African Journal of Biotechnology Vol. 5 (19), pp. 1827-1835, 2 October 2006
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2006 Academic Journals

Full Length Research Paper

Supported liquid membrane extraction of 17β- estradiol and its metabolites in a variety of biological matrices

Titus A.M. Msagati* and Mathew M. Nindi

Department of Chemistry, University of Botswana, P. Bag UB00704, Gaborone, Botswana.
*School of Chemistry, Faculty of science and Agriculture, University of KwaZulu-Natal, Durban, 4001.

Accepted 9 May, 2005

A sample purification and enrichment technique involving the use of supported liquid membrane (SLM) has been developed for the selective extraction of 17β- estradiol and its metabolites, namely 17β-estriol and estrone in various biological matrices and water. The biological matrices in which extraction was done included bovine kidney and liver tissues, milk and urine. The liquid membrane used to trap these compounds was made of 5% tri-n-octylphosphine oxide (TOPO) dissolved in a mixture of di-n-hexylether and n-undecane (1:1, v/v). Separation and detection of the analytes obtained after SLM enrichment was done by liquid chromatography-electrospray mass spectrometry (LC-ESI-MS). The extraction efficiencies (E) for 1 ng/L estradiols mixture spiked in various biological matrices were in the order of 61–80%, 52–74%, 67–89% and 61–82% for kidney tissue, milk, urine and liver tissue, respectively. For spiked water sample, the extraction efficiencies were of the order 82–96% from a 1 ng/L sample mixtures. LC-ESI-MS gave the detection limits of 0.3 ng/L, 1.8 ng/L and 2.4 µg/L for 17β-estradiol, estrone and 17β-estriol, respectively.

Key words: Supported liquid membrane, biological matrices, liquid chromatography, electrospray, mass spectrometry.

INTRODUCTION

Normally, extracts from matrices, whether of biological or environmental origin, contain many diverse compounds included with the possible analytes of interest. To exclude these interfering molecules, a variety of sample pre-treatment techniques are usually employed. Supported liquid membrane (SLM) is among these techniques which have gained much popularity due to the high selectivity it offers Chimuka et al., 1998). (The technique has been used as a sample preparation alternative to many others in the extraction of different compounds in a variety of matrices (Jönsson and Mathiasson, 1999a, b). Audunson (1986, 1988) first reported the use of SLM in the determination of amines and after that the technique has been used in many other applications such as enrichment of metals in natural waters (Djane et al., 1997; Ndung'u et al., 1998, 1999). The technique has also been used in monitoring of certain classes of veterinary drugs in biological matrices (Msagati and Nindi, 2001, 2004a, b) and in the enrichment of herbicides in natural water samples (Chimuka et al., 1997). In this work supported liquid membrane was used for the enrichment of 17β-estradiol and its metabolites in various biological matrices. 17β-estradiol and its metabolites are important estrogenic hormones, which are being used and abused in human medicine and veterinary agro-industries as growth promoters (Draisci et al., 1998). Estrogenic steroid hormones influence growth, development, differentiation and function of reproductive system's peripheral tissues and also play an important role in bone maintenance, and in cardiovascu-lar as well

*Corresponding Author E-mail: msagati@ukzn.ac.za. Fax: +27 031 260 3091. Tel: +27 031 260 3097.

Abbreviations: %E, extraction efficiency; DL, detection limit; ESI, electrospray; ESI-MS, electrospray ionization – mass spectrometry; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; MS, mass spectrometer; PTFE, polytetrafluoro ethylene; RSD, relative standard deviation; SIM, selected ion monitoring; SLM, supported liquid membrane; SPE, solid phase extraction; TOPO, tri-n-octyl phosphine oxide; UHP, ultra high purity, and UV/DAD, ultra violet/Diode array detector.

as in the central nervous system (Shimada et al., 1998). The rampant use of these hormones and related synthetic compounds, such as those used in contraceptive formulations has been reported very often and residues of these compounds have been encountered in wastewater effluents and various other biological matrices (Lopez de Alda and Barceló, 2001a). This suggests a wide application of such compounds in human and veterinary medical practices, which may lead to their presence in foodstuffs of animal origin and thereby risking the health of consumers (Draisci et al., 1998, Lopez de Alda and Barceló, 2001a). Human breast cancer and endometrium cancer have been reported to be promoted by prolonged exposure to either endogenous or synthetic estrogens or their metabolites (Shimada et al., 2001; Hu et al., 1992). The concentrations of these compounds, however low they may be, have the potential to turn on and trigger estrogenic responses, which may alter the norms in the reproduction and development systems within an individual (Lopez de Alda and Barceló, 2001a). This shows the need to monitor the presence of these hormonal compounds in foodstuffs of animal origin to safeguard the health status of consumers. However, the residue analysis of these anabolic compounds in biological matrices is problematic due to the fact that the parent compounds are easily metabolized. This necessitates the development of a multi-residue method for a simultaneous determination of the parent compound and its metabolites in a various target organs such as, kidney, liver and also animal products like milk, as well as animal by-products like urine (Hooijerink et al., 1998).

A variety of analytical methods and techniques to determine these compounds are available in the literature. These include spectrophotometry and single sweep polarography (Hu et al., 1992), immunoassays and radioreceptor assay (Arts et al., 1998) and LC-UV/DAD after solid phase extraction (SPE) sample clean-up (Lopez de Alda and Barceló, 2001b). However, neither LC-UV/DAD nor any of these other methods meet the selectivity and sensitivity requirements to monitor estrogens in the biomatrices or wastewater treatment plants (López de Alda and Barceló, 2001a). Also most of the SPE cartridges available are for single use only and this may increase the cost of analysis due to the continuous replacement of cartridges. Approaches such as gas chromatographic (GC) and GC-mass spectrometry (GC-MS) have been used more often (Hooijerink et al., 1998, Choi et al., 2000). One shortcoming of this method is that, it requires derivatisation prior to gas chromatographic analysis, as the compounds are not volatile (Hooijerink et al., 1998, Choi et al., 2000). Furthermore, both GC and GC-MS are limited to factors such as high molecular weight of the compounds to be analyzed (López de Alda and Barceló, 2001a).

We therefore propose the use of supported liquid membrane (SLM) as a sample clean up and enrichment technique in the determination of 17β-estradiol and its metabolites in a variety of biological matrices. Among the advantages of using SLM are that, the system uses very minimal amount of organics and therefore is an environmental friendly technique. Also, unlike SPE, SLM has the reusability capability (the same membrane can be used over and over) and this conserves expenditures. Moreover, the use of LC-ESI-MS for separation and detection makes the technique more attractive unlike GC which requires derivatization prior to separation.

MATERIALS AND METHODS

Reagents

Compounds used in this study, 17β-estradiol, 17β-estriol and estrone, were from Sigma (St. Louis, MO, U.S.A). The structures, molecular weights and CAS numbers of these compounds are given in Table 1. All organic solvents, methanol and diethyl ether used were of HPLC grade, from BDH laboratory supplies (Poole, U.K.) and were filtered through a 0.45 µm organic membrane filter, type HVLP, Millipore (Dublin, Ireland). Ultra high purity water was processed though a Millipore Quantum Ultrapure Ionex Gradient A10 purification system (Millipore SA-67120) Molsheim-France. The aqueous solvents were filtered through cellulose nitrate membrane filters with 0.45 µm pore size and 47 mm diameter. All weight measurements were taken using the electronic ultramicro balance, Sartorius supermicro S4, Sartorius GmbH (Goettingen, German).

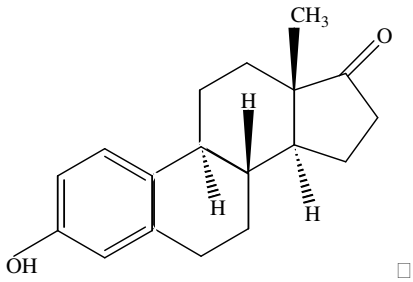
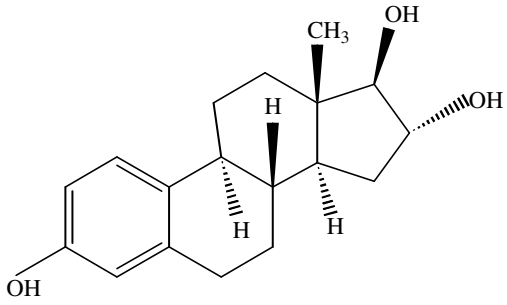
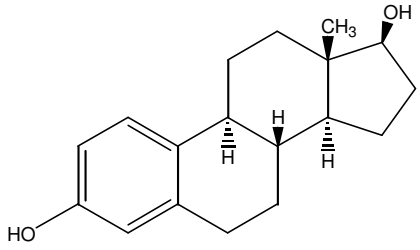
Preparation of compounds

Approximately 1 milligram of each of the compounds was dissolved in a corresponding volume of methanol to make a stock solution of 1000 mg/L. From this, diluents of different concentrations were prepared in methanol/water (1:1, v/v). The exact weights of the compounds were as follows; 17β-estradiol (1.2696 mg), 17β-estriol (1.1250 mg) and estrone (1.4950 mg). From this, diluents of different concentrations were prepared in methanol/water (1:1, v/v).

Sample treatment with supported liquid membrane

The SLM set-up device (fabricated at Department of Analytical Chemistry, Lund University, Lund, Sweden) used in all the experimental work is similar to that described in our previous publications (Msagati and Nindi, 2001, 2004a, b). The membrane used was prepared by impregnating a Millipore filter, made of Teflon, FG type with a pore size of 0.2 µm with 5% TOPO in 1:1 di-n-hexylether:n-undecane for 24 min. After assembling the membrane in the separator, both the donor and acceptor channels were flushed with water to remove the excess solvent membrane from the surface. A peristaltic pump, Minipuls 3 (Gilson, Villiers-Le-Bel, France), was used to pump and control the flow rates (0.1ml/min) of the solutions for both the donor and acceptor phases. The flow system was made up of acid resistant tubes (Elkay Products,Shrewsbury, MA, USA); with internal diameters of 1.2 mm and 0.60 mm for the donor and acceptor, respectively, connected by 0.5 mm internal diameter PTFE tubing and Alex screw fittings. The sample and buffer were emerging in a PTFE tee connection and then mixed in coil tubing before entering the donor channel. The donor and accept of buffer used were Na_2HPO_4/NaH_2PO_4 at pH 6.1, while the acceptor buffer was Na_2HPO_4/NaOH, pH 13.

Table 1. Chemical structures, molecular weights and CAS numbers of 17β-estradiol and its metabolites.

Compound	Molecular weight	CAS number
 estrone	270	53-16-7
 17β-estriol	288.40	50-27-1
 17β-estradiol	272.39	50-28-2

Membrane enrichment

Different concentrations of 17β-estradiol and its metabolites spiked in different biological matrices and the buffer (Na_2HPO_4/NaH_2PO_4 pH 6.1) were delivered with a peristaltic pump to the extraction system. A total donor flow rate of 0.1 ml/min and sample to buffer ratio was 2:1 was used, while the extraction time was 60 min. The compounds were enriched at a stagnant acceptor solution (Na_2HPO_4/NaOH, pH 13). Most interferents, which should be charged due to the pH conditions in the donor buffer channel, passed through the donor channel to the waste while the neutral molecules comprising the analytes of interest distributed between these two phases. After a sample processing time of 60 min the system was washed by pumping through the donor solution for 5 min, while the acceptor solution was kept stagnant and then the system was left to stand for 10 min to allow the diffusion of analytes from the membrane to the acceptor phase. The acceptor solution containing the analytes was then transferred quantitatively into 2 ml volumetric flasks using a peristaltic pump. The acceptor channel was then swept with the acceptor solution for 5 min to clear the system of any trace of analyte before the commencement of the next extraction. A 20 µl aliquot of the enriched sample was then introduced into the HPLC system.

Preparation of spiked bovine liver and kidney tissues samples of a mixture of 17β-estradiol and its metabolites

Finely sliced liver (after removal of the gall bladder) and kidney carcasses (~ 5 g) from the local abattoir were minced and spiked with a known concentration of a mixture of estradiol and its metabolites. The concentration of a mixture ranged from 0.1 ng/L to 1 mg/L. The spiked samples were homogenized by using a blender and then extracted with 10 ml ethyl acetate containing 20 µl of 0.5 M H_2SO_4, with shaking in an incubator for 2 h at room temperature. The organic phase containing these compounds was washed with UHP H_2O and evaporated. The residues were dissolved in 1 ml of MeOH-H_2O (40:60, v/v) and then stored at 4°C before enrichment and/or clean up with SLM.

Preparation of spiked milk and urine samples of estradiol anabolic compounds mixtures

A 10.0 ml aliquot of milk or urine quantitatively spiked with a mixture of estradiol compounds was transferred into 25 ml centrifuge tubes and then treated the same way as was for liver and kidney tissues.

Table 3. Characteristic ions for 17β-estradiol and its metabolites under ESI-MS.

ESI – MS Data for 17β-estradiol metabolites (%Energy = 0)			
Compound	**Mol. mass**	**MS ion mode**	**Characteristic ions observed**
17 β -estradiol	272	NI	271 $[M – H]$; 290 $[M + H_2O]^+$
		PI	273 $[M + H]$; 290 $[M + H_2O]^+$
Estrone	270	PI	271 $[M + H]^+$, 293 $[M + Na]^+$, 253 $[(M + H) – H_2O]^+$
17 β -estriol	288.4	PI	289 $[M + H]^+$

Where NI = Negative ion, and PI = Positive ion.

Table 2. Gradient elution of anabolic 17β-estradiol and its metabolites.

Time (min)	Flow rate (µl/min)	A	B
0.00	100	40	60
55.00	100	100	0

A = 85% methanol + 15% 25 mM acetic acid in water;
B = 85% 25 mM acetic acid in water + 15% methanol.

High performance liquid chromatographic separation of estradiol anabolic compounds

A Hewlett Packard Series 1100 system HPLC with DAD detector, binary pump, thermostatted column compartment, vacuum degasser system, manual injector, and controlled by an HP ChemStation (Hewlett Packard, Waldbronn, Germany) was used for separation of estradiol compounds. Samples were pumped in a gradient elution as shown in Table 2. The mobile phase was; A = 85% methanol + 15% 25 mM acetic acid in water; B = 85% 25 mM acetic acid in water + 15% methanol. The column used was a Waters XTerra microbore column 150 mm x 2.1 mm x 3.5 µm, at a flow rate of 100 µl/min and the injection volume was 20 µl.

ESI-MS of 17β-estradiol and its metabolites

MS detection was operated under electrospray ionization (ESI) positive mode. The direct infussion was carried out under full scan conditions (m/z 150 to 350). The samples 17β-estradiol, estrone and 17β-estriol (Table 3), were introduced to the electrospray source, by direct infusion using a syringe pump Harvard Apparatus 22, South Natick (Massachusetts, U.S.A). 50 µl of dissolved samples was mixed with 200 µl of buffer and the resulting solution infused at 3 – 5 µl/min. The buffer used was made using UHP water and 25 mM acetic acid/methanol, 1:1 (v/v). The spectra were obtained using a Finnigan LCQ Deca Quadrupole Ion Trap mass spectrometer and this system use Xcalibur software (San Jose, California, USA).

RESULTS AND DISCUSSION

Extraction from biomatrices

Solvent extraction involving the use of water immiscible solvent such as ethyl acetate was employed to extract estradiols from sample matrices. Ethyl acetate played a role in deproteinization while methanol was used to remove fats in sample matrices.

Optimization of the SLM system

The pH of the donor phase was investigated by varying between pH 2 to pH 13, as the pH for acceptor solution was kept constant. Figure 1 shows the variation of extraction efficiencies with respect to pH of the donor buffer solution, whereby the optimal pH value was found to be about 6.1. Estradiols like other estrogens do ionize under alkaline conditions (Hurwitz and Liu, 1977; Choi et al., 2000) and therefore, at pH ranges above neutral points they are negatively charged and also at low pH ranges they are positively charged. Therefore, there is an intermediate range where these compounds are neutral and hence optimally extractable. That region corresponded to an optimum donor pH of 6.1. This was in agreement with the theoretical predictions, that the donor pH should be at least 2 pH units below the lowest pKa value of the acidic analyte (Knutsson et al., 1996). pKa value for 17β-estradiol, estrone and 17β-estriol are 10.46, 10.34 and 10.38, respectively (Huwitz and Liu,1977).

ESI-MS for 17β-estradiol and its metabolites

A summary of the fragmentation pattern of estrogens is shown in Table 2. Generally, the compounds formed were mostly deprotonated ions, $[M - H]^+$, under negative ion mode and protonated ions, $(M + H)^+$, under positive ion mode. In addition to this 17β-estradiol formed hydrated molecules, $[M + H_2O]^+$ ion, under the same conditions. The two 17β-estradiol metabolites (17β-estriol and estrone) formed protonated, $[M + H]^+$, ions with estrone also forming sodiated, $[M + Na]^+$, and dehydrated, $[(M+H) – H_2O]^+$, ions (Figure 2). Under these conditions, all compounds gave very light fragmentation showing very few predominant ions. MS conditions that give light fragmentation and few predominant ions are preferred in order to get maximum

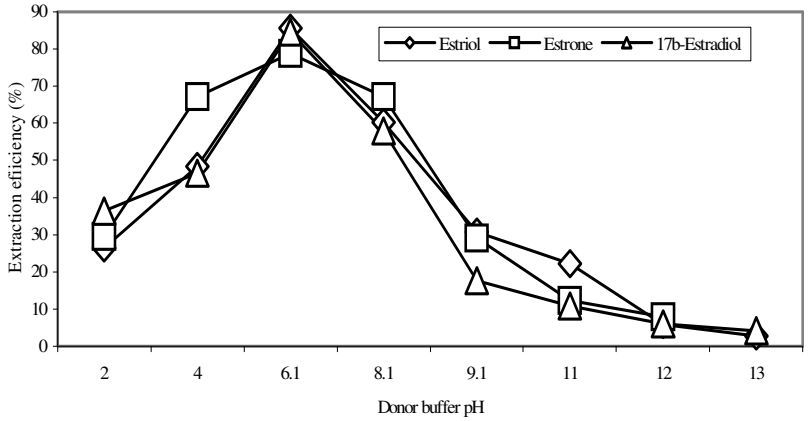


Figure 1. Donor optimization in the extraction of 17β-estradiol and its metabolites: Membrane = di-n-hexyl ether:n-undecane (1:1) with 5% TOPO; extraction time = 60 min, flow rate = 0.1 ml/min; acceptor solution = Na_2HPO_4/NaOH, pH 13.

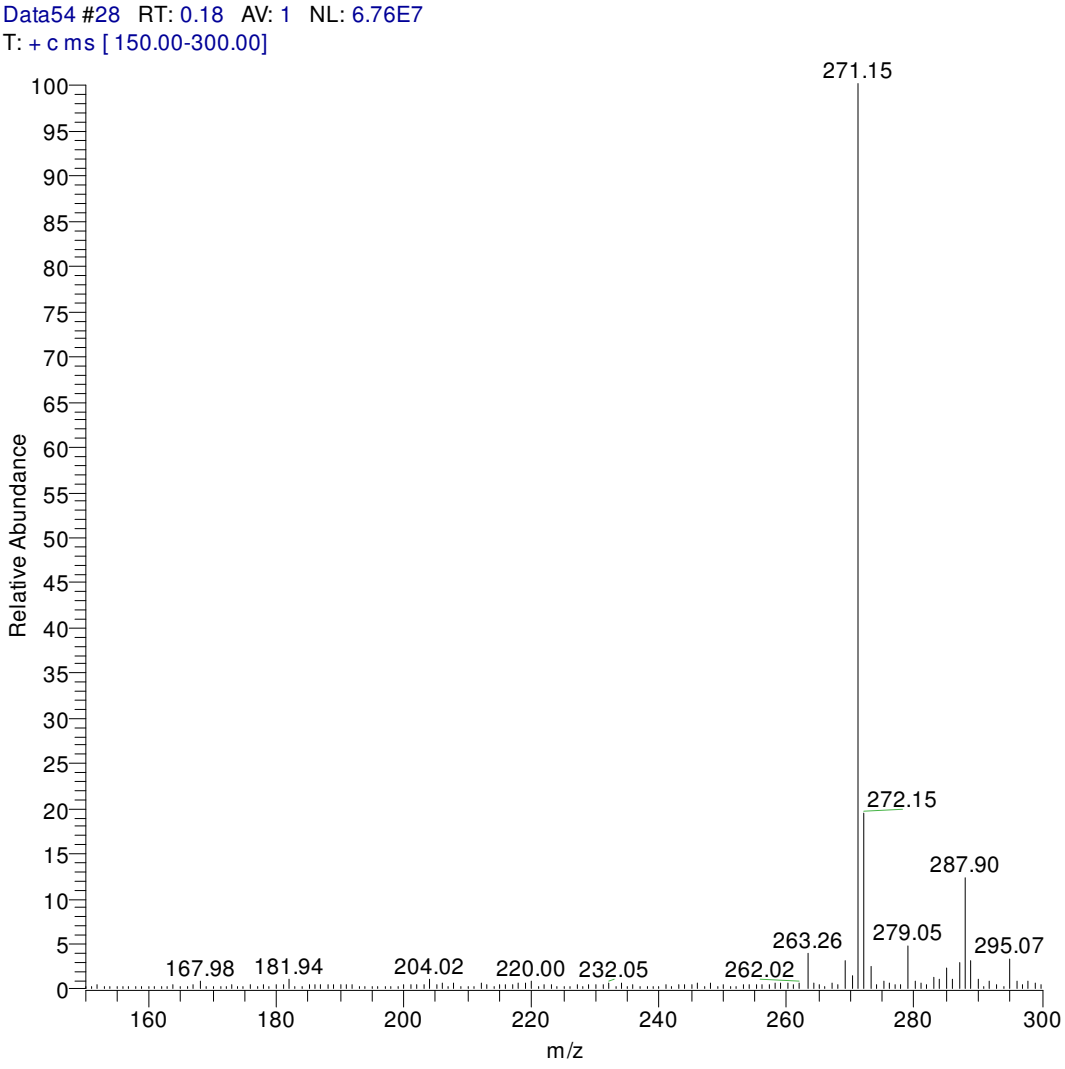


Figure 2. ESI-MS spectrum for estrone.

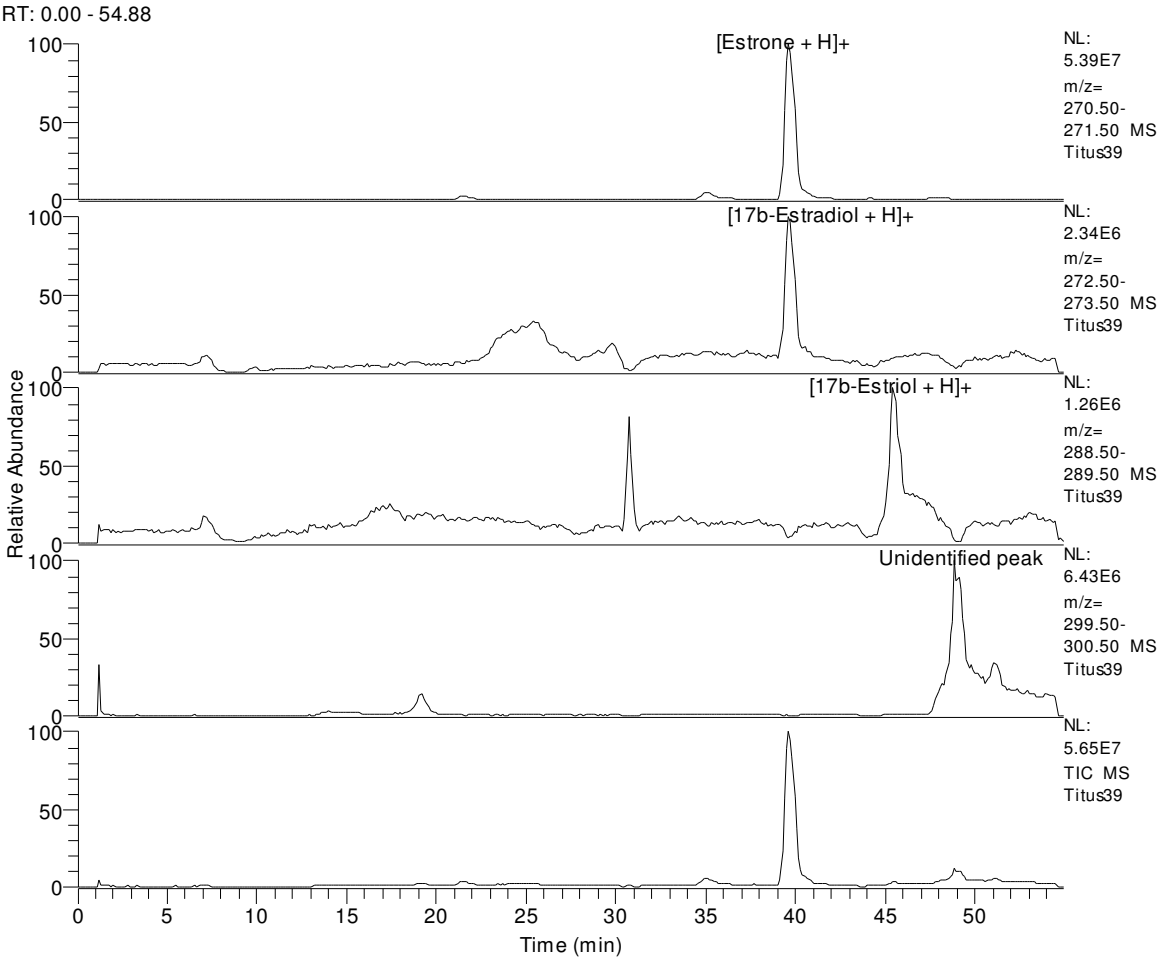


Figure 3. SLM/LC-ESI-SIM-MS of a mixture of estradiols in milk. Mobile phase contained 75% 25 mM acetic acid at concentrations 0.1 µg/L.

and sufficient sensitivity to enable the determination of the analytes at low concentrations, which they can be found in the various natural environments (Lopez de Alda and Barcelo, 2001b). Possible fragmentation peaks that are characteristic to anabolic estrogenic compounds studied were compiled and were used for the selected ion monitoring (SIM) techniques.

LC-ESI-MS of 17β-estradiol and its metabolites

In the separation of 17β-estradiol and its metabolites, the importance of acidic mobile phase additives was demonstrated when the compounds in the mixture failed to separate in their absence. However, when 25 mM acetic acid was added to the mobile phase, it resulted in separation improvement as the components started to resolve. When 25 mM acetic acid was increased from 75 to 85%, two of the compounds that had co-eluted (Figure 3), were completely separated from each other (Figure 4). This was probably due to the fact that, the inclusion of buffers increases the polarity and hence the selectivity and retention factor, i.e. the ratio of the distribution of the solute in the stationary phase to that in the mobile phase. LC-ESI-MS provided the best approach for the monitoring of estrogenic compounds. LC-ESI-MS not only allowed simultaneous monitoring of estrogens, but also provided useful means of structural confirmation by using selected ion monitoring (SIM). In the separation of 17β-estradiol and its metabolites, the hydrophobic interaction between the stationary phase and the lipophilic backbone of the estradiols seem to have played an essential role in achieving acceptable resolution. The use of a microbore column was important since the eluent was pumped at lower flow rates in comparison with analytical column. Low flow rates are attractive due to their reduced dispersion in the microcolumns, and also lead to higher instantaneous concentration of the solute observed.

Linearity in the SLM extraction

Linearity was investigated experimentally by spiking

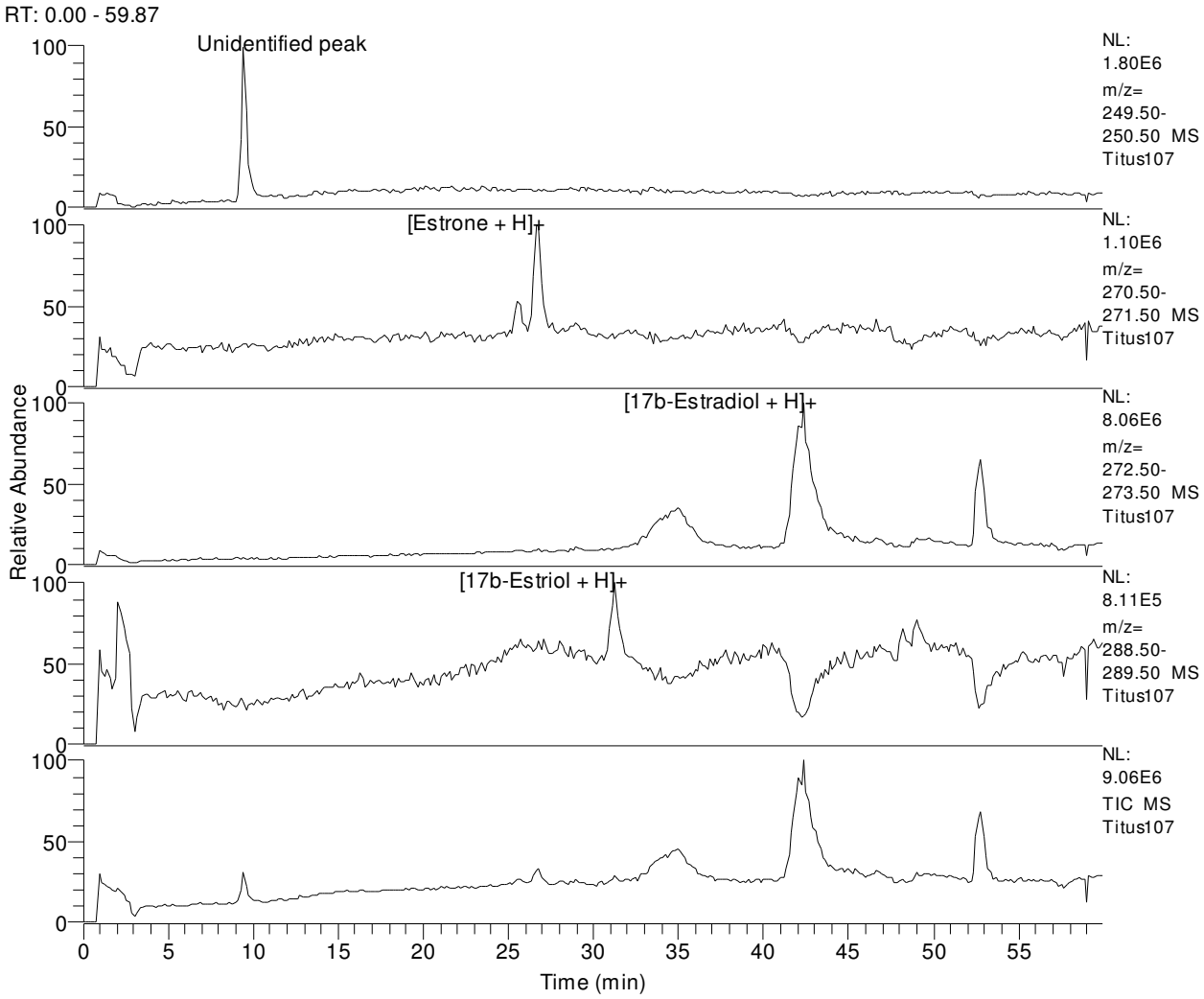


Figure 4. SLM/LC-ESI-SIM-MS of a mixture of estradiols in milk. Mobile phase contained 85% 25 mM acetic acid at concentrations 0.01 µg/L.

standard solutions at different concentrations ranging from 0.1 ng/L to 1 mg/L in a water sample matrix and then enriched in SLM, and quantified by LC-MS technique. From the linearity data obtained it was shown that, the correlation coefficients and y-intercepts of the linear regression line for the responses versus concentration plots gave acceptable linearity. This was evident from the values of correlation coefficients obtained, which were in the order 0.99.

Method precision and accuracy

Repeatability of the results from LC-ESI-MS was investigated by performing analyses of mixtures prepared in water samples at concentrations ranging from 1 ng/L to 1 mg/L. Each concentration was enriched in SLM at optimal conditions and each time a new membrane device was used. From the results it was observed that the %RSD obtained ranged between 0.47 to 2.13%. These %RSD values show good precision of the method developed. On the other hand, accuracy was evaluated by spiking a range of known concentrations in both water samples, which were considered as a blank matrix and also in other biological matrices.

The percentage recovery was determined and the results in spiked water samples were higher than those in biological matrices.

Quantitation

Table 4 shows the results of quantification of the compounds spiked in water and various biological matrices as detected using LC-MS technique. A suitable calibration curve obtained by external standard method was used for quantitation purposes in both cases. It was found that the amount recovered from milk was relatively

Table 4. Comparison of SLM enrichment for 17β-estradiol and its metabolites in a variety of biological matrices.

	Extraction efficiencies at concentration ranging, 1 ng/L – 1 mg/L				
	Milk	**Urine**	**Kidney**	**Liver**	**Water**
17β-Estradiol	74-52	89-67	80-61	82-63	96-82
17 β -Estriol	56*-39	67*-45	60*-44	63*-45	79-51
Estrone	63-42	81-55	69-50	71-52	85-63

*Concentration range = 1 βg/L – 1 mg/L in comparison with 1 ng/L – 1 mg/L.

lower than in the other matrices [52 – 74% for 17β-estradiol, 40 – 56% for 17β-estriol and 42 – 63% for estrone (concentration ranging ng/L – 1 mg/L)]. This might have been due to the complex nature of this matrix, which is known to be rich in fats, lipids and proteins, which in this case are regarded as interferents. The amount recovered from liver and kidney tissues appeared not to vary significantly [kidney: 61 – 80% for 17β-estradiol, 44 - 60% for 17β-estriol and 50 – 69% for estrone; liver: 61 – 82% for 17β-estradiol, 43 – 63% for 17β-estriol and 52 – 71% for estrone] due to the similarity in terms of the interfering substances that may be found in these biological matrices. Extraction from the urine matrix was slightly higher than in other biological matrices possibly due to little interfering species found in them as compared to the other biomatrices. The extraction efficiencies from urine matrix were found to be 67 – 89% for 17β-estradiol, 45 - 67% for 17β-estriol and 55 – 81% for estrone. Quantification from samples spiked in water was the highest (82 -96% for 17β-estradiol, 51 – 79% for 17β-estriol and 63 – 85% for estrone).

Detection limit

The detection limit (DL) refers to the lowest analyte concentration that produces a response detectable above the noise level of the system, generally three times the noise level. This parameter was investigated in all biological matrices. The detection limits obtained from liver and kidney tissues were 0.3 ng/L for 17β-estradiol, 1.8 ng/L for estrone and 2.4 µg/L for 17β-estriol. For milk matrix the DL values were 2.5 ng/L for 17β- estradiol, 5.8 ng/L for estrone and 9.6 µg/L for 17β-estriol.

Linear range determination in the SLM extraction process

Linear range in the extraction process refers to the concentration interval over which accuracy, linearity and precision is acceptable, and it was calculated using data obtained from linearity and accuracy studies. This was done by plotting percent relative standard deviation, (%RSD) versus concentrations. The results show that the linear range was between 0.01 µg/L and 0.1 mg/L.

Conclusion

A study on the analysis and detection of 17β-etradiol and its metabolites using LC-ESI-MS after SLM enrichment in spiked water and a variety of biological matrices have been carried out. The potential of incorporating sample SLM to LC-MS as a monitoring technique for veterinary anabolic compounds was demonstrated. The results of the ESI-MS spectra obtained provided valuable information data obtained could be used for ion selected monitoring (SIM) technique.

ACKNOWLEDGEMENTS

This work was financially supported by UB-Research and Publication. Private financial assistance in support of TAM by JTMN was greatly appreciated.

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