k^{th} recovery efficiency resulting from the ith concentration level and the jth compound; μ is a constant; α is the effect of the ith level of concentration in a given matrix; β is the effect of the jth level of compound in the same matrix; $\alpha\beta$ is the interaction between the ith level of concentration in a given matrix and jth level of benzimidazole compound in the same matrix; and e is the error function (uncertainty) associated with the observation Y.

Thus, the two chemometric techniques (PCA and ANOVA) were used to reveal the nature of interactions between benzimidazole compounds and matrix components, that is, establishment of whether the effect of interaction is additive or parallel and hence the impact of these interactions on the extractability and hence limit of quantification of the benzimidazole compounds in various biomatrices.

EXPERIMENTAL

Benzimidazole anthelmintics

The benzimidazole anthelmintics (albendazole, fenbendazole, mebendazole, oxibendazole and thiabendazole) were from Sigma (St. Louis, USA). The standard stock solutions were prepared by dissolving ~1 mg of benzimidazole compound in 1 ml of the solvent comprising of 1:1, v/v of methanol and formic acid in water (95:5, v/v) to give a final solution concentration of 1000 mg/L. The structures and molecular weights of the benzimidazole anthelmintics compounds studied are shown in Figure 1.

SLM procedure

SLM was used in this work for the extraction of benzimidazoles from a variety of biological matrices and the extracts were detected using LC-MS. SLM involve a three phase system (Msagati and Nindi, 2001; Msagati and Ngila, 2003). An aqueous sample phase (feed/donor) is separated from an aqueous receiver (acceptor) phase by a layer of organic solvent impregnated in the porous

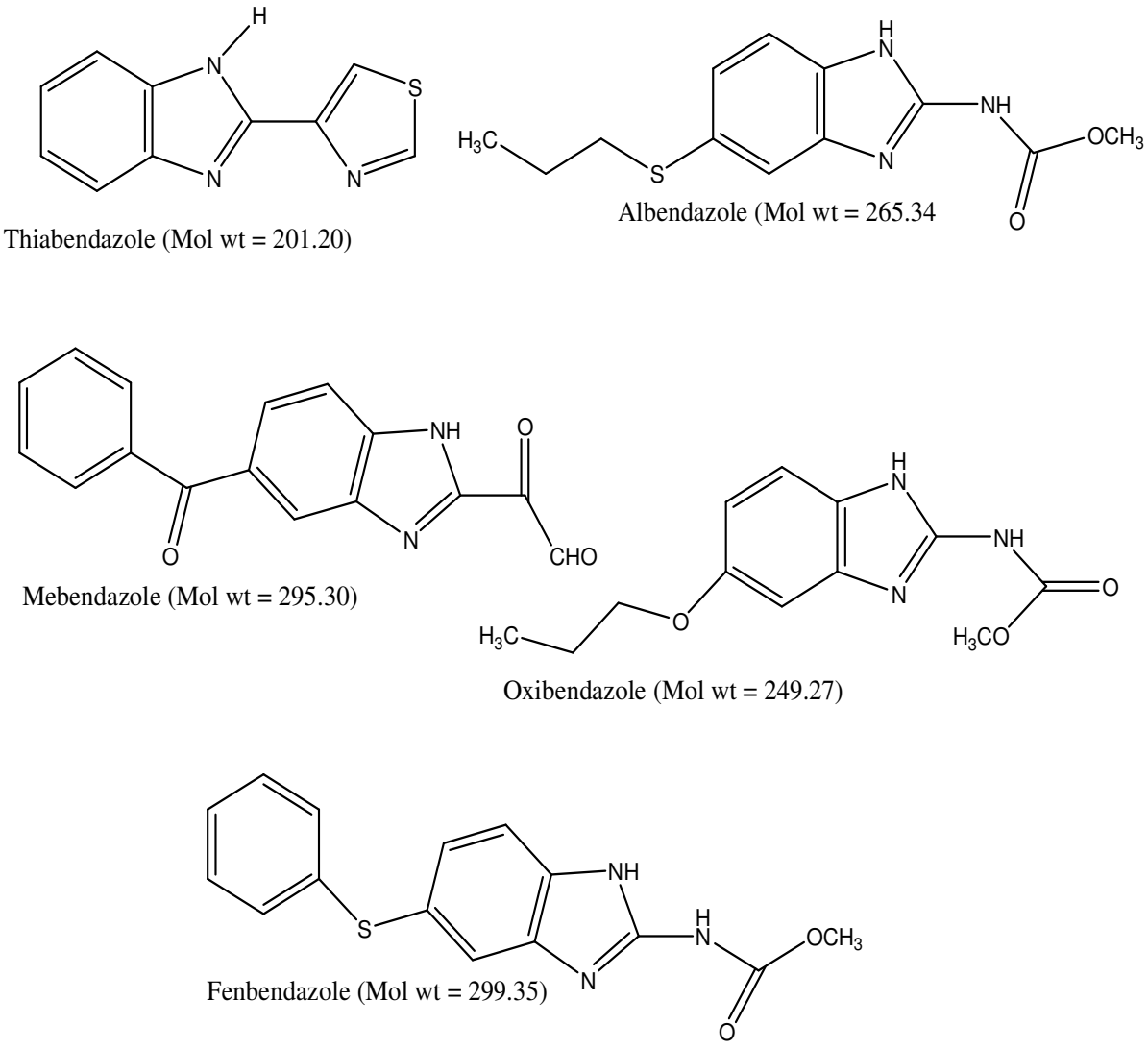

Figure 1. Structures and molecular weights of benzimidazole anthelmintics studied.

membrane. In order to enhance extractability and selectivity of solutes in SLM, the conditions (sample pH, extraction time and flow rate) in the aqueous sample phase were adjusted such that the analyte of interest is forward-extracted out of the sample phase into the organic phase and back-extracted out of the organic phase into the receiver phase, in a concerted fashion (Msagati and Nindi, 2001; Msagati and Ngila, 2003; Msagati, 2005; Msagati and Nindi, 2006). The SLM experiment in this work incorporated a chemical extractant or a carrier (tri-n-octylphosphine oxide – TOPO) to enhance the process of selective transport of analyte components across the membrane interface. The extracts were determined by LC-MS.

RESULTS AND DISCUSSION

The results and discussion given here is restricted to PCA and ANOVA studies and no attempt shall be made to discuss data obtained with SLM/LC-MS, as we have previously reported them (Msagati and Nindi, 2001; Msagati, 2005; Msagati and Nindi, 2006). In this paper, PCA and a two-way analysis of variance (ANOVA) were applied, as they provide an efficient evaluation for all the observed behaviour during the SLM process of benzimidazoles, in a variety of biomatrices at different concentration levels. The principal component analysis has been instrumental in scaling down (compress) the data, in order to understand better, how various factors interact.

Inter-matrix variation studies

PCA studies of the SLM extractability of benzimidazole anthelmintics from liver tissues

The PCA analyses of the extractability of various benzimidazole compounds in the liver matrix has

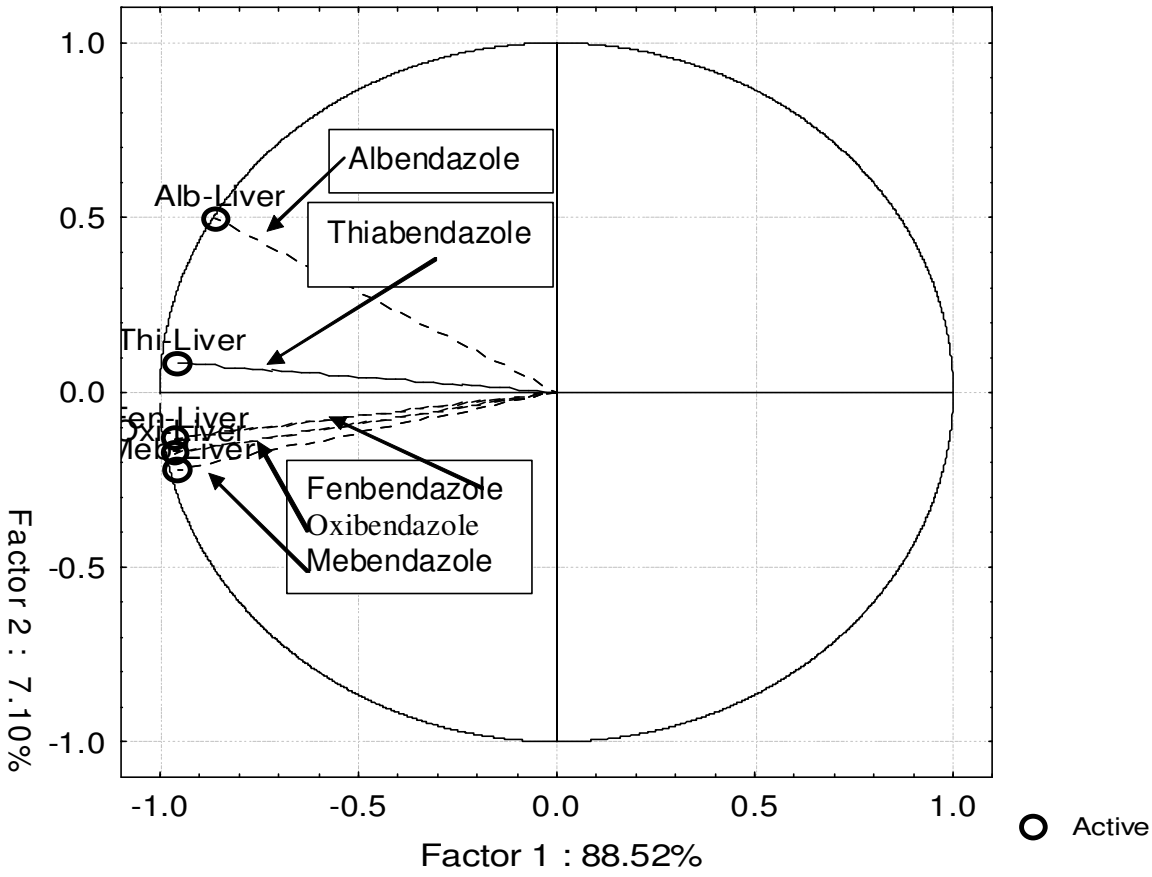

Figure 2a. PCA of benzimidazole's physico-chemical properties influence on the SLM extractability of the compounds in the liver.

revealed that, albendazole and thiabendazole interact with the components of this matrix in more or less the same way. Thiabendazole seems to be interacting differently from all other benzimidazole anthelmintics studied. Structurally, the compounds (except thiabendazole) (Figure 1) contain the same side chain, joining the imidazolium ring (NH-CO-OCH_3); presumably, this is the functional group responsible for the interaction with the molecules found in the liver matrix. Alternatively, the similarity in terms of their extractability may be based on the fact that, the vast chemistries in the liver matrix offer similar kinds of interaction to these compounds and at the same magnitude, hence affect their extractability in a similar proportion.

However, the effect of the concentration levels (cll-benzimidazol) was found to be more pronounced than the effect of the physico-chemical properties of the compounds (cpl-benzimidazol) (Figures 2b and c). The more pronounced effect of concentration levels in the liver matrix as compared to the effect of chemical properties, might be attributed to the saturation of the active sites for binding within the matrix, such that at a certain level of concentration, these active sites were exhausted and therefore easier to extract as the drug molecules were in free forms (unbound). Before the saturation of the active sites in the matrix, there exist a competition between the matrix molecules and the organic membrane for the drug molecules, but in the absence of competition, all the free drug molecules will tend to cross the organic membrane and be trapped in the stagnant acceptor.

PCA studies of the SLM extractability of benzimidazole anthelmintics from kidney tissues and urine

The results of the extraction of benzimidazole anthelmintics from kidney tissues show a similar pattern as that for liver in that, only thiabendazole displayed a different behaviour (Figure 3a). The explanation for this similarity is that, there is commonality in terms of chemical properties (Figure 1) in that, thiabendazole with no side chain interacts differently from other benzimidazole compounds in the same matrices and hence different extractability trend. However, in urine

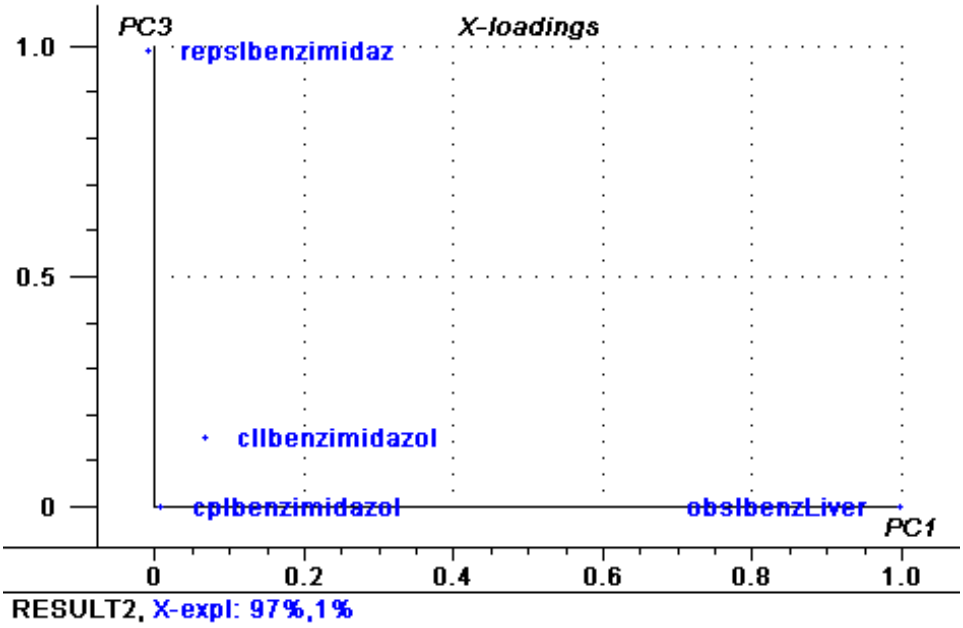

Figure 2b. PCA of combined benzimidazole's physico-chemical properties and concentration levels' influence on the SLM extractability of the compounds in the liver.

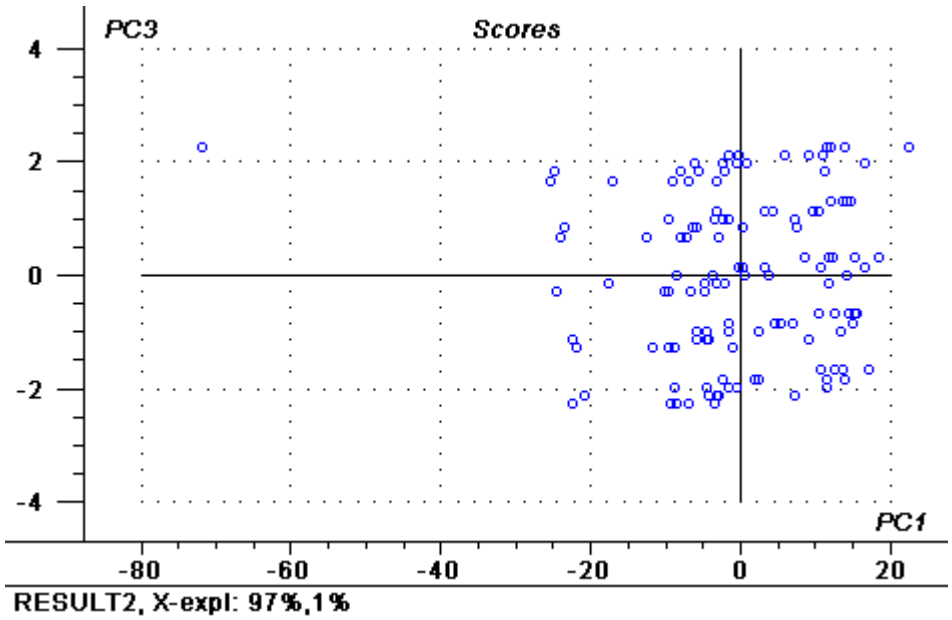

Figure 2c. PCA scores for the combined benzimidazole's physico-chemical properties and concentration levels' influence on the SLM extractability of the compounds in the liver.

matrix, both the chemical properties and levels of concentrations seem to affect weakly the extractability of the compounds (Figures 3b and c); with the kidney matrix, there is commonality in terms of chemical properties between fenbendazole and oxibendazole, as they seem to interact the same way with milk matrix as they show similar extractability trend. The same can be concluded for albendazole and mebendazole; with the kidney matrix also, both the chemical properties and levels of concentration affects weakly and therefore, have little influence on the extractability of the compounds.

PCA studies of the SLM extractability of benzimidazole anthelmintics from milk PCA results for benzimidazoles in the milk matrix indicated poor extractability for all the compounds. This may be due to the complex nature of the milk matrix itself, as it is composed of very

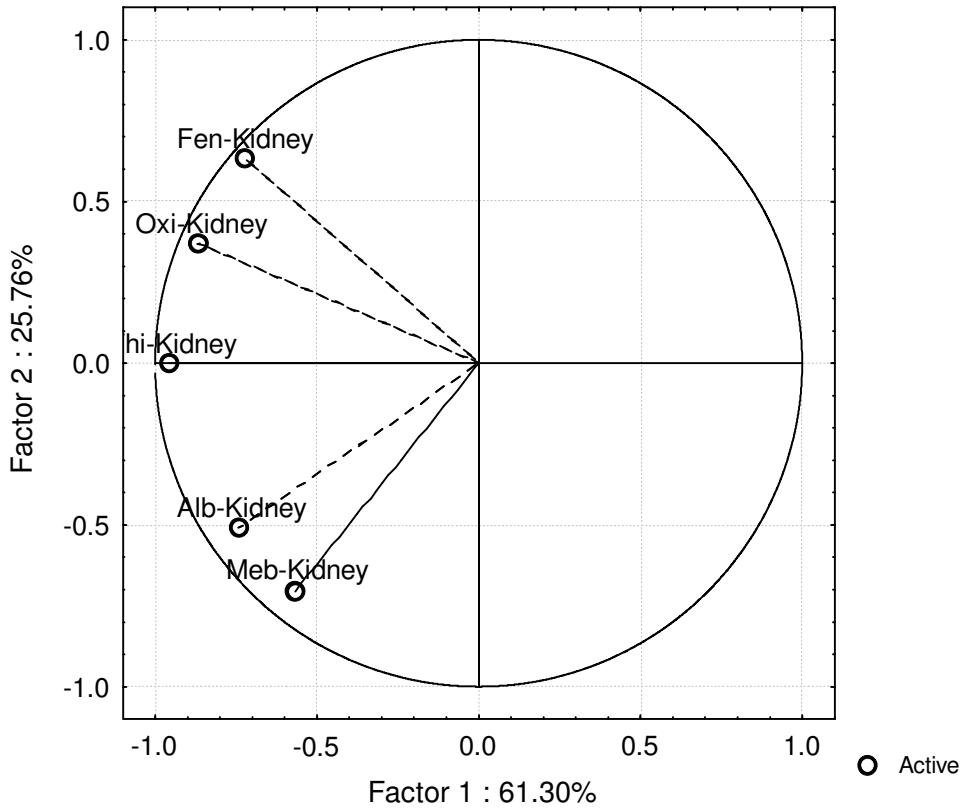

Figure 3a. PCA of benzimidazole's physico-chemical properties influence on the SLM extractability of the compounds in kidney tissues.

hydrophobic molecules, such as fats and lipids, which complicate tremendously to the difficulties in the SLM extraction process of benzimidazole anthelmintic compounds 2-5.

Inter-analyte variation

Analysis of variance (ANOVA) studies of the SLM extractability of benzimidazole anthelmintics from various matrices

A two way-ANOVA enabled the efficient evaluation of whether different concentration levels affect the efficiency of supported liquid membrane performance, that is, the extractability of benzimidazole compounds for a particular matrix under study. It also allowed the evaluation of whether the nature (chemical properties) of different compounds behaved differently as they diffused across the hydrophobic membrane during extraction when concentration levels, were held constant or varied. The results of the ANOVA tests on the extractability of benzimidazole compounds across the supported liquid membrane are shown in Tables 1 to 3. The results are in agreement with the test proposed model (in Equations 3 and 4).

Two-way ANOVA studies of the SLM extractability of benzimidazole anthelmintics from liver tissues

The results in Table 1 show that the proposed model (defined in Equations 1 and 2) is suitable in describing the relationship between the extraction efficiency, the nature of compounds and concentration levels ($P < 0.05$). Furthermore, there is a significant difference between the concentration levels and the compounds at $p < 0.05$, as well as a significant interaction between concentration levels and compounds.

In order to determine which compounds are significantly different from each other in terms of their extractability in the supported liquid membrane, the least square difference (LSD) which is, a multiple comparison test, was carried out. The results (Table 2) reveal that thiabendazole is significantly different from all the other compounds ($P < 0.05$), with the exception of mebendazole. Further, it was observed that, there was no difference between oxibendazole and fenbendazole; fenbendazole and mebendazole, implying that any of these compounds could have been used to give the same extraction efficiency.

Table 3 shows the multiple comparison tests for the concentration levels, whereby all the concentration levels are significantly different from each other ($P < 0.05$). This

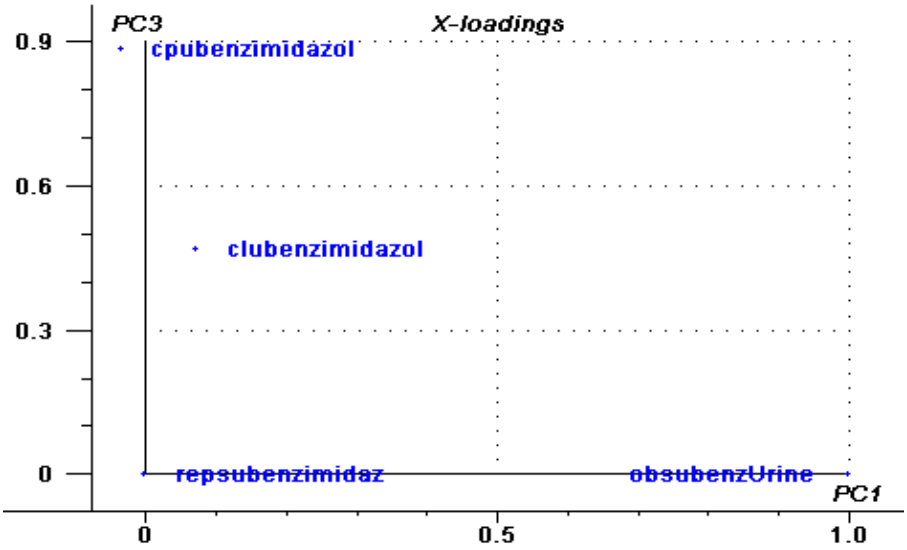

Figure 3b. PCA of combined benzimidazole's physico-chemical properties and concentration levels' influence on the SLM extractability of the compounds in urine.

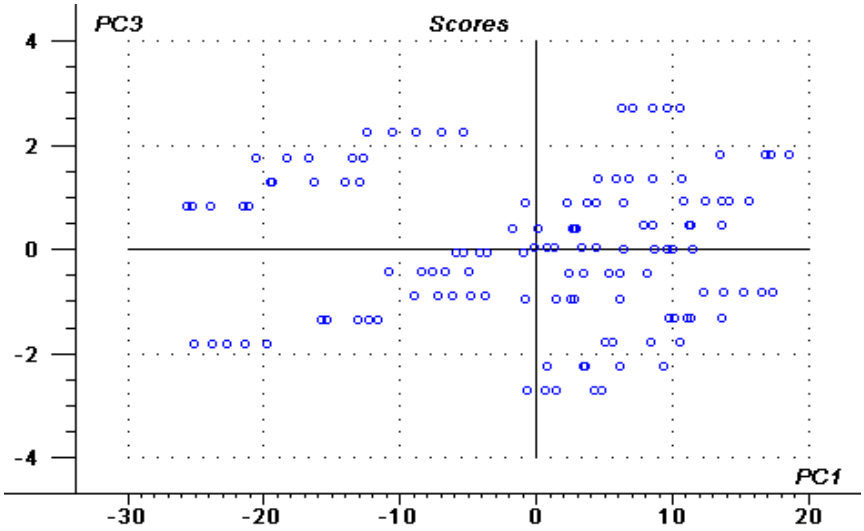

Figure 3c. PCA scores for the combined benzimidazole's physico-chemical properties and concentration levels' influence on the SLM extractability of the compounds in urine.

Table 1. Tests of between-subjects effects in the SLM extraction of benzimidazoles from liver tissues. Dependent variable: concentrations in spiked liver.

Source	Type III sum of squares	df	Mean square	F	Sig.
Corrected model	13935.594[a]	24	580.650	9.256	0.000
Intercept	669033.568	1	669033.568	10664.663	0.000
β benzimidazoles liver	4467.361	4	1116.840	17.803	0.000
α benzimidazoles liver	7000.454	4	1750.113	27.898	0.000
αβ benzimidazoles liver * benzimidazoles liver	2467.779	16	154.236	2.459	0.004
Error	6273.368	100	62.734		
Total	689242.530	125			
Corrected total	20208.962	124			

R^2= 0.690 (adjusted R^2 = 0.615).

Table 2. Multiple comparisons within and between benzimidazole compounds extracted from liver tissues at 95% level of confidence using Least Square Difference (LSD) approach.

(I) Benzimidazoles	(J) Benzimidazoles	Mean difference (I-J)	Std. error	Sig.	95% CI	
					Lower	Upper
Thiabendazole	Oxibendazole	10.0680(*)	2.24024	0.000	5.6234	14.5126
	Mebendazole	2.1280	2.24024	0.344	-2.3166	6.5726
	Albendazole	-7.6120(*)	2.24024	0.001	-12.0566	-3.1674
	Fenbendazole	6.1600(*)	2.24024	0.007	1.7154	10.6046
Oxibendazole	Thiabendazole	-10.0680(*)	2.24024	0.000	-14.5126	-5.6234
	Mebendazole	-7.9400(*)	2.24024	0.001	-12.3846	-3.4954
	Albendazole	-17.6800(*)	2.24024	0.000	-22.1246	-13.2354
	Fenbendazole	-3.9080	2.24024	0.084	-8.3526	0.5366
Mebendazole	Thiabendazole	-2.1280	2.24024	0.344	-6.5726	2.3166
	Oxibendazole	7.9400(*)	2.24024	0.001	3.4954	12.3846
	Albendazole	-9.7400(*)	2.24024	0.000	-14.1846	-5.2954
	Fenbendazole	4.0320	2.24024	0.075	-0.4126	8.4766
Albendazole	Thiabendazole	7.6120(*)	2.24024	0.001	3.1674	12.0566
	Oxibendazole	17.6800(*)	2.24024	0.000	13.2354	22.1246
	Mebendazole	9.7400(*)	2.24024	0.000	5.2954	14.1846
	Fenbendazole	13.7720(*)	2.24024	0.000	9.3274	18.2166
Fenbendazole	Thiabendazole	-6.1600(*)	2.24024	0.007	-10.6046	-1.7154
	Oxibendazole	3.9080	2.24024	0.084	-0.5366	8.3526
	Mebendazole	-4.0320	2.24024	0.075	-8.4766	0.4126
	Albendazole	-13.7720(*)	2.24024	0.000	-18.2166	-9.3274

Based on observed means. * The mean difference is significant at the 0.05 level.

Table 3. Multiple comparisons within and between various concentration levels in SLM extraction of benzimidazole compounds from liver tissues at 95% level of confidence using Least Square Difference (LSD) approach.

(I) Conc. level for liver (µg/L)	(J) Conc. level for liver (µg/L)	Mean difference (I-J)	Std. error	Sig.	95% CI	
					Lower	Upper
1000	100	-6.0000(*)	2.24024	0.009	-10.4446	-1.5554
	10	-11.0840(*)	2.24024	0.000	-15.5286	-6.6394
	1	-16.3400(*)	2.24024	0.000	-20.7846	-11.8954
	0.1	-21.2720(*)	2.24024	0.000	-25.7166	-16.8274
100	1000	6.0000(*)	2.24024	0.009	1.5554	10.4446
	10	-5.0840(*)	2.24024	0.025	-9.5286	-0.6394
	1	-10.3400(*)	2.24024	0.000	-14.7846	-5.8954
	0.1	-15.2720(*)	2.24024	0.000	-19.7166	-10.8274
10	1000	11.0840(*)	2.24024	0.000	6.6394	15.5286
	100	5.0840(*)	2.24024	0.025	0.6394	9.5286
	1	-5.2560(*)	2.24024	0.021	-9.7006	-0.8114
	0.1	-10.1880(*)	2.24024	0.000	-14.6326	-5.7434

Table 3. Contd.

1	1000	16.3400(*)	2.24024	0.000	11.8954	20.7846
	100	10.3400(*)	2.24024	0.000	5.8954	14.7846
	10	5.2560(*)	2.24024	0.021	0.8114	9.7006
	0.1	-4.9320(*)	2.24024	0.030	-9.3766	-0.4874
0.1	1000	21.2720(*)	2.24024	0.000	16.8274	25.7166
	100	15.2720(*)	2.24024	0.000	10.8274	19.7166
	10	10.1880(*)	2.24024	0.000	5.7434	14.6326
	1	4.9320(*)	2.24024	0.030	0.4874	9.3766

Based on observed means. * The mean difference is significant at the 0.05 level.

may be attributed to the fact that, the stripping solution (acceptor solution) which was used to entrap the compounds after they had diffused across the supported liquid membrane, was stagnant and hence, was subject to saturation by higher concentrations. Moreover, the compounds differ in their ionizability (different pKa values). This property is important when optimizing the pH of donor and acceptor pH.

Two-way ANOVA for the SLM extractability of benzimidazole anthelmintics from kidney tissues

The results of a two-way ANOVA analysis for the SLM extractability of benzimidazoles from the kidney tissues showed that, there was significant difference at 95% confidence level ($p = 0.05$) for all the compounds with an exception of albendazole and fenbendazole which showed similar extractability properties. The two compounds differ in the structures of their side chain, in which the albendazole has a propyl group attached to the sulfur atom, whereas in fenbendazole, it is a benzene ring. This difference in chemical functionality might have influenced their ability to cross through the hydrophobic membrane, as well as their extractability behavior, towards the stripping solution applied for these two compounds; with regard to the effect of concentration levels, a two-way ANOVA showed that there was significant difference at all concentrations, except between 100 and 1000 µg/Kg, as well as between 1 and 10 µg/Kg. This could have been caused by the carry over effects during the extraction process, which may cause the analyte leftovers from previous enrichment to be carried over into the subsequent experimental run. Thus, carry over effects will render 100 µg/Kg to behave as 1000 µg/Kg and 1 µg/Kg as 10 µg/Kg.

Two-way ANOVA for the extractability of benzimidazole anthelmintics from milk

The results from milk matrix showed that there was significant difference between the extractability of oxibendazole and albendazole and also between oxibendazole and fenbendazole. The pKa values which are an indication of their ionizability may be responsible for the trend observed. The pKa value for albendazole is 2.80, while those of fenbedazole and oxibendazole are between 5 and 6. It is worth noting that the compounds that ionize well usually extract better using liquid membranes. The effect of concentration levels on the extractability of benzimidazoles from milk samples, showed significant difference at 95% confidence limit (CL) for extraction at 10 and 100 µg/L. This again could be the consequence of carryover effects during theextraction process.

Two-way ANOVA scheme studies of the extractability of benzimidazole anthelmintics from the urine matrix

The general trend of extractability of the benzimidazole compounds in urine matrix showed significant difference at 95% confidence level for all the compounds at different concentration levels, except for thiabendazole. The nature of substituent bonding to the thiazolidine and imidazolium rings may account for this, since these are the only two compounds in which C atom is connected to a C atom in the thiazolidine ring of the benzimidazole structure. In the other compounds studied, N atom is bonded to the thiazolidine ring.

Conclusions

In this study, it was shown that, the ease of extractability of benzimidazole anthelmintic compounds using SLM was greatest for urine, followed by liver, then kidney and lastly milk. This study has also demonstrated the potential of multivariate principal component analysis (PCA) and a two-way ANOVA in predicting the optimization of crucial parameters mostly chemical properties and concentration level for the extractablity of benzimidazole anthelmitics. A two-way ANOVA has indicated that at 95% confidence level, the ionizability of compounds (exemplified by the pKa and pH values),

concentration level and the chemistry of the matrix strongly influence the extractability of benzimidazole compounds using supported liquid membrane. The bonding of the substituent groups such as C-N or C-C proved to be significant (CL 0.05) in the extraction of benzimidazoles in urine matrix. Complex matrix such as milk significantly (CL 0.05) affected the extractability of benzimidazoles, due to its strong interaction with analyte molecules. Furthermore, PCA has shown how various factors come into play to influence the extraction process.

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