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Liquid membrane extraction and trace enrichment of selected pesticides in agro-processed fruit juice and Ethiopian honey wine (*Tej*) samples

MSc. Graduate Project

By

Samuel Kassahun

February 2010

Liquid Membrane Extraction and Trace Enrichment of Selected Pesticides in Agro-processed Fruit Juice and Ethiopian Honey Wine (*Tej*) Samples

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DECLARATION

This project is my original work except where due reference has been made in the acknowledgments. This work has not been submitted for a degree in any other University.

Signature......................................

Date..

This project has been submitted for examination with my approval as university advisor.

Negussie Megersa (PhD.)

Signature......................................

Place and date of submission: Department of Chemistry
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Abbreviations	Description
%	Percent
L	Liter
mL	Milliliter
μL	Micro liter
g	Gram
kg	Kilogram
mg	Milligram
μg	Microgram
^{o}C	Degree Celsius
min	Minute
HPLC	High performance liquid chromatography
UV	Ultraviolet
DAD	Diode array detector
cGC	Capilary gas chromatography
GC	Gas chromatography
MIP	Molecularly imprinted polymer
PTFE	Polytetrafluoroethylene
V/V	Volume by volume
ΔC	Change in concentration
ΔP	Change in pressure
ΔE	Change in electric potential
SD	Standard deviation
RSD	Relative standard deviation
LOD	Limit of detection
LOQ	Limit of quantification

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Abstract

Supported liquid membrane (SLM) was optimized for extraction of selected triazine herbicides at microgram level in different type of agro-processed fruit juices and home-made Tej samples prior to HPLC-UV analysis. By appropriate optimization of the SLM parameters, the extraction efficiency and enrichment factor of these herbicides can be improved with good repeatability of the results. For samples spiked at 100 µg L^{-1} *the detection limit ranges from 2.61 µg L*$^{-1}$ *for ametryn in Tej to 12.32 µg L*$^{-1}$ *for terbutryn in orange juice. The optimized SLM extraction method was linear in pineapple juice at concentration range of 50 µg L*$^{-1}$*- 500 µg L*$^{-1}$ *with correlation factors greater than 0.99 and average % RSDs less than 5.50% for triplicate analysis. The method was highly selective towards the selected triazine herbicides in all the sample matrices considered, showing no interfering peaks at the retention times of the target analytes and giving well resolved chromatographic peaks. The results suggest that SLM extraction is an alternative method for trace enrichment of wide variety of pesticides from liquid and semi-liquid matrices and could be used as a routine method for trace extraction and enrichment of pesticides from such matrices.*

1. Introduction

1.1. Pesticides

A pesticide is any substance or mixture of substances, natural or synthetic, formulated to control or repel pests. The term pest includes insects, weeds, mammals and microbes. Pesticides are usually chemical substances, although they can sometimes be biological agents such as bacteria or virus [1]. The active portion of pesticide, the active ingredient, is generally formulated by the manufacturer as emulsifiable concentrates or in solid particles. Many commercial formulations have to be diluted with water before use and contain adjuvants to improve pesticide retention. There are different classes of pesticides according to their type of use, target pest, and chemical property. The main pesticide groups are herbicides, insecticides, and fungicides [1, 2].

The use of chemical pesticides in forestry, railway, and protection against disease transmitting insects and in agriculture is very common all over the world. At present, around 2.5 million tons of pesticides are produced annually. The effective use of pesticides for agricultural purposes has improved the quality and quantity of food production. Advances in pesticide technology contributed for sustaining and improving the health and well-being of the ever-growing human population [2].

On the other hand, pesticides comprise widely varying classes of compounds with very different chemical and physical properties. Some are persistent in the environment and some degrade readily into various types of transformation products, again varying in the degree of toxicity and distribution in a similar manner as their parent compounds. One recent estimate indicates that only 0.1 to 5% of the herbicides applied reach the weeds targeted, and that only 0.003% of the insecticides used are consumed by the insects targeted. These estimates suggest that the remaining quantities of pesticides are released to the environment where they can adversely affect the non-target organism and the ecosystem as a whole [3].

There are also many pathways for exposure: in drinking water from contaminated wells, in food from household pesticide use, and from residues on plants as they are picked, or machinery as they are being handled or repaired, from pesticide drift as it is being sprayed, from spills during transport and from dermal exposure during mixing and application [2, 3].

At present, due to the possible toxic effects of pesticides on human health and on the environment, there are strict regulations for their registration and use all over the world. The registration of pesticide for its application on particular crop requires data on physicochemical properties, analytical methods, efficacy, toxicology, ecotoxicology, and fate and behavior in the environment [1]. The maximum residue levels (MRLs) in different foods have been established according to good agricultural practices, the observed toxic effects of the pesticide and the amount of food consumed. MRLs are proposed by the Joint FAO/WHO meeting on pesticide residues (JMPR) and recommended for adoption by the Codex committee on pesticide residues [4].

1.2. Herbicides and Their Classification

Herbicides are the main group of pesticides used worldwide, followed by insecticides and fungicides. Herbicide is a chemical product which can be used to destroy or inhibit the growth of weeds or unwanted plants. Based on the chemical structure, herbicides can be classified in ten groups: These are (1) Phenoxyacids; (2) Substituted Urea and Uracil; (3) Bipyridium; (4) Dinitroaniline; (5) Amides; (6) Carboxylic and Benzoic acids; (7) Carbamates; (8) Phenols and Diphenyl ether; (9) Heterocyclic nitrogen containing; and (10) Miscellaneous herbicides. Based on their mode of action herbicides can be classified as amino acid synthesis inhibitors, cell division inhibitors and photosynthesis inhibitors. Herbicides can also be classified as soil- or foliage-applied compounds; they can be total or selective in their action. Herbicides are applied either as pre-plant to prepare crop beds for sowing or post-plant for on-going weed control [1, 2].

The intensive application of herbicides has resulted in the contamination of the atmosphere, agricultural products, ground and surface waters. Consequently, this has resulted in the direct or indirect pollution of biological systems, food and food products. Knowledge of physicochemical properties, *i.e.*, vapor pressure, octanol/water partition coefficient (log K_{ow}) and solubility in water allows the prediction of the fate and behavior of such chemicals in the environment [1- 3].

1.2.1. Properties of Triazine Herbicides

A number of important herbicides contain three heterocyclic nitrogen atoms in ring structures (see Figure 1) and are therefore called triazines. They are herbicides with great consumption all over the world. Triazine herbicides are used to control broadleaf and grassy weeds by inhibiting photosynthesis [5]. They are one of the largest groups of herbicides which are most commonly used as selective pre- and post-emergence weed control for variety of crops including fruits and vegetables. Selectivity is gained by the inability of target plants to metabolize and detoxify the herbicide [6]. They are also the herbicides most frequently found in environmental samples owing to their relative persistence to abiotic and biotic breakdown with easy transport, producing detectable levels in drinking water, foods and fish [7]. Due to their water solubility, they may leach into the ground water, as well be transported in surface run off. Photodegradiation and volatilization of these herbicides is of little significance under most field conditions [8]. They have half-lives of the order of months, due to their low solubility in water and due to their strong sorption on carbonous materials and clays. This can be the origin of important environmental and toxicological problems because these compounds can be bioaccumulated along the trophic chain, especially in fruits and oils and finally in humans [9]. Their extensive use causes pollution of food and drinking water, by parent pesticide and their degradation products [10, 11].

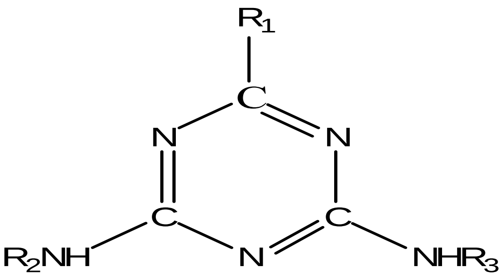

Figure 1. The general structure of triazines; where R_1 can be chloride, alkoxy or alkylthio substituent, R_2 and R_3 are alkyl substituent

1.2.2. Toxicity of Triazine Herbicides

According to the 2007 joint FAO/WHO report on pesticide residue on food, the concentration of individual pesticide should not exceed a maximum admissible concentration of 0.01 mg kg^{-1} for a single triazine herbicide and 0.04 mg kg^{-1} for total concentration of triazines [12]. Also according to European Union Directive, the maximum limit concentration for atrazine is set at 0.1 µg L^{-1} for single pesticide and 0.5 µg L^{-1} for total pesticide in drinking water [13]. However, for other matrices including food samples the triazine tolerance level is much higher and ranges between 100 and 250 µg kg^{-1} in the EPA Tolerance Index [14].

In 2002, USEPA published a report that evaluated a series of structurally related triazine pesticides for inclusion in a common mechanism group (*i.e.*, atrazine, simazine, propazine and their chlorodegradates) based on their ability to induce similar toxic effect through a common mechanism of toxicity [15]. These herbicides are determined to cause neuro-endocrine related developmental and reproductive side effects, through their ability to induce aromatase activity in human adrenocortical carcinoma cell line at concentrations of sub micro-molar range [7, 16, 17].

Epidemiologic studies have been documented long term exposures to triazine herbicides with increased risk of ovarian cancer in female farm workers in Italy and of breast cancer in the USA [7]. Upon chronic exposure atrazine may cause weight loss, cardiovascular damage, retinal degeneration, and mammary tumors in rats [8]. Ametryn is classified as toxicity class III of EPA, which is slightly toxic. Ametryn is moderately toxic to fish, highly toxic to crustaceans and moderately to highly toxic to mollusks [18].

1.2.3. Physico-Chemical Properties of Triazine Herbicides

The physico-chemical properties of triazines are determined mainly by the substituent at 2-position, generally for chlorine (the name ending with-azine), for methoxy (the name ending with-tone), and for alkylthio (the name ending with-tryn). They are solids, with a low vapour pressure at room temperature and have water solubilities in the range 5 - 750 mg L^{-1}. The properties of chloro-triazines considerably differ from those of the other two groups, whereas the methylthio- and methoxy- derivatives behave similarly. Substituent at positions 4 and 6 exert substantially smaller effects on the derivative properties [2].

As can be seen in table 1, triazine herbicides are weakly basic substances, the basicity increases with the order of substituent in the 2-position; *i.e.*, $Cl < SCH_3 < OCH_3$. The size and the degree of branching of N-alkyl groups at the 4 and 6 positions also have a distinct but less pronounced effect; in which increasing basicity parallels increasing the electron-donating power of the substituent [2, 19]. Dialkylamino-triazines have low solubility in water, the 2-chloro-triazines being less soluble than the 2-methylthio and 2-methoxy analogs. Aqueous solubility is, over a wide range, practically independent of the pH of the solution. However, a pronounced increase in solubility is observed at pH values where strong protonation occurs. Structural modifications of the substituent at either 2 or 4 and 6 positions of the dialkylamino-triazines significantly affect solubility at all pH levels [19].

Triazine herbicides undergo different kinds of reactions such as hydrolysis at C-2 position, N-dealkylation at C-4 and C-6 positions, and photochemical degradations at all the ring carbons. The process governing these degradations can be either chemical, photolytic, microbial, or a combination of all. Triazines are deactivated in tolerant plants by three different biotrasformation pathways; *viz*, benzoxazinone-mediated hydrolysis, N-dealkylation by cytochrome P450s and glutathione conjugation [19, 20]. Triazine herbicides are known to be metabolized in various mammals and chickens. In humans, triazine metabolism is catalyzed primarily by cytochrome P450 (CYP) enzyme [7]. Bacterial strains representing several genera have been isolated that can completely mineralize atrazine. The bacterium *pseudomonas sp*. mineralizes high concentrations (*ca*. 500 mg L^{-1}) of atrazine under both growth and non-growth conditions, using the herbicide as the sole nitrogen source [20].

Table 1. Physical constants of triazine herbicides [2]

Common name	**IUPAC nomenclature**	**Solubility in water (ppm) (25 °C)**	**pK_a**	**Density (g cm^{-3})**	**Absorption maxima (nm)**	
					λ_1	**λ_2**
Atrazine	2-chloro-4-ethyaminol-6-isopropylamino-*s*-triazine	33	1.68	1.187	222	263
Simazine	2-chloro-4,6-bis(ethylamino)-*s*-triazine	6.2	1.65	1.302	222	263
Ametryn	2-methylthio-4-ethylamino-6-isopropylamino-*s*-triazine	185	4.1	1.190	222	-
Terbutyrn	2-methylthio-4-ethylamino-6-tert-butylamino-*s*-triazine	25	4.3	-	223	-

1.3. Analysis of Triazine Herbicides in Food Samples

The use and development of new analytical techniques in food science runs parallel with the increased consumer concern about the quality and safety of the food they eat. Legislative limits for contaminants in food are decreasing as government and consumers demand better quality and higher standards [21]. Methods of analysis must be sensitive enough to cover not only the allowed use of permitted substances down to legislative limits but also the detection of trace contaminants and banned substances, especially for compounds that may be potentially genotoxic or carcinogens [22]. It has to be borne in mind that theoretically each food or food product can be contaminated by herbicides. Therefore sensitive and selective methods need to be developed for the measurement of herbicide residues in any food and food products [23].

The determination of pesticides in food and environmental samples at low concentrations is always a challenge, especially when the components of interest exist at trace levels. Several authors have developed and validated methods for trace level triazine herbicide determination in food based on chromatographic, spectrophotometric and electrochemical methods of analysis. Generally all the methods of chemical analysis employed common steps that must be properly coordinated from sampling to data analysis, as shown in Figure 2 below [24].

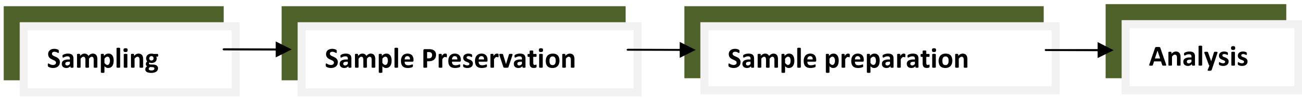

Figure 2. Steps in chemical analysis [24]

1.3.1. Sample Preparation of Triazine Herbicides in Food Samples

Sample preparation is a step meant to isolate components of interest from a sample matrix into a sample suitable for analysis. The analytes of interest are separated from the matrix and are pre-concentrated to improve the selectivity, sensitivity, reliability, accuracy and reproducibility of the analysis. Some of the commonly encountered steps in sample preparation are described in Figure 3 below. However, they depend on the analyte, sample matrix, concentration level, and analytical technique to be employed [22, 24].

The determination of trace residues of triazines in complex matrices such as food often requires extensive sample preparation prior to instrumental analysis. Since triazine herbicides and their degradation products are found in various complex matrices at very low concentrations pre-concentration, clean-up and separation of the compounds is highly desirable prior to analysis. The detection level of these residues depend more on the isolation and enrichment procedure chosen than on the method used for final determination. Selection of the procedure mainly depends on the sample character, the presence of other trace compounds and the concentration of the analyte [22, 24, 25].

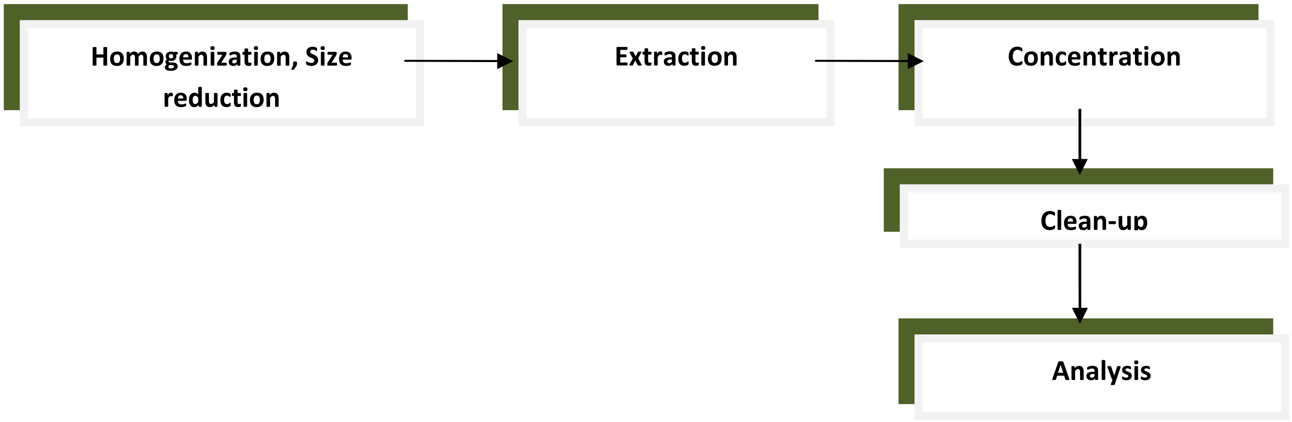

Figure 3. Steps in sample preparation [24]

Any sample preparation method must first fulfill the condition of selective extraction and enrichment of analytes of interest [22]. As herbicide pollutants are generally present in trace level, they have to be selectively separated from the sample matrix and enriched before analysis. The selection of most effective extraction and enrichment procedure is vital for reliable measurement of herbicide concentration in food samples [23]. The selective extraction of triazines is based on differences in their chemical and physical properties. These typically include molecular weight, charge, solubility, polarity and volatility differences. [26].

1.3.1.1. Liquid-Liquid Extraction (LLE)

Liquid-liquid extraction is the most widely used and traditional technique for extraction of organic analytes from aqueous solution. The basic principle of LLE is the partition of the sample between two immiscible solvents, in which the analyte and matrix have different solubility [27].

Sample preparation of triazine herbicides from olive oil samples was performed by AramendÍa *et al*. [28] using acetonitrile/*n*-hexane mixture as extraction solvent and clean-up by Florisil columns prior to analysis. Water/1,2-dichloroethane solvent mixture was used for LLE of atrazine, propazine and prometryn from aqueous samples by Juarez *et al*. [29] for cyclic voltammetry determination.

LLE is thoroughly studied, optimized and widely practiced technique. However, it is less attractive because it is tedious, time consuming, difficult to automate and forms emulsion. In addition, the toxicity, environmentally unfriendly, limited selectivity of the solvents makes it less preferable technique [24, 30].

1.3.1.2. Solid Phase Extraction (SPE)

Solid phase extraction (SPE) involves a liquid-solid partition, where the extracting phase is a solid sorbent. It has been used extensively to remove and concentrate trace organic pollutants from liquid samples. Analytes are retained on the solid sorbent and selectively eluted with an appropriate solvent or solvent system [23, 24]. Varieties of sorbents are available using different mechanisms for extraction/retention of analytes (polar, non-polar, ion exchange, affinity). The separation can be employed both off and on-line coupled to the analytical instrument [27, 30].

Five triazine herbicides were adsorbed on a graphitized carbon black cartridge for extraction from breast milk samples by Balduini *et al.* [23]. Dispersive-SPE with a primary-secondary amine sorbent has been used by Feng Ji *et al.* [31] for extraction of six triazine herbicides from fruit juices and vegetables. Bjarnason *et al.* [32] used a coupled-column system consisting of a combination of a molecularly imprinted polymer (MIP) and a C_{18}-silica column, for selective triazine detection in the reversed phase-HPLC mode from apple samples.

The advantages of SPE include; avoiding clean-up step, high enrichment factors, simple operation and easy automation [23, 31, 32]. The drawbacks of SPE are the less uniformity of the packing resulting in poor efficiency, difficulties with reproducibility of automated systems and it is limited to semi-volatile compounds [24, 27, 30].

1.3.1.3. Solid Phase Micro-Extraction (SPME)

In solid-phase microextraction (SPME), a fused silica fiber is coated with a stationary phase. The fiber is exposed to an aqueous or gaseous sample until equilibrium is established between the analyte in the sample and on the fiber [3]. The analyte is then desorbed from the fiber into a suitable analytical instrument (GC and HPLC) for separation and quantification [27].

A novel molecularly imprinted polymer (MIP) coated SPME fiber was prepared with prometryn as the template molecule. Special selectivity to other triazines which have similar structure to prometryn was discovered with the MIP-coated fibers and applied to the determination of five triazines in the spiked soybean, corn and lettuce samples [33]. The system propazine|methacrylic acid was used as a model for the preparation of molecularly imprinted fibers and its ability to selectively rebind triazines was evaluated by Turiel *et al*. [34].

The main advantage, compared to SPE, is that SPME does not suffer from plugging or channeling and it also eliminates usage of organic solvents. Disadvantages include relatively long equilibration time and losses of volatile analytes when transferring the SPME unit to the instrument [27, 30, 35]

1.3.1.4. Supercritical Fluid Extraction (SFE)

Supercritical Fluid Extraction (SFE) utilizes the unique properties of supercritical fluids to facilitate the extraction of organics analytes. Supercritical fluids have good solvating power, high diffusivity, low viscosity and minimal surface tension [24]. Due to its high purity, nonflammable, non-toxic, inert, and easily accessible critical point, carbon dioxide has been widely used as supercritical fluid in SFE. Supercritical solvents such as N_2O and $CHClF_2$ are rarely used due to environmental concerns [27].

Atrazine, simazine, and ametryn were extracted from tomato samples using SFE by Lanças *et al*. [36]. El-Saeid *et al*. [37] extracted atrazine from spiked fruit juices and vegetables using SFE with an automated restrictor and a solid phase sorbing trap. A combined method using SFE followed by cGC has been developed by Cserháti *et al*. [23] for the determination of 10 triazine herbicides in egg. SFE is fast, solventless, easy to automate, and environmentally friendly extraction technique. Selectivity of extraction can be achieved by tuning the strength of the extraction fluid and alteration of operating conditions [35 – 37]. The costs of the instrumentation, limited sample size and its matrix dependent nature are the main drawbacks [38, 39].

1.4. Membrane Based Sample Preparation Techniques

A membrane is a selective barrier through which different gases, vapors and liquids move at varying rates [1]. In membrane extraction, the membrane facilitates the two phases coming into contact with each other without direct mixing. Molecules move through the membrane by the process of diffusion and are driven by concentration, pressure or electrical potential gradients (see Figure 4). The use of membrane techniques allow the simultaneous enrichment and selective extraction at trace levels with small amount of solvent [40]. The interesting aspect of this technique is that both the donor and acceptor can flow continuously leading to the development of real-time monitoring techniques [24].

Membrane based extraction from liquid samples play a special role among the solvent-free procedures and provide remarkable clean-up efficiency. Membrane extraction techniques can be readily automated and connected on-line to chromatography systems and other instruments. Both inorganic and organic analytes can be extracted in a wide polarity range [24, 40].

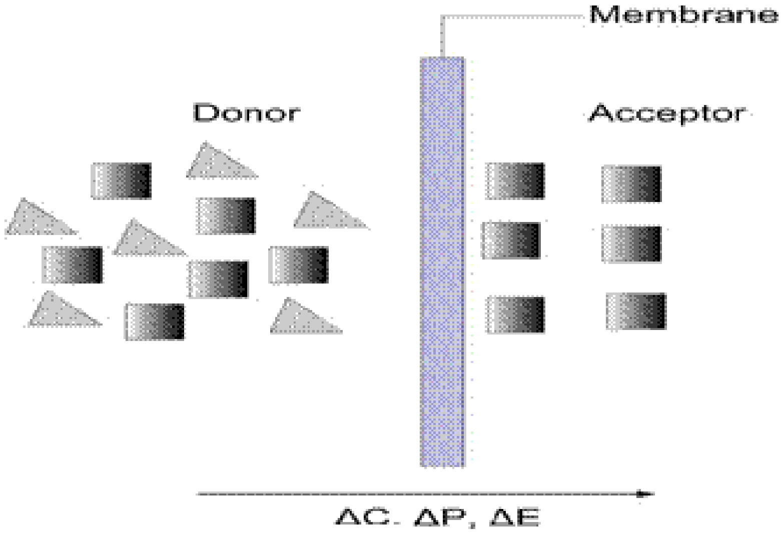

Figure 4. Permeation across a membrane; ΔC, ΔP, ΔE are the concentration, pressure and electrochemical gradients respectively, along which analytes migrate [40].

1.4.1. Membrane Classification

There are a large number of membrane types, with different structures and separation mechanisms. The large variety of membranes can be classified from different points of views. Classification can be made by origin (*i.e.*, biological or synthetic), structure, application area and separation mechanism (one, two and three phase system). Synthetic membranes can be classified based on a number of properties. The basic classification scheme is highlighted in Figure 5 [24, 40].

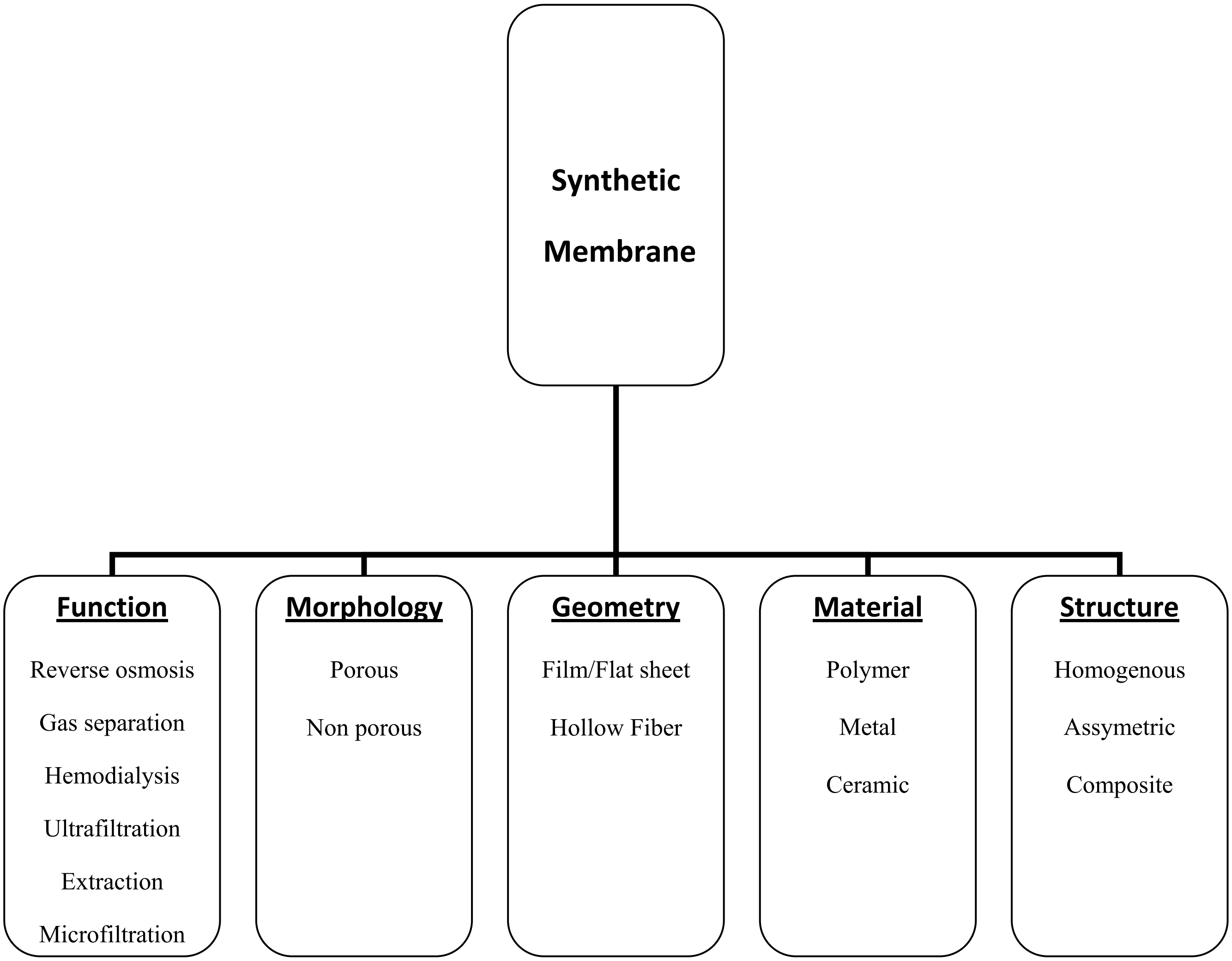

Figure 5. Classification of synthetic membranes [40].

1.4.2. Liquid Membrane Extraction

Liquid membranes have been used extensively in the extraction of organic molecules such as pesticides and metals in water and wastewater matrices as well as other organic pollutants. Liquid membrane extraction techniques may be applied in many ways, employing different types of membranes and a variety of configurations or designs [41].

1.4.2.1. Two-Phase Liquid Membrane Extractions

In two-phase liquid membrane extraction systems both the membrane and the acceptor phase consist of an organic solvent. The extraction efficiency is limited by the partition coefficient, as for conventional LLE, but can be performed in a flow system. For larger partition coefficient, stagnant acceptor can be used to obtain considerable enrichment. Whereas with smaller partition coefficient it might be necessary to use flowing acceptor with small flow rate to successively remove the extracted analytes and maintain diffusion through the membrane [42, 43]. Two-phase liquid membrane extraction is mainly suitable for hydrophobic analytes that are non-dissociable and non-charged. These compounds are easily extracted from water to an organic solvent, but they cannot be back-extracted into a second aqueous phase [44].

1.4.2.2. Three-Phase Liquid Membrane Extractions

With three-phase liquid membrane extraction techniques the analytes are transferred through the membrane to the aqueous acceptor phase. The analyte molecules are irreversibly trapped after entering the acceptor phase, for example by pH changes (for acids or bases) or by some complexation reactions. This trapping results in a transport of analyte molecules from the donor to the acceptor phase. Trapping is crucial for the success of the three-phase liquid membrane extraction. By means of suitably selected acceptor conditions, it is possible to tune the mass transfer process and to obtain the desired selectivity and degree of enrichment for a number of useful applications [44].

1.4.2.3. Transport Mechanisms in Liquid Membranes

The permeation of ions or molecules through a liquid membrane can be realized to take place in a stepwise manner, as in Figure 6. The following steps are occurring in the process: (i) analyte diffusion from the bulk donor phase to membrane surface; (ii) analyte sorption into the membrane; (iii) analyte diffusion through the membrane; (iv) desorption of analyte from the membrane, and (v) analyte diffusion from the membrane surface to the bulk of the acceptor [2].

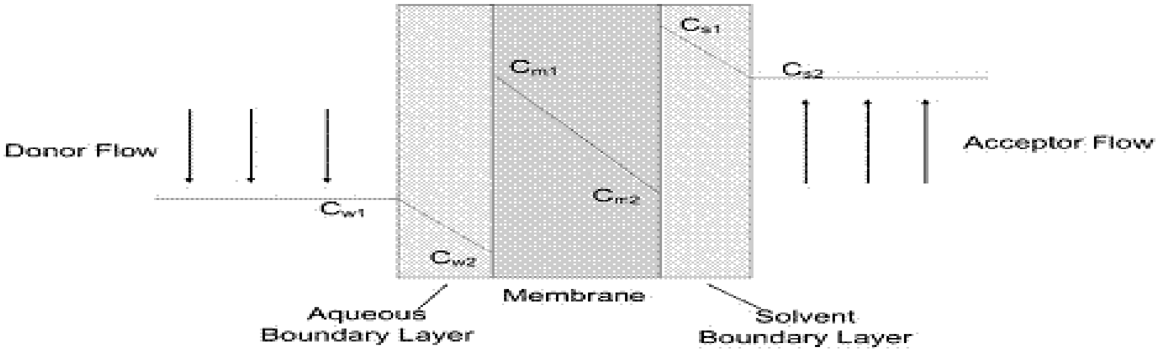

Figure 6. Concentration profile in liquid membrane extraction process; where C_w, C_m and C_s refer analyte concentration in water, membrane and the extractant phase, respectively [40].

The selective separation and thus permeation in the liquid membrane is influenced mainly by the composition of the membrane solvent and classified as follows;

1. ***Passive Transport.*** In this process the separation is based on the difference in solubility of the components in the membrane phase and diffusion through the membrane based on concentration gradient. The analytes are trapped in the acceptor phase by reacting with appropriate chemical species not soluble in the membrane [2].
2. ***Carrier Mediated Transport.*** A mobile carrier, which more or less selectively binds to the analyte, is incorporated into the membrane liquid. Two requirements of the mobile carrier are: first, the carrier and its complex must be soluble in the membrane liquid but not in the adjacent aqueous phase. Secondly; the complex formation must be strong on the donor side but weak on the acceptor side, where the carrier releases the analyte [2, 25].

1.5. Supported Liquid Membrane Extraction

The supported liquid membrane (SLM) extraction technique is an alternative approach for selective extraction of trace quantities of potential pollutants from various matrices of environmental and biological origin [2]. In SLM extraction technique an organic solvent or a mixture of solvents is immobilized in a thin porous polytetrafluoroethylene (PTFE) membrane to form a barrier between two aqueous phases in a flow system. Compared to polymeric membranes, this offers high-mass transfer rates and the possibility for wide variety of organic liquids and additives without compromising the stability of the membranes [42, 45].

The most important applications of SLM extraction technique are in the simultaneous sample pre-concentration and clean-up of ionisable pesticides from environmental waters containing complex matrices of varying properties [2]. Selective extraction of these pollutants has been demonstrated for different classes of pesticides including chlorophenols, sulfonylureas [46], phenoxy acids [47], and triazine herbicides and their degradation products [6, 11].

1.5.1. SLM Modules

The membrane module designs (hollow fiber, flat sheet, and spiral) are based on the membrane geometry. One important property is the ratio between membrane surface area and its volume. This ratio should be high to get large enrichment factors especially for analytical purposes. This ratio is highest for the hollow fiber module followed by the spiral module and lowest for the flat module [40 - 42].

1.5.1.1. Flat Sheet and Spiral Modules

Classically, flat-sheet porous PTFE or polypropylene membranes are used as support for the membrane liquid and mounted in holders permitting one flow channel on each side of the membrane as shown in Figure 7. Such membrane units are typically operated in flow systems and in principle applicable to all versions of membrane extraction for analytical sample preparation or sampling. Such a setup can be easily interfaced with different analytical instruments such as HPLC and various spectrometric instruments, and thereby provide good possibilities for automated operations [42, 48].

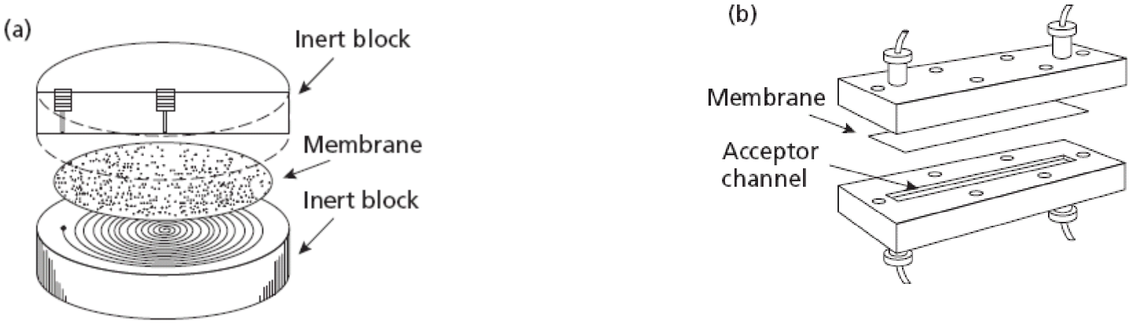

Figure 7. SLM modules (a) spiral and (b) flat sheet [48].

1.5.1.2. Hollow Fiber Modules

Porous polypropylene hollow fibers are used in a disposable way, minimizing carryover problems and reducing costs. On the other hand, manual manipulations are needed, limiting the possibility for automation. With these devices the extraction can be carried out in a static mode, either in large sample volumes, where the extraction is not intended to be complete, or in small volumes aiming for complete extraction. Usually stirring is applied to increase the speed of mass transfer. Some typical practical arrangements are shown in Figure 8 [3, 44].

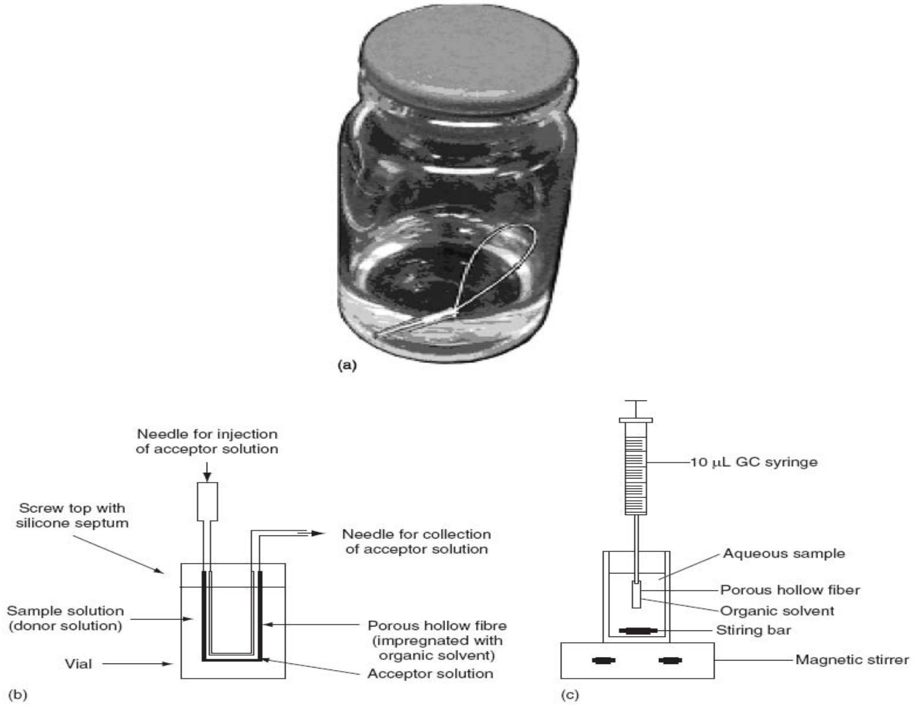

Figure 8. Hollow-fiber modules for membrane extraction (a) Hollow-fiber loops for equilibrium extraction (b) Liquid-phase microextraction, and (c) Syringe-based hollow fiber LPME [44].

1.5.2. Principles of SLM Extraction

In SLM extraction, the analytes are partitioned from the aqueous sample stream into the organic membrane and are then re-extracted into the aqueous acceptor phase. The driving force is the difference in analyte concentration between the donor and acceptor phases. In order to maintain the concentration gradient across the two phases the solutes must be able to exist in two forms: in a non-ionic form in the donor side to be extracted into the membrane and in an ionic form on the acceptor side to be irreversibly trapped. The uncharged analytes are extracted from the flowing aqueous donor stream into the organic phase followed by a second extraction into the stagnant acceptor phase. The volumes of the stagnant strip solutions that are used normally much smaller than the sample solution volumes pumped through and this ensures high pre-concentration factors [26, 42, 48].

The liquid membrane solvents have low water solubility, low volatility, low viscosity, long term stability, and high efficiency for the analyte. The most commonly used solvents are long chain hydrocarbons like *n*-undecane and more polar compounds like di-*n*-hexylether, di-octyl phosphate and others depending on the type of contaminants analyzed [2, 3, 49].

By careful choice of the pH in the donor and the acceptor phases, as well as the composition of the liquid membrane, selective extraction and enrichment can be achieved in one step for the diffusing species. The technique is well suited for ionisable compounds such as medium to weak acids and bases which may be shifted in their aqueous/organic partitioning ratio by pH adjustments [43, 49, 50]. The hypothetical extraction mechanism is described in Figure 9.

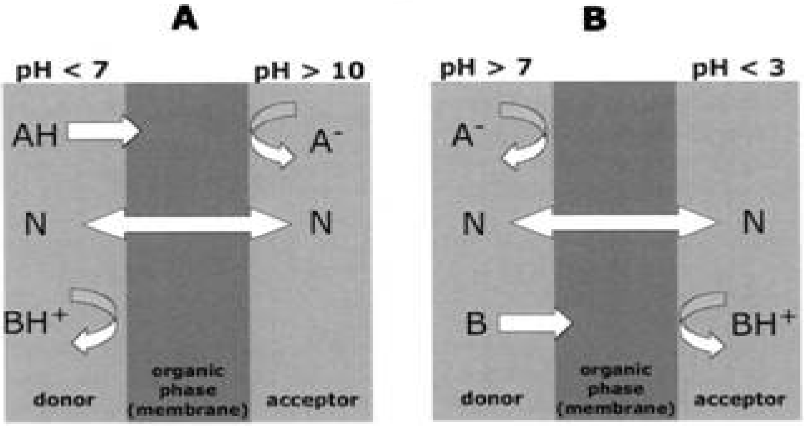

Figure 9. Analyte extraction using SLM extraction technique: **A**: extraction of analytes with acid groups; **B**: extraction of analytes with basic groups [43].

1.5.3. Theory of SLM Extraction

The process of diffusion in liquid membranes is governed by Fick's first law of diffusion:

$$J = -D\frac{\partial \phi}{\partial \chi} \qquad (1)$$

Where; *J* denotes the extent of flux of the analyte molecules per given area and time (g cm^{-2} s^{-1} or mol cm^{-2} s^{-1}). *D* (diffusion coefficient) gives the diffusibility measure of the analyte across the membrane with units of $cm^2 \cdot s^{-1}$ and is proportional to the velocity of the diffusing particles. The concentration gradient is given by the ratio of the change in concentration ($\partial\Phi$) to the change in position ($\partial\chi$), *i.e.*, $\frac{\partial\phi}{\partial\chi}$. The term *D* depends on the temperature, viscosity of the fluid and the size of the particles [40, 42, 44].

Integrating the Fick's equation gives:

$$J = D\frac{\left(C_{is} - C_{il}\right)}{l} \tag{2}$$

Where: C_{is} is the concentration of analyte *i* at the outer membrane interface C_{il} the concentration of analyte *i* in acceptor phase *l* is the membrane thickness. Factors which control the *D* term such as temperature and partition coefficient are normally adjusted to optimal conditions in order to increase the flux across the membrane [42, 44].

Partition coefficient (*K*) is another important factor which controls the diffusion (flux) of analyte molecules across the membrane. It is defined by the equation below:

$$K = \frac{C_{membrane}}{C_{matrix}} \tag{3}$$

Where C_{membrane} is the analyte concentration in the membrane and C_{matrix} is the analyte concentration in sample matrix [41, 42, 44].

The rate of extraction, *i.e.*, $\frac{\partial\phi}{\partial\chi}$, the flux of analyte molecules from donor to acceptor is proportional to the concentration difference (ΔC) of the diffusing species (uncharged analyte molecules) through the membrane.

$$\Delta C = \alpha_D C_D - \alpha_A C_A {}^{K_A}\!/_{K_D} \tag{4}$$

C_D and C_A denote concentration in the donor phase (sample) and acceptor phase respectively. α_D and α_A are the fractions of the analytes that are in uncharged form in the donor and acceptor phases respectively. K_A and K_D are the partition coefficients for the analytes in the acceptor and donor phases respectively. For maximum performance, the extraction conditions are set up so that α_D is close to 1 and α_A is a very small value (< 0.0005). C_A is zero in the beginning of the extraction and increases during the operation, usually to values greater than those of C_D. Thus, ΔC will decrease as the extraction is going on and eventually approach zero, which leads to the expression for maximum concentration enrichment factor:

$$E_{e\max} = \left(\frac{C_A}{C_D}\right)_{\max} = \left(\frac{\alpha_D K_D}{\alpha_A K_A}\right) \tag{5}$$

If K_D is assumed to be equal to K_A, which is the case if the composition of the two aqueous phases is similar (except for pH), then:

$$E_{e\max} = \left(\frac{C_A}{C_D}\right)_{\max} = \frac{\alpha_D}{\alpha_A} \tag{6}$$

The rate of mass transfer is constant only when ΔC is constant, i.e., when $\alpha_D C_D >> \alpha_A C_A\, K_A/K_D$. Thus, for the purpose of analytical applications of SLM extraction, it is necessary that α_A is very low. For a monoprotonic basic analyte, the fraction of non-ionised molecules (α_A) is related to the pH of the acceptor solution by:

$$\alpha_A = K_A \big/ \left(\left[H^+\right] + K_A\right) \tag{7}$$

From equations 6 and 7, the maximum concentration enrichment factor can thus be estimated. For compounds with high values of α_A, as calculated from equation 7, the resulting low maximum enrichment factors were easily attained. It was found that, if $\alpha_A < 0.0005$ the change of mass transfer rate and thus extraction efficiency is negligible. This leads to the rule of thumb; for basic analytes the pH in the acceptor phase should be at least 3.3 units lower than the pK_a of the less basic analyte and vice versa [42, 49, 51].

1.5.3.1. Extraction Efficiency

Extraction is usually evaluated in terms of extraction efficiency (E) which is the fraction of analyte that is collected in the acceptor to the amount present in the sample. It is a measure of the rate of mass transfer through the membrane. It is constant at specified extraction time, flow rate, phase composition and ionic strength.

$$E = \frac{n_A}{n_S} = \frac{(C_A V_A)}{(C_S V_S)} \tag{8}$$

Where n_S and n_A are the number of moles input from the sample during the extraction and those collected in the acceptor, respectively. C_A and C_S are the corresponding concentrations in the acceptor and sample. V_A is the volume of the stagnant acceptor phase and V_S is the volume of the extracted sample that has passed through the donor channel [42, 44].

The most efficient extractions are obtained at low donor flow-rates, because a low flow-rate increases the residence time of an analyte molecule in the donor channel. Extraction efficiency theoretically approaches unity as the flow-rate approaches zero. Extraction efficiency increases with organic-aqueous or octanol-water partition coefficient (log K_{ow}) of the analyte. Too low values lead to insufficient extraction into the membrane, and too-high values lead to insufficient transfer of analyte molecules from the membrane into the acceptor. It is actually best to work at high flow-rates for compounds with high log K_{ow} values [42, 48, 51].

1.5.3.2. Enrichment Factor

One of the most important parameters used to express the extent of membrane extraction process is the concentration enrichment factor (E_e). It is an important parameter since it gives an idea of the detection limit of the compounds to be extracted. The concentration enrichment factor (E_e) is related to the extraction efficiency as:

$$E_e = \frac{C_A}{C_S} = E\left(\frac{V_S}{V_A}\right) = E\left(\frac{F_D t}{V_A}\right) \tag{9}$$

From equation 9, it is seen that an increase in donor flow-rate (F_D) increases E_e. However, the increase in E_e also depends on how E depends on the flow rate. When the donor flow-rate is increased, an increasing amount of analyte is in to the system, which is offset by a decrease in E. This change usually results in an increase of the E_e with donor flow-rate for a given time. It follows from equation 9 that even if the extraction efficiency E approaches 1, the enrichment factor can never become larger than the volume ratio (V_S/V_A) [42, 44].

The concentration enrichment factor (E_e) is especially influenced by the organic-aqueous partition coefficient (log K_{ow}) of the analytes. For polar compounds with low log K_{ow} values, the dissolution into the membrane limits the extraction efficiency and not much gain is achieved at higher donor flow rates. If the analytes are hydrophobic enough (i.e. log $K_{ow} \geq 2.0$) it is preferable to work at high donor flow-rates, thus gaining high enrichment factors and in short analysis time. On the other hand, if the analytes are polar (log $K_{ow} < 2.0$), it is beneficial to use lower donor flow-rates, increasing the contact time for compounds that have poor dissolution into the membrane resulting in longer extraction times since each degree of enrichment takes more time [42].

1.6. Ethiopian Honey Wine (*Tej*)

Tej is a honey wine (or honey mead) made from fermented honey and a special kind of hops called Gesho (*Rhamnus prinoides*), a species of buckthorn that grows native only to Africa [52]. Sometimes, widely for commercial purposes, mixture of honey and sugar could be used for its preparation. In cases where sugar is used as part of the substrate, natural food coloring is added so that the beverage attains a yellow color similar to that made from honey [53].

Figure 10. *Tej* being served with *'Berele'* for drinking [52].

Tej is a home-processed honey wine, in fact, considered to be the "national drink." Some make it at home for weddings, cocktails and other celebrations; women traditionally prepare gallons of it. *Tej* is also commercially served in *'Tej bet'* (*Téj* House, similar to a Coffee House), or special bars set up strictly to sell *Tej* by a special flask called *'Berele'* (Be-re-lé) as in Figure 10. The neighborhood *'Tej Bet'* is a very popular gathering place in Ethiopia (Figure 11) [52, 54]. Ethiopia is the largest honey producer in Africa where 70% of the honey sold goes to the making of *Tej*, according to an April 2007 report by an Ethiopian research agency [55, 56]. Other reports describe that the amount of Ethiopian honey used for *Tej* is as high as 80% [57].

Figure 11. Ethiopians drinking *Tej* with *'Berele'* at a local *'Tej Bet'* [52].

Good quality *Tej* is yellow, sweet, effervescent and cloudy due to the content of yeasts. The flavor of *Tej* depends upon the part of the country and the climate where the bees have collected the nectar [58]. The alcohol content of the *Tej* that one buys in a *'Tej Bet'* varies widely from 7.0% to 10.9% according to studies [59]. The longer the fermentation process the more the sugar from the honey will turn to alcohol. *Tej* left to be fermented for months can almost take on the taste and color of liquor (but still with a sweet undercurrent). The fermentation is spontaneous and depends on the microflora naturally present in the substrates, on utensils and equipment used [52, 59]. Both the leaves and the stem of the *Gesho* plant can be used in the making of *Tej*. Most Ethiopians prefer the one made from the stem. *Tej* made of the stem is pale yellow in color and tastes at once sweet and spicy. *Tej* made from the leaves on the other hand, is amber and usually has more of a pungent flavor [52]. The detailed preparation procedure of *Tej* is given in section 2.4 of this thesis.

Fusel oil content of *Tej* samples varies between 13.6 to 27.4 g/l00 L. Fusel oils contribute to the flavor and odor of *Tej.* Fusel oil is a collective name of isopentyl alcohol, 2-methyl-1-butanol, isobutyl alcohol, propyl alcohol, esters and aldehydes [53]. Mean total carbohydrate content ranges between 1.49 to 3.73 mg mL^{-1}; mean total lipid content is less than 1.00 mg mL^{-1}; mean protein content ranges between 0.33 and 4.66 mg mL^{-1} and mean values for reducing sugars ranges between 0.46 and 2.09 mg mL^{-1} [53, 59].

With intensive application of herbicides by the flower farms and other farms producing flowering crops, vegetables and fruits cause a possible contamination of the honey by residues of the herbicides through the nectars collected by the bees. Bee farmers may also use selective herbicides to prevent their flowers from weeds. As described above, the larger percent of honey product in Ethiopia is used for *Tej* preparation. Consequently the *Tej* produced from the honey product can be contaminated by the herbicide residues and hence there is a need to develop an analytical technique for extraction and quantification of herbicide residues in *Tej* samples.

1.7. Objectives

1.7.1. General Objective

To develop a liquid membrane extraction and trace enrichment method for selected pesticide residues in agro-processed fruit juice and Ethiopian honey wine (*Tej)* samples.

1.7.2. Specific Objectives

The specific objectives of the present study are:

1. To optimize SLM parameters for trace level extraction and HPLC-UV determination of selected triazine herbicide residues in spiked agro-processed fruit juice and home made *Tej* samples.
2. To determine extraction efficiency and concentration enrichment factor of the developed method in spiked agro-processed fruit juice and home made *Tej* samples.
3. To evaluate analytical performance of the developed method, in the selected sample matrices.

2. Experimental

2.1. Chemicals and Reagents

Standards of atrazine, ametryn and terbutryn used in this study were from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The organic solvents used for immobilizing into the membrane were di-*n*-hexyl ether (Sigma-Aldrich Chemie GmbH, Germany) and *n*-undecane (Fluka Chemie GmbH, Germany). Potassium dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate (K_2HPO_4), used for preparation of the phosphate buffers, were from Fluka (Fluka Chemie GmbH, Germany). HPLC grade methanol and acetonitrile, used as a mobile phase for chromatographic separation, were from Techno Pharmchem (Techno Pharmchem, Bahadurgarh, India). Other chemicals used in this study, including hydrochloric acid, phosphoric acid, sodium hydroxide and potassium hydroxide was all from Riedel-de Haën (Riedel-de Haën, Germany).

Stock solutions of 100 mg L^{-1} of each triazine standard were prepared by dissolving 2.5 mg of each triazine standard in small amount of acetonitrile (less than 1 mL) and by diluting to 25 mL with de-ionized water. The prepared stock solutions of the triazine standards were stored in a refrigerator when not in use. Working solutions of triazine standards mixture for calibration were obtained by diluting the stock solution with de-ionized water. A series of triazine standard mixture solutions were prepared, for calibration at five points in the concentration range of 0.1 to 2.0 mg L^{-1}, by diluting from the 10 mg L^{-1} triazine standards mixture solution each day.

A 3.5 x 10^{-3} mol L^{-1} phosphate buffer at pH = 7.0, for use in mobile phase, was prepared by dissolving 118 mg of K_2HPO_4 and 146 mg of KH_2PO_4 in 500 mL de-ionized water. The prepared mobile phase buffer was filtered through a 0.22 µm filter (Millipore) and degassed for one hour using ultrasonic bath, before being used for chromatographic separation. A 0.01 mol L^{-1} phosphate buffer at pH = 7.0 was also prepared, as donor phase buffer in the membrane enrichment, by dissolving 250 mg of KH_2PO_4 and 275 mg of K_2HPO_4 in 250 mL de-ionized water.

2.2. Apparatus

Gilson Minipuls 3 peristaltic pump (Gilson Medical Electronics, Villiers-Le-Bel, France) with acid resistant tubing (Acid-Flexible; Elkay Products, Shrewsbury, MA, USA) used for pumping the donor and the acceptor solutions to the SLM unit. Analytical balance (Mettler Toledo, Mettler instrument AG, Switzerland) used for weighing the standards and the buffer salts. Digital pH meter (HANNA instruments, Melbourne, Australia) used for pH measurement of the buffer solutions and sample solutions. Ultrasonic bath (Decon F5100b, England) was used for degassing the mobile phase solutions. Agilent Technologies 1200 series HPLC (Agilent Technologies, Germany) with C_{18} analytical column (Techsphere 5ODS, 250 mm x 4.6 µm internal diameter; HPLC Technology, Macclesfield, Cheshire, UK) was used for chromatographic separation and HPLC analysis the extracted of analytes. Whatman number 1 and number 42 filter papers (whatman® Schleicher & Schuell, England) were used for filtration of extraction sample solutions. Mobile phase buffer solutions were filtered through 0.22 µm filter paper (Millipore, Germany). Porous membrane filter paper (Milipore FG, Germany) was used as a membrane support by soaking with the organic membrane solvents. Hot plate with magnetic stirrer (Model 04803-02, USA) was used for rapid mixing and dissolution of the buffer salts in the preparation of buffer solutions.

2.3. Preparation of Ethiopian Honey Wine (*Tej*)

The fermentation pot is seasoned by smoking over smoldering gesho (*Rhamnus prenoides*) stems and olive wood, before starting the actual preparation procedure. One part of honey is mixed with 3 parts of water (V/V) and placed into a 5000 mL pot covered with a cloth and let to begin fermentation on its own for three days. On the third day of the fermentation process wax and scum are removed by decantation. Then 1000 mL portion of the fermented solution is mixed, in another pot, with 500 g of chopped gesho (*Rhamnus prenoides*) stems. To remove dust particles, the chopped gesho (*Rhamnus prenoides*) stems are peeled and washed with clod water before mixing with the 1000 mL portion of the fermented solution. The prepared mixture is boiled for about 10 min and then returned back to the remaining portion of the honey water fermented solution, in the fermentation pot. The gesho (*Rhamnus prenoides*) stems are removed after two weeks, from the day the honey and water are mixed to ferment, and then left to ferment for another two more weeks. The mixture was stirred daily, for more than one hour, through out the fermentation process. Finally the well fermented and consumable *Tej* was filtered through a woven cloth, to remove sediment and gesho residues [52, 58].

2.4. Sample Treatment

All agro-processed fruit juices were made from water and natural fruit juice concentrates of pineapple, orange and apple with no preservative, no artificial color and no additive. The selected samples of fruit juices are commercially available and are produced by Al Buheira Lacnor, Sharjah, United Arab Emirates. The collected fruit juice samples were stored in refrigeration at 4 ^{o}C. The samples were equilibrated to room temperature before being used for spiking analysis.

The *Tej* sample was home-made with fresh honey purchased from bee farmers of South Western Shoa area. Detailed preparative procedure is presented in the above section. No sugar, no food colorant, no flavoring materials were used in the preparation procedure of the *Tej* sample. The prepared *Tej* sample was stored in a refrigerator below 4 ^{o}C and was also equilibrated to room temperature before being used for spiking analysis.

From the 1000 mL fruit juice sample the 100 mL portion was used for pH measurement, 100 mL portion was used for unspiked sample extraction, and another 100 mL portion was used for spiking with triazine standards mixture. The same procedure was performed on the 5000 mL of the prepared *Tej* but 200 mL portion was used for spiking with triazine standards mixture.

The unfiltered fruit juice and *Tej* samples were spiked with 1 mL of 10 mg L^{-1} triazine standard mixture to give 100 mL of 0.1 mg L^{-1} spiked fruit juice samples. All the extraction samples were filtered, before SLM extraction, first with whatman number 42 filter paper and further filtered with whatman number 1 filter paper to prevent clogging of the tubes and the membrane pores of the SLM unit. Triplicate extractions were performed for all fruit juice samples and five extractions were also performed for the *Tej* samples.

2.5. Membrane System

A similar membrane unit configuration with the previous SLM works [10, 45, 49] is used and consisted of two circular polytetrafluoroethylene (PTFE) blocks (diameter = 120 mm, thickness = 8 mm) with machined grooves (depth = 0.25 mm, width = 1.5 mm, length = 250 cm) each with a total volume of about 0.95 mL. The grooves formed channels that are arranged in the form of Archimedes' spirals. The donor channel of the PTFE block was equipped with an O-ring, outside the grooves, for sealing the flow system. Both sides of the holder were backed with aluminum blocks of 6 mm thickness in which threads for the clamping screws were machined to make the assembly stable.

The liquid membrane support was Millipore FG (Millipore) with an average pore size of 0.2 µm, a total thickness of 175 µm of which about 115 µm is polyethylene backing and a porosity of 70%. The liquid membrane was prepared by immersing the membrane support in the organic solvent to be immobilized for a period of 30 min. The soaked membrane was placed between the two PTFE blocks, with the rough side of the membrane facing the donor side and the whole construction was clamped together tightly and evenly with six screws. Thus the two channels are separated by the liquid membrane, forming the donor (feed) and the acceptor (receiving) compartments [10, 11, 49].

Two peristaltic pumps were used to control the flow rates of the donor and acceptor phases independently. The tubes used for pumping solutions were acid-resistant, with internal diameters of 2 mm for the donor and 1 mm for the acceptor. The various parts of the flow system were connected with 0.8 mm internal diameter PTFE tubing and Altex screw fittings. The sample and buffer in the donor stream were merged in a PTFE tee connection and then mixed in a coil (1.0 m x 0.8 mm internal diameter coiled PTFE tubing) before entering the donor channel of the membrane device [10, 11].

2.6. Chromatographic System

All chromatographic analyses were performed using Agilent Technologies® 1200 series HPLC, quaternary pump, equipped with an auto sampler and degasser. Chromatographic separations of the analyte compounds were performed on a C_{18} analytical column and detections were made using UV-DAD detector at 235 nm. The whole part of the HPLC system was interfaced with computer; all the LC parameters and chromatograms were monitored and collected on computer (Hp Compaq, intel®, *Pentium 4 HT*) using Chemstation software (Agilent Chemstation, Agilent Technologies®). The chromatograms were integrated, analyzed and calibration curves were prepared by the same software.

2.7. Membrane Enrichment and Chromatographic Separation

After installation of the membrane enrichment system, as described above, both the donor and the acceptor channels were flushed with de-ionized water to remove excess of the organic membrane solvent from the membrane surface. The acceptor channel was pumped with 1 mol L^{-1} HCl acceptor solution, until the acceptor channel is filled with the acceptor solution [11, 45].

The sample and the donor phase buffer solution (0.01 mol L^{-1} phosphate buffer at pH = 7.0) were pumped with a peristaltic pump, with a total donor phase flow rate of 1.0 mL min^{-1} (sample to buffer volume ratio of 1:1). Sample and buffer were mixed in the tee connection and the mixing coil before being delivered to the extraction system [10, 11]. The extraction samples (both the fruit juice and *Tej* samples) and the donor buffer solution were mixed at equal volume in 100 mL volumetric flask to assure complete mixing of the samples with the buffer solution before being pumped through the tee connection.

After 30 min delivery of the sample and the donor phase buffer solution, the flow system was rinsed by pumping of the pure donor phase buffer solution for 10 min while the acceptor phase was kept stagnant. The system was left to stand for another 10 min to allow equilibrium diffusion of the analytes from the non polar organic membrane to the acidic acceptor phase. At the end of the 10 min equilibration time, the contents of the acceptor channel were quantitatively transferred into a 10 mL calibrated glass tube by displacement with the acidic acceptor solution (1.0 mol L^{-1} HCl), at the flow rate of 0.4 mL min^{-1} to a final volume of 2.0 mL [11, 45]. To prevent the possible acidic hydrolysis of the extracted triazines, the collected extracts were adjusted to pH = 7.0 with 200 µL of 7.0 mol L^{-1} sodium hydroxide solution before being transferred to the sample vials for injection with an auto sampler [10].

A 20 µL aliquot of the enriched acceptor phase solution from the sample vials was injected into the HPLC system. For a reversed-phase chromatographic separation of the triazine mixture a mobile phase consisting of 50% acetonitrile and 50% phosphate buffer (3.5 x 10^{-3} mol L^{-1} at pH = 7.0) was utilized. All separations are performed at the mobile phase flow rate of 1.0 mL min^{-1} for 20 min. The chromatograms were recorded by UV-DAD at 235 nm with a reference at 360 nm.

A series of triazine standards mixture solutions, from the stock solutions of the standards, were prepared every week for calibration of the HPLC method. The calibration graphs were prepared daily in the concentration range of 0.1 to 2.0 mg L^{-1} at five points based on triplicate injections and for measurement of peak area. Calibration curves of correlation coefficient greater than 0.999 are prepared for each triazine herbicide in all the HPLC methods used in this study.

3. Results and Discussion

3.1. Optimization of Membrane Solvent

For systems with flowing donor and stagnant acceptor, two mass transfer conditions can be distinguished: membrane-controlled extraction and donor-controlled extraction. In the membrane-controlled conditions, the rate-limiting step is the diffusion of the analyte compound through the membrane. With donor-controlled conditions, extraction is limited by the diffusion in the donor phase and thus depends on the diffusion coefficient in the donor phase [44]. The donor-controlled extraction conditions prevail when donor-membrane partition coefficients (K_D) is larger than 10. The mass transfer is mainly membrane-controlled when donor-membrane partition coefficient (K_D) is less than 1. In donor-controlled conditions it is found that the value of the partition coefficient has no large influence on the extraction efficiency or the enrichment factor, as long as it is reasonably large. On the other hand, the rate at which equilibrium is reached will be influenced by the partition coefficients. Further, too large partition coefficients are not favorable, as the transfer of analyte out of the membrane into the acceptor phase in those cases may become less efficient [2, 44]. Hence, one of the most important parameters that can influence the efficiency and selectivity of extraction is the composition of the membrane solvent through which permeation will occur [10].

For fairly polar compounds such as atrazine, a more polar membrane is desirable. However polar liquid membrane solvents tend to be more water soluble with less stability and less selectivity; a compromise must be done between membrane stability and selectivity [45]. It was observed that in other studies from the literature, addition of carrier had not significantly improved extraction efficiencies of the parent triazine herbicides considered in this study [10, 11]. Hence, no carrier addition was applied in all the membrane solvent compositions.

Starting from 100% (V/V) *n*-undecane, the membrane solvent composition has been varied by increasing the amount of di-*n*-hexylether to 50% (V/V). The values of extraction efficiencies (E) obtained for the selected triazine herbicides with different composition of membrane solvent are shown in Figure 12 below. The results show the obvious influence of the composition of the immobilized organic membrane solvent on the extraction efficiency. The extraction efficiency of atrazine rapidly increases with the amount of di-*n*-hexylether, while the extraction efficiency for ametryn and terbutryn show very slight increase. Increasing the amount of di-*n*-hexylether beyond 50% may further increase the extraction efficiency of atrazine except shortening the lifetime of the liquid membrane, which might be due to gradual dissolution of the more polar di-*n*-hexylether into the aqueous flow system. Higher extraction efficiencies and better membrane stabilities were observed for liquid membrane solvent composition of 50% (V/V) di-*n*-hexylether and 50% (V/V) *n*-undecane. Membrane solvent composition of 50% di-*n*-hexylether/50% *n*-undecane was used for the extraction of trace level triazine herbicides from spiked fruit juice and *Tej* samples.

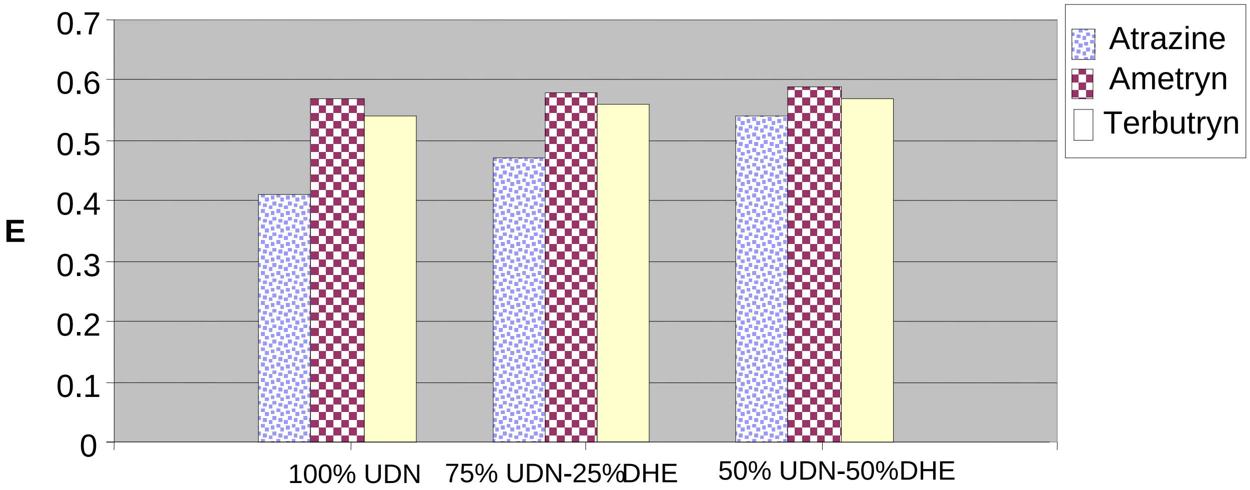

Figure 12. Effect of the membrane solvent composition on extraction efficiency (E). UDN = *n*-undecane and DHE = di-*n*-hexylether.

3.2. Optimization of Acceptor pH

When SLM technique is applied to basic or acidic analytes, the extraction efficiency of the target analytes is highly influenced by the pH of the stagnant acceptor phase [60]. The acceptor pH is seen to be critical and is ideally set so that analyte molecules are completely ionized and trapped [49]. According to the theoretical treatment by Jönsson *et al*. [51], for maximum enrichment factor of basic analyte compounds, the acceptor phase pH should at least be 3.3 units below the lowest pK_a value of the target analytes.

For compounds investigated in this work the lowest pK_a value is that of atrazine ($pK_a = 1.68$), and hence the lowest pH should be less than -1.62. To get closer to the theoretical lowest acceptor phase pH, acceptor solution of 1 mol L^{-1} HCl was used giving a pH value of approximately 0.0 for the acceptor phase. The above condition could not be fully met for atrazine, leading to incomplete trapping, which limits the time linearity of the extraction efficiency as well as the maximum enrichment factor attainable. The degree of trapping could be increased by using more acidic acceptor solution (more than 1 mol L^{-1} HCl). The disadvantage with this approach is decreased stability for both the membrane and the analytes [49]. Triazines are known to undergo acidic hydrolysis [19]. However, it has been shown in earlier study by Chimuka *et al.* [49], even under incomplete trapping condition maximum enrichment factors for atrazine can be reached at the acceptor pH = 0.0.

In order to observe the influence of increasing the pH of the acceptor phase beyond the theoretically recommended pH, the acceptor phase pH was varied from 0.0 to 2.0 while the pH of the donor phase was kept at pH = 7.0. Acceptor phase HCl concentrations of 1 mol L^{-1}, 0.3 mol L^{-1}, 0.1 mol L^{-1}, 0.03 mol L^{-1} and 0.01 mol L^{-1} were used. The results in Figure 13 show a good agreement with the developed theory that the amount extracted increases with increase in the degree of trapping, which can also be increased by using more acidic acceptor solution for basic analytes. For the more basic analytes ametryn and terbutryn larger increase in extraction efficiency was not observed beyond pH = 1.0, but a steep rise in extraction efficiency for the less basic analyte atrazine was observed up to pH = 0.0, which is in good agreement with the theoretical derivations [51]. Furthermore, with all other parameters being equal, the more basic compound should have the highest enrichment factor [49]. However, terbutryn is slightly more basic than ametryn but its enrichment factor was observed to be less. This reverse might be due to difference in partition coefficients of the two compounds into the membrane liquid.

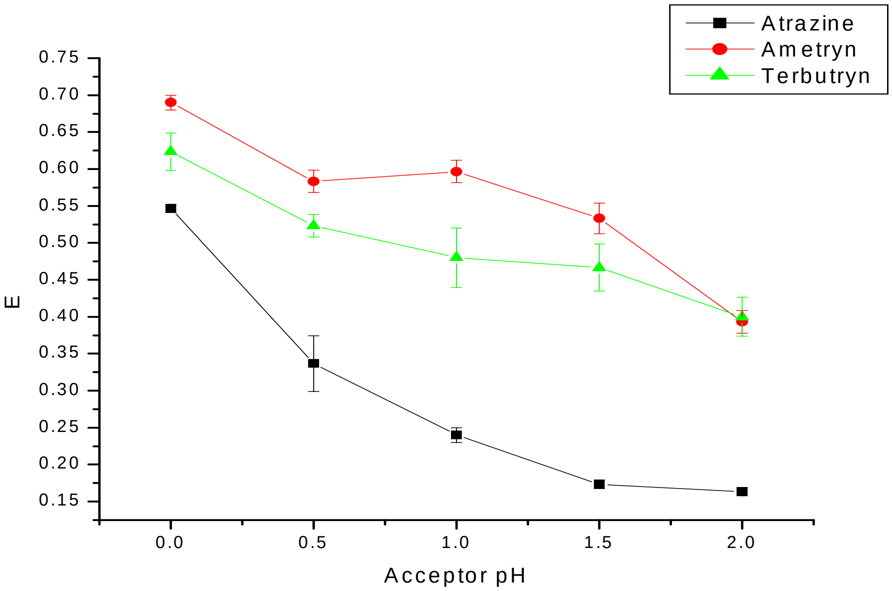

Figure 13. Effect of increasing the acceptor phase pH on extraction efficiency for 30 min extraction of 0.5 mg $L^{-1}$of each triazine herbicide.

3.3. Optimization of Donor pH

According to the theoretical consideration by Jönsson *et al*. [42], for basic compounds such as triazines, the pH of the flowing donor phase should be at least 2 pH units more than the highest pK_a value to facilitate dissolution into the membrane. The highest pK_a value is that of terbutryn ($pK_a = 4.3$) and the lowest pK_a value is that of atrazine ($pK_a = 1.6$), for which lowest extraction efficiency is expected below pH = 4.0 because most of the analytes will get protonated and rarely cross the non polar organic membrane.

Solutions of the herbicides containing 0.5 mg L^{-1} of each of the triazines under study were mixed at the tee connection, with the donor phosphate buffer before entering the donor channel of the SLM unit. The donor phase phosphate buffer solutions ranging in pH from 3.0 to 7.0, all at buffer concentration of 0.01 mol L^{-1} were freshly prepared buffer solutions from H_3PO_4-KH_2PO_4 (pH = 3.0), KH_2PO_4 (pH = 4.0), and KH_2PO_4-K_2HPO_4 (pH = 6.0 and 7.0). The results in Figure 14 show significant increase in extraction efficiencies for all triazines with increase in donor phase pH. Highest extraction efficiency values were observed at donor phase pH = 7.0 and the lowest values were observed below pH = 4.0 for all the triazines, which is in good agreement with the theoretical derivations [51]. Beyond donor phase pH = 7.0 the results were not reproducible, shown by very large percent relative standard deviation values (*i.e.*, %RSD > 10%) for triplicate (n = 3) measurements, and hence were not considered in the optimization. These are due to alkaline hydrolysis reactions of triazines at more alkaline conditions (*i.e.*, pH > 7.0) in the donor phase [19]. Donor phase of pH = 7.0 was optimum pH and used for all experiments through out this study.

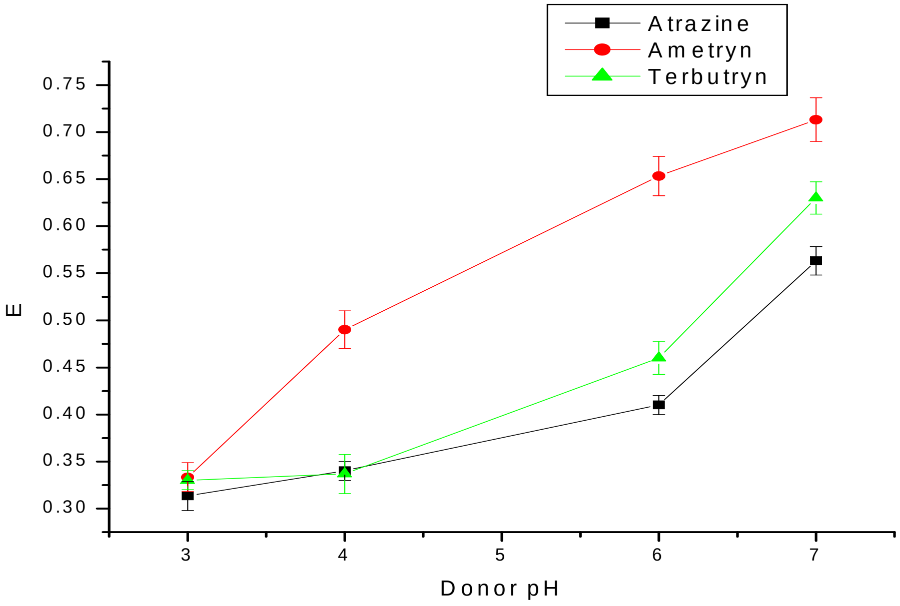

Figure 14. Effect of increasing the donor phase pH on extraction efficiency for 30 min extraction of 0.5 mg L^{-1} of each triazine herbicide.

3.4. Optimization of Donor Flow Rate

One of the advantages of liquid membrane extraction is the possibility of increasing the amount of sample passing the donor channel per unit time, especially when large sample volumes are available, so as to increase the total amount of analyte accumulated in the stagnant acceptor solution. This is primarily useful when the extraction system is limited by the mass transfer in the donor channel, *i.e.*, if the partition coefficient between the organic membrane liquid and the aqueous donor phase is relatively large ($K_D > 1$). Under these circumstances the decrease in the extraction efficiency with increase in donor flow rate is small, while the enrichment factor (E_e) may increase with the donor flow rate. Further experimental benefits of such a procedure are shortening of the extraction time used and also lowering of the detection limit with the same extraction time [10].

The influence of the donor flow rate on the extraction efficiency and also on the enrichment factor (E_e) of the triazines was investigated by varying the donor flow rates from 0.5 to 5.0 mL min^{-1}. Highest extraction efficiency (E) values for all the triazines are obtained at lower donor flow rates as shown in Figure 15. This is mainly because a low donor flow rate increases the residence time of analyte molecules in the donor channel, which provides longer contact time for complete equilibration with the liquid membrane. On the other hand, increasing the donor flow rate increases the amount of analytes introduced into the extraction system often increasing the amount of analytes accumulated in the acceptor per unit time [48]. Extractions at higher donor flow rate for a fixed time increases both the enrichment factor (E_e) and the limit of detection [2]. The results shown in Figure 16 are in good agreement with this theoretical consideration. The problems associated with increasing the donor flow rate are the decrease in the lifetime of the membrane and reduced extraction efficiency for the subsequent extractions [10]. This colud be due to dissolution of the membrane liquid into the flowing large volume of the aqueous donor phase. In order to gain relatively large extraction efficiency from a limited sample volume at longer life time of the membrane, a donor phase flow rate of 1.0 mL min^{-1} was chosen for all experiments through out this study.

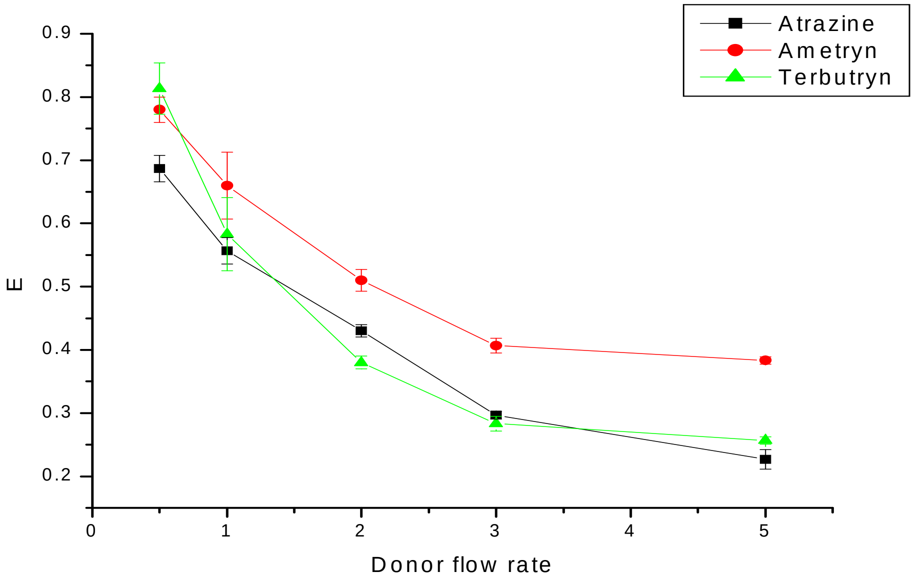

Figure 15. Effect of increasing donor phase flow rates on extraction efficiency for 30 min extraction of 0.5 mg $L^{-1}$of each triazine herbicide.

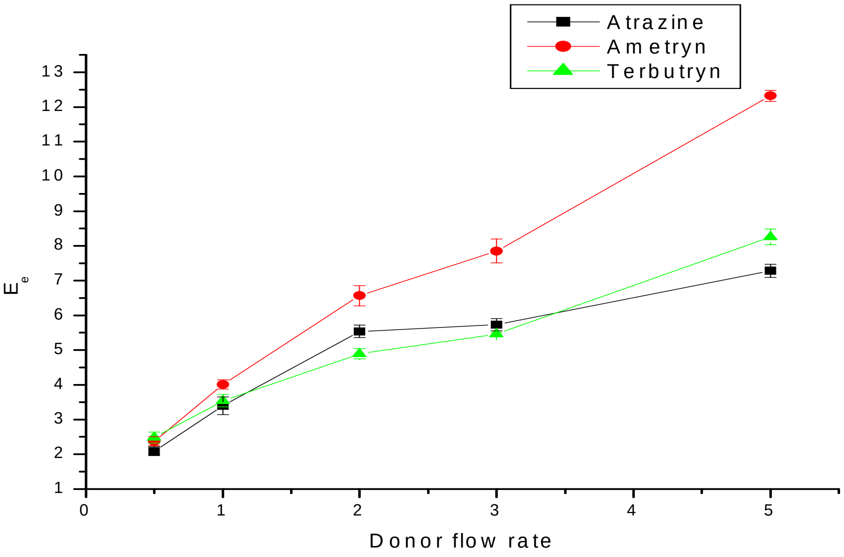

Figure 16. Effect of increasing the donor phase flow rates on enrichment factor (E_e), for 30 min extraction of 0.5 mg $L^{-1}$of each triazine herbicide.

3.5. Application

The optimized liquid membrane extraction method was applied for the extraction and enrichment of the triazine herbicides in spiked fruit juice and *Tej* samples. The pH of the sample matrices were studied before SLM extraction; pH = 4.5 (pineapple juice at 20 ^{o}C), pH = 4.7 (orange juice at 21 ^{o}C), pH = 4.3 (apple juice at 21 ^{o}C) and pH = 4.1 (*Tej* at 21 ^{o}C). The sample pH study results show a pH ranging from 4.1 to 4.7. The pH was adjusted by the donor buffer since ametryn and terbutryn will get protonated at these sample pH values.

A 0.1 mg L^{-1} triazine standards mixture spiked 100 mL fruit juice and 200 mL *Tej* samples were mixed with the phosphate buffer solution and extracted for 30 min with the optimized liquid membrane extraction method. HPLC analyses were performed on final extracts and the extraction efficiency and enrichment factor of each triazine in all sample matrices were calculated. The obtained extraction efficiencies and enrichment factors of the triazine herbicides in all of the sample matrices considered here are given in table 2 below. Good repeatability of the results was observed from the lower percent relative standard deviation (% RSD) values.

Lower extraction efficiencies and enrichment factors for all the triazine herbicides were obtained for orange juice. This might be due to the more viscous or turbid nature of the orange juice which will result in accumulation of the triazine herbicides in the solid particles of the juice during filtration and liquid membrane extraction steps. Larger extraction efficiencies and enrichment factors of all the triazines were obtained for the clearer and less viscous apple juice and also for the large volume (*i.e.* 200 mL) spiked *Tej* samples. The extraction efficiency and enrichment factors for ametryn were larger in all the sample matrices. This might be due to the larger aqueous solubility of ametryn, since the sample matrices are mainly aqueous in composition which also improves its availability for extraction into the membrane solvent.

Table 2. Extraction efficiency (E), enrichment factor (E_e), standard deviation (SD) and percent relative standard deviation (% RSD) values for extraction efficiency of the triazine herbicides, at 0.1 mg L^{-1} spiking and after 30 min SLM extraction. Analytes are listed in their order of chromatographic elution.

Matrice	Analyte	E	E_e	SD	% RSD
Pineapple	Atrazine	0.55797	3.69333	± 0.02014	3.61 (n = 3)
	Ametryn	0.68610	4.53667	± 0.03835	5.59 (n = 3)
	Terbutryn	0.59380	3.93000	± 0.03764	6.33 (n = 3)
Orange	Atrazine	0.44067	2.76333	± 0.03157	7.16 (n = 3)
	Ametryn	0.59007	3.70667	± 0.01714	2.90 (n = 3)
	Terbutryn	0.47637	3.00000	± 0.02320	4.87 (n = 3)
Apple	Atrazine	0.61857	3.98000	± 0.04394	7.10 (n = 3)
	Ametryn	0.75493	4.85667	± 0.02720	3.60 (n = 3)
	Terbutryn	0.69533	4.48000	± 0.01525	2.19 (n = 3)
Tej	Atrazine	0.59638	3.99200	± 0.03743	6.28 (n = 5)
	Ametryn	0.73408	4.91200	± 0.06711	9.14 (n = 5)
	Terbutryn	0.71034	4.75600	± 0.07588	10.68 (n = 5)

3.6. Linearity of the Analytical Method

Linearity is the method's ability to obtain results which are either directly or after mathematical transformation, proportional to the concentration of the analyte within a given range. Linearity is determined by calculating the regression line using a mathematical treatment of the results (*i.e.*, least mean squares) versus analyte concentration [61]. The optimized liquid membrane method shows better linearity than other literature results [22].

The linearity of the optimized liquid membrane extraction method was evaluated by spiking 100 mL of pineapple juice with the triazine standards over concentration ranges. Linear calibration curves were obtained for each of the triazine herbicides at concentration range of 50 µg L^{-1} to 500 µg L^{-1} with correlation coefficients greater than 0.99 as shown below in Figures 17 – 19. Average percent relative standard deviation (% RSD) values were calculated by taking the mean of percent relative standard deviations (% RSDs) obtained at each concentration level for triplicate (n = 3) measurements. Average percent relative standard deviation (% RSD) values of 3.2% (n = 3) for atrazine, 5.0% (n = 3) for ametryn, and 5.4% (n = 3) for terbutryn were obtained using peak area as a response variable. The regression equations and average percent relative standard deviation (% RSD) values for each of the triazines in pineapple juice matrices are given in table 3 below.

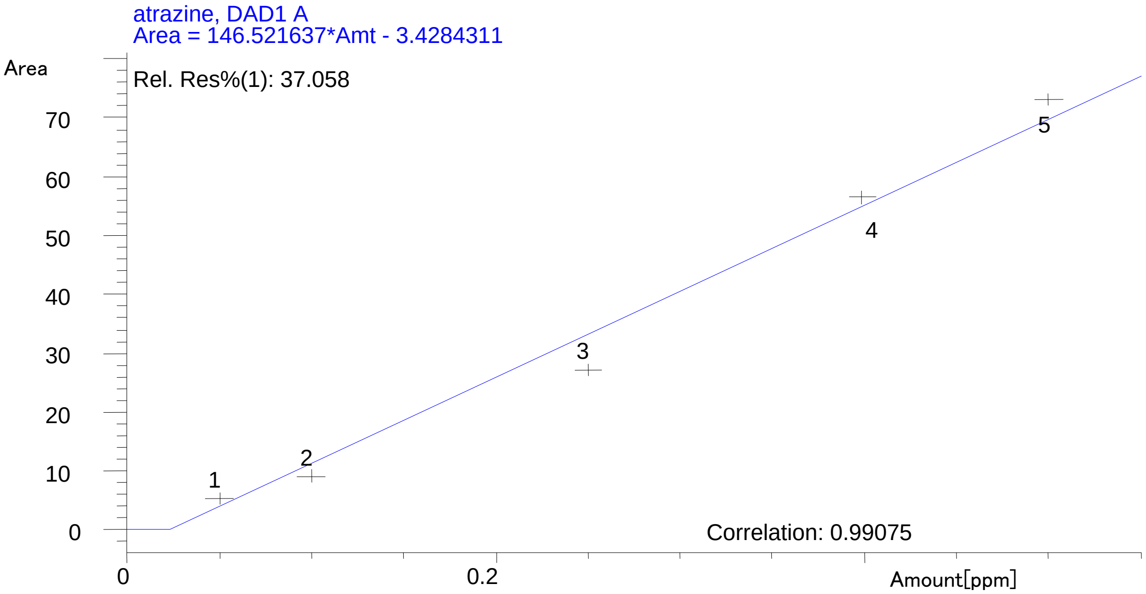

Figure 17. Linearity curve of atrazine in pineapple juice for spiking at concentration range of 0.05 mg L^{-1} – 0.5 mg L^{-1} based on triplicate (n = 3) extractions.

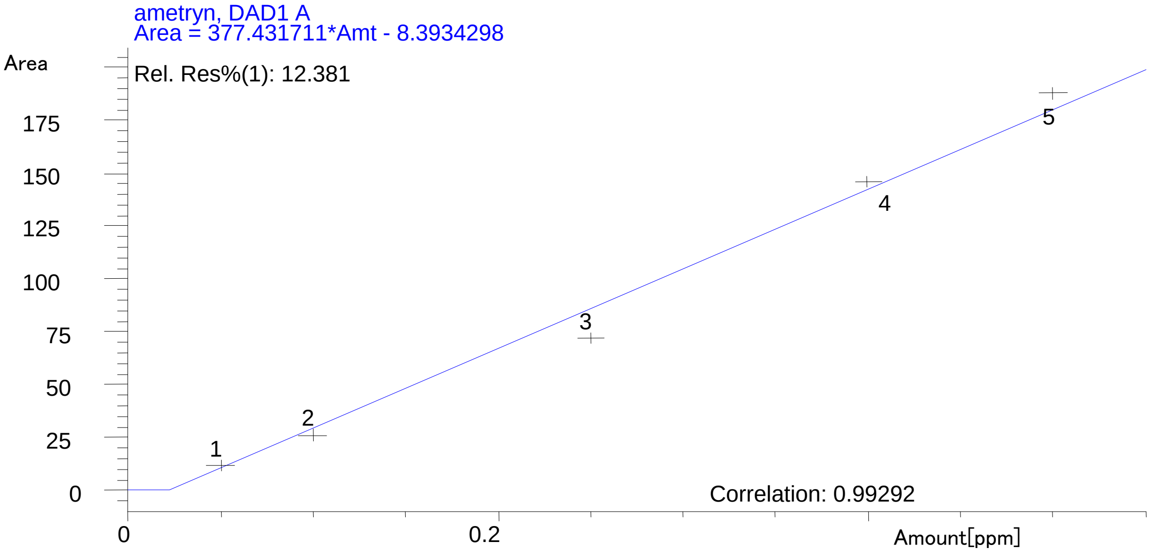

Figure 18. Linearity curve of ametryn in pineapple juice for spiking at concentration range of 0.05 mg L^{-1} – 0.5 mg L^{-1} based on triplicate (n = 3) extractions.

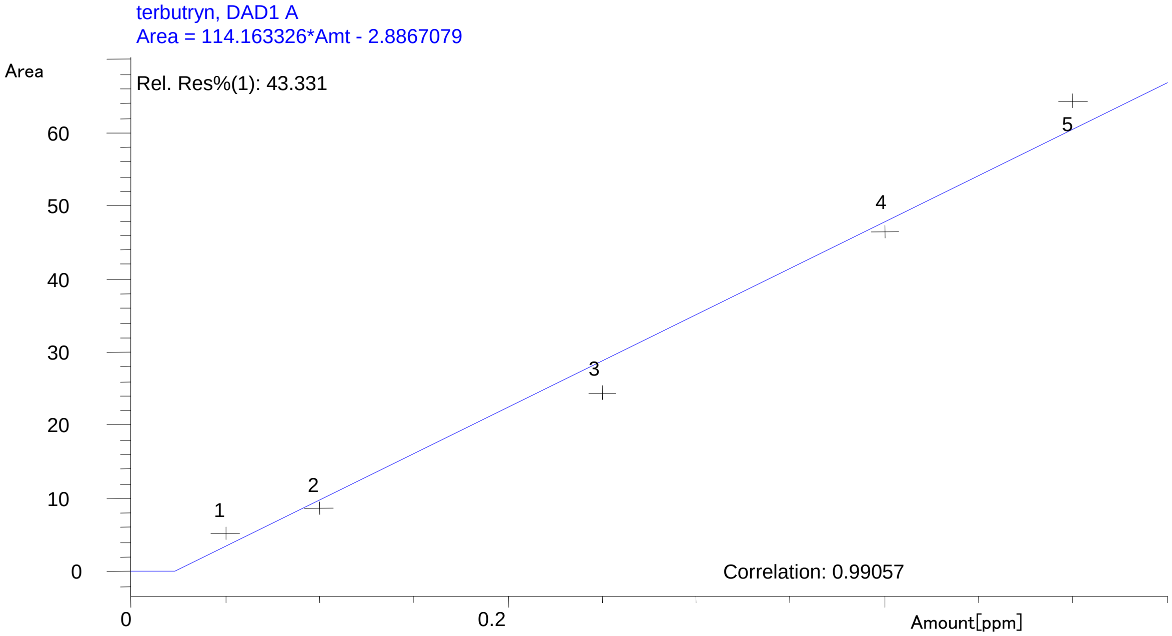

Figure 19. Linearity curve of terbutryn in pineapple juice for spiking at concentration range of 0.05 mg L^{-1} – 0.5 mg L^{-1} based on triplicate (n = 3) extractions.

Table 3. Linearity of the analytical method for triplicate (n = 3) extractions of the triazine herbicides in pineapple juice, after 30 min SLM extraction.

Matrice	Analyte	Conc. range (mg L^{-1})	Regression equation	Correlation coefficient	Average % RSD
Pineapple	Atrazine	0.05 – 0.5	Y = 146.5216X – 3.4284	0.9907	3.20 (n = 3)
	Ametryn	0.05 – 0.5	Y = 377.4317X – 8.3934	0.9929	5.00 (n = 3)
	Terbutryn	0.05 – 0.5	Y = 114.1633X – 2.8867	0.9906	5.41 (n = 3)

3.7. Method Detection and Quantification Limits

Limit of detection is the concentration derived from the smallest response or signal that can be detected with reasonable certainty for a given analytical procedure. Limit of quantification is regarded as the lower limit for precise quantitative measurements, as opposed to qualitative detection [62]. Limit of quantification (LOQ) and limit of quantification (LOQ) values were calculated by analyzing blank spiked samples, and they were determined as the lowest concentrations of the analytes that produce chromatographic peaks. The LOD and LOQ values were determined using peak height measurements, since accurate peak area measurements are difficult due to tailing or fronting peaks at lower concentrations. In chromatographic analysis; LOD and LOQ are usually defined as concentrations that give a signal to noise ratio of 3:1 and 10:1 respectively, and are expressed as the concentration of the analyte [60]. The LOD and LOQ values calculated accordingly for the triazine herbicides, in the different matrices considered here, were given in table 4 below.

The lowest LOD and LOQ values are obtained for ametryn in all the matrices, with the highest being for terbutryn. This might be due to the higher partition coefficient of ametryn to the liquid membrane solvent. Lower detection limits for all the triazines were observed in apple juice and *Tej* sample matrices. The detection limits for atrazine in all the matrices are lower than the values obtained in other similar works [22].The detection limits, for all the triazines studied here, were well below the maximum residue limits set by the regulatory bodies for food matrices [13, 14].

Table 4. Limit of detection and quantification of the triazines in the different matrices with regression equation and correlation coefficient, at 0.1 mg L^{-1} spiking and after 30 min SLM triplicate extractions (n = 3) for fruit juice and five extractions (n = 5) for *Tej* samples.

Matrice	Analyte	LOD (µg L^{-1})	LOQ (µg L^{-1})	Regression equation	Correlation Coefficient
Orange	Atrazine	10.40	34.67	Y = 68.081X – 0.626	0.99996
	Ametryn	4.78	15.92	Y = 97.685X – 0.085	0.99990
	Terbutryn	12.32	41.05	Y = 58.406X – 0.117	0.99995
Pineapple	Atrazine	9.00	30.00	Y = 54.604X – 0.875	0.99956
	Ametryn	4.46	14.86	Y = 93.984X – 1.599	0.99935
	Terbutryn	11.7	39.00	Y = 59.272X – 0.059	0.99932
Apple	Atrazine	4.92	16.42	Y = 98.741X – 0.922	0.99996
	Ametryn	2.75	9.18	Y = 179. 115X – 0.398	0.99992
	Terbutryn	10.64	35.46	Y = 79.301X – 0.062	0.99996
Tej	Atrazine	4.97	16.56	Y = 98.081X – 0.622	0.99989
	Ametryn	2.61	8.70	Y = 177.685X – 0.088	0,99990
	Terbutryn	10.69	35.61	Y = 68.416X – 0.127	0.99997

3.8. Selectivity of the Analytical Method

Selectivity is the ability to extract and measure accurately and specifically the analyte in the presence of the other components that may be expected to be present in the sample matrix under the stated conditions [61]. According to the latest IUPAC recommendation; 'selectivity refers to the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other compounds of similar behavior' [63]. To obtain a high selectivity membrane extraction has a clear advantage over other sample preparation techniques, as all the compounds that reach the analytical instrument selectively cross the membrane to be enriched in the acceptor phase [44].

By carefully applying all the optimized liquid membrane extraction and HPLC separation conditions and by implementing the optimum sample treatment and sample storage conditions, the selectivity of the method can be enhanced. The selectivity of the optimized liquid membrane extraction method in fruit juice and *Tej* sample matrices can be evaluated from the final chromatograms obtained for the extracts. No interfering peaks were observed at the retention times of all the triazines, which can easily be compared from the chromatograms of the unspiked and spiked sample extracts. Similar chromatograms are shown in Figures 20 – 23, all the triazine peaks are clearly separated and can easily be quantified by integration of their peak areas. This can be attributed to the rejection of potentially interfering solutes from entering the acceptor compartment of the SLM unit.

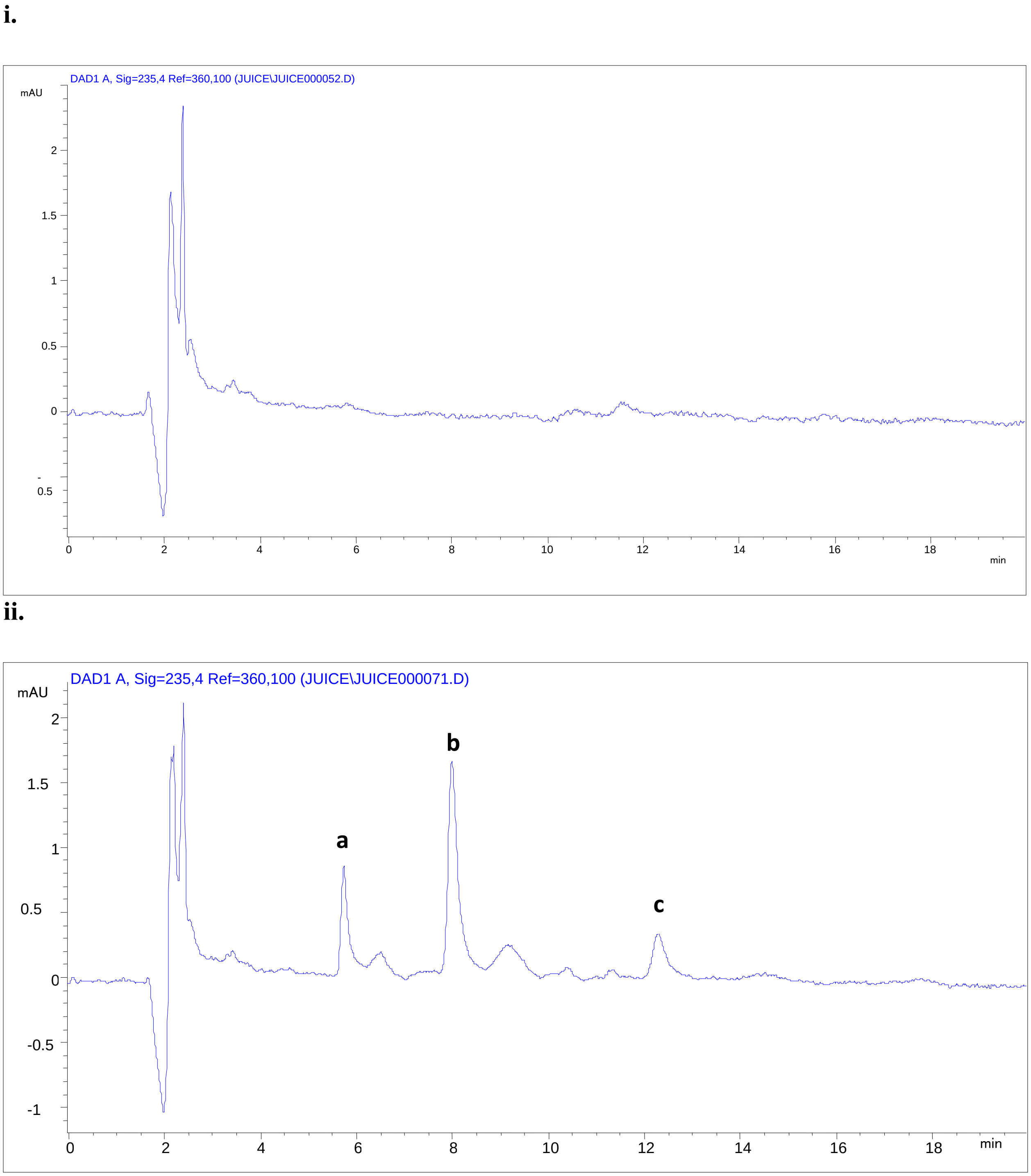

Figure 20. HPLC-UV chromatograms for 20 µL injection of extracts obtained after 30 min SLM enrichment of (i) unspiked pineapple juice extract and (ii) 0.1 mg L^{-1} triazine standard mixture spiked pineapple juice extract. **a.** atrazine **b.** ametryn **c.** terbutryn

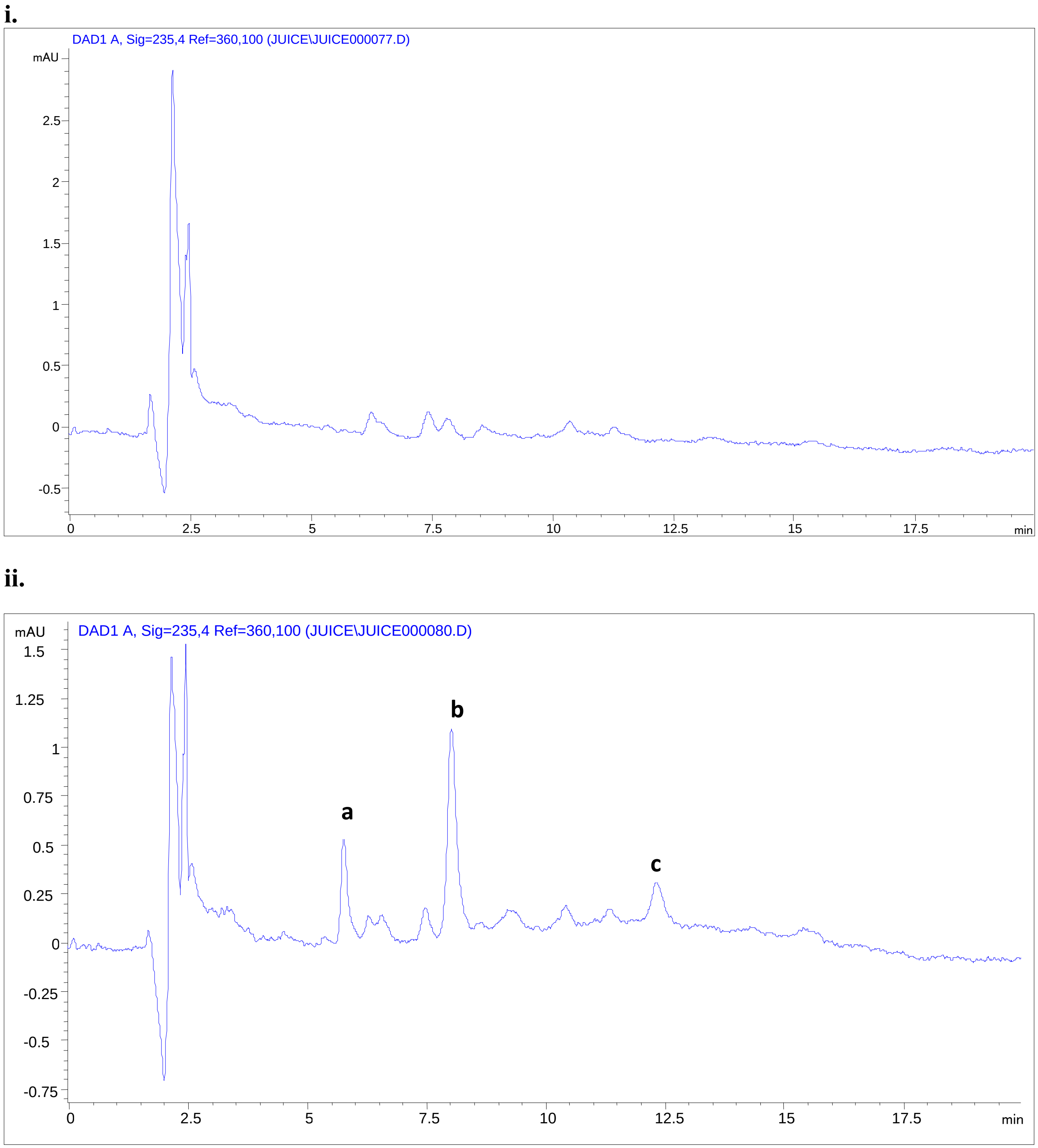

Figure 21. HPLC-UV chromatograms for 20 µL injection of extracts obtained after 30 min SLM enrichment of (i) unspiked orange juice extract and (ii) 0.1 mg L^{-1} triazine standard mixture spiked orange juice extract. **a.** atrazine **b.** ametryn **c.** terbutryn

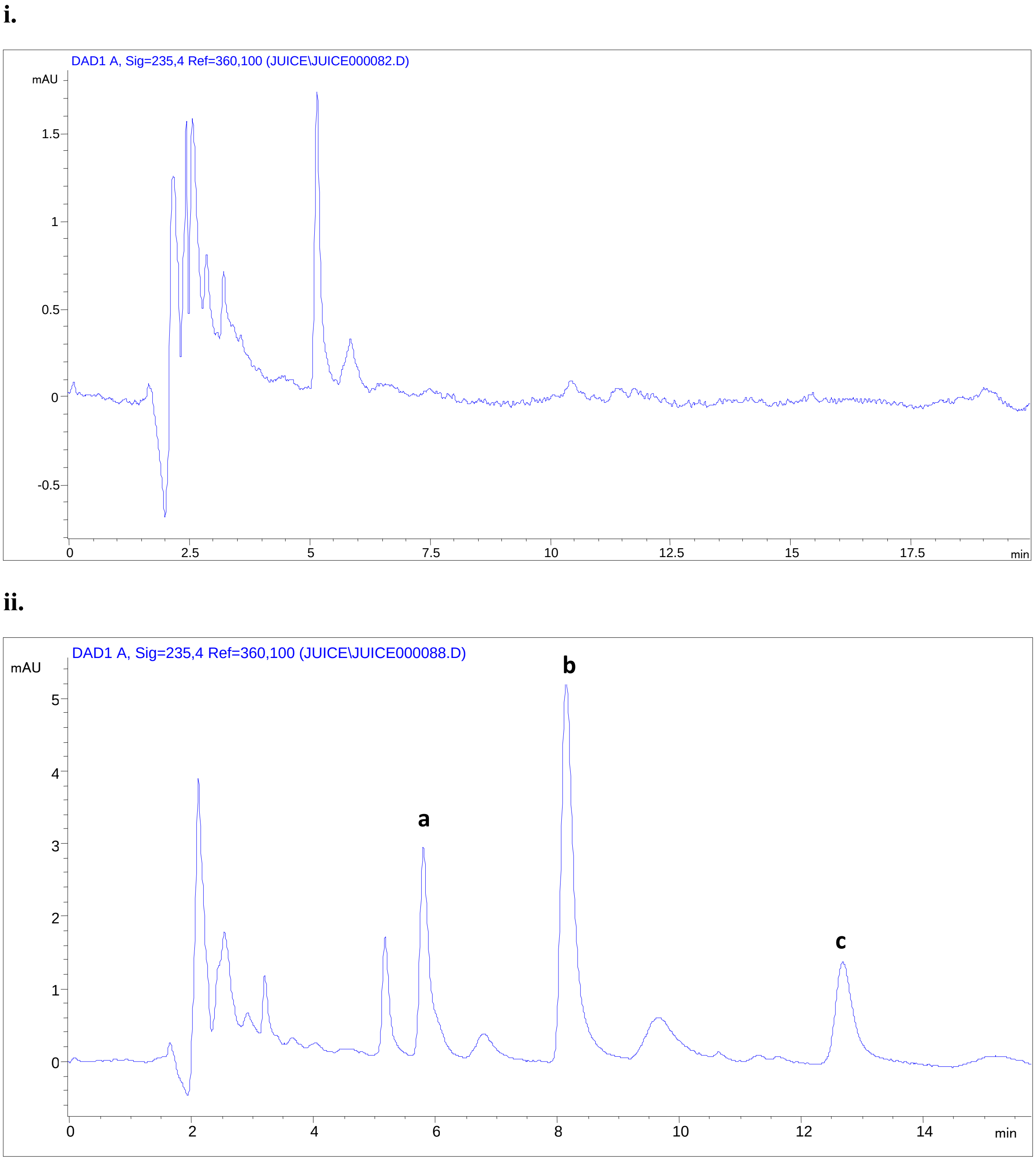

Figure 22. HPLC-UV chromatograms for 20 µL injection of extracts obtained after 30 min SLM enrichment of (i) unspiked apple juice extract and (ii) 0.1 mg L^{-1} triazine standard mixture spiked apple juice extract. **a.** atrazine **b.** ametryn **c.** terbutryn

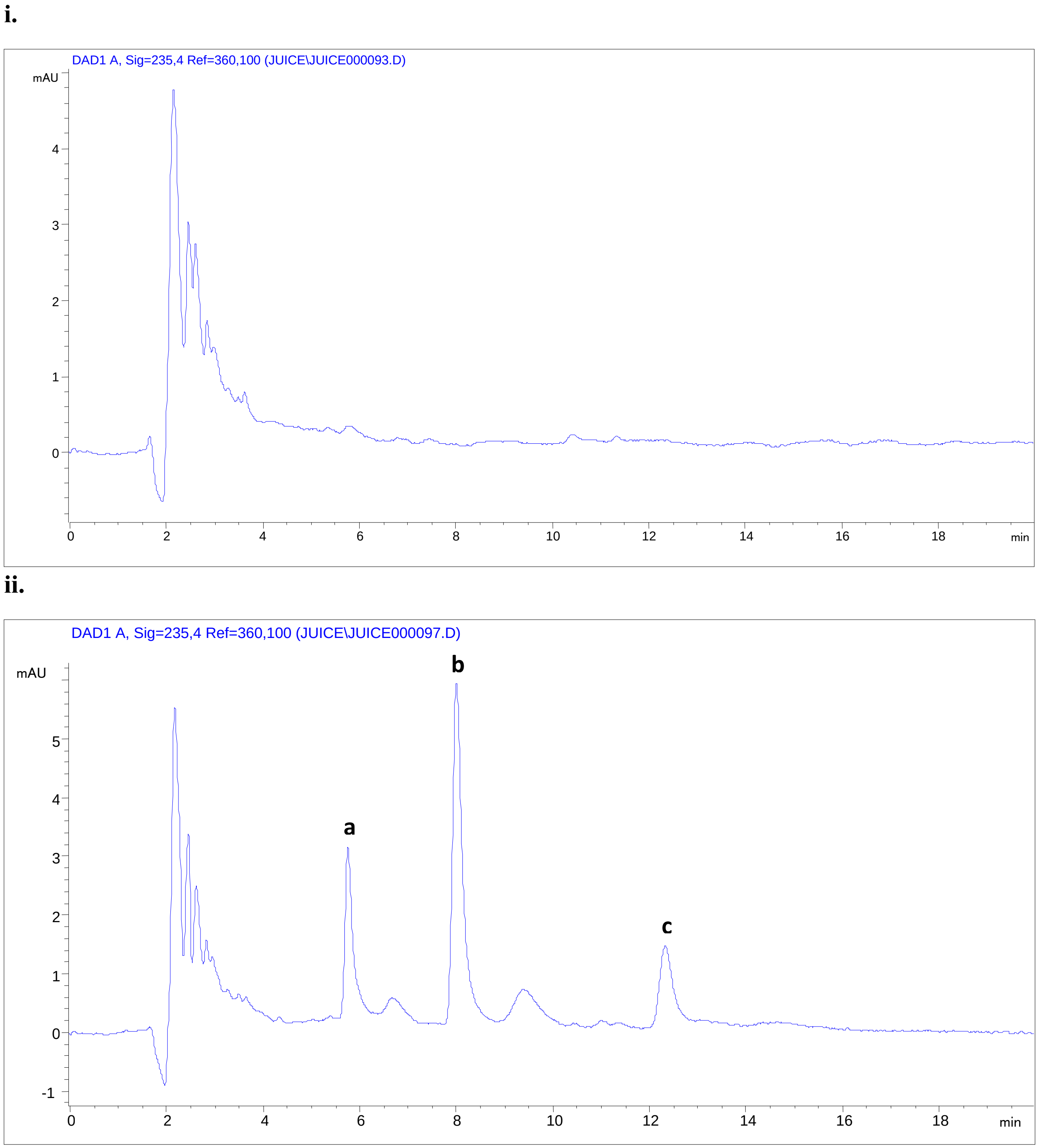

Figure 23. HPLC-UV chromatograms for 20 μL injection of extracts obtained after 30 min SLM enrichment of (i) unspiked *Tej* extract and (ii) 0.1 mg L^{-1} triazine standard mixture spiked *Tej* extract. **a.** atrazine **b.** ametryn **c.** terbutryn

4.0. Conclusions and Recommendations

It has been demonstrated that the optimized; solventless, environmentally friendly and less expensive supported liquid membrane extraction method can be successfully applied to the extraction and enrichment of trace level of triazine herbicides from fruit juice and *Tej* samples. In comparison to other methods, SLM extraction needs no clean up procedure, since it allows selective extraction and enrichment of analytes, giving cleaner extracts free of interferents for HPLC-UV analysis.

Good extraction efficiencies and enrichment factors for all the triazines have been obtained, with good repeatability in all the sample matrices. The method shows good linearity for all the triazines in pineapple juice, with linear curves of correlation coefficients greater than 0.99 and average percent relative standard deviation (% RSD) values less than 15%. The detection limits range from 2.61 $\mu g\ L^{-1}$ for ametryn in *Tej* to 12.61 $\mu g\ L^{-1}$ for terbutryn in orange juice. The quantification limits range from 16.42 $\mu g\ L^{-1}$ for ametryn in apple juice to 41.05 $\mu g\ L^{-1}$ in orange juice. Both the detection and quantification limits obtained are below the maximum residue limits set by EPA and other regulatory bodies for food matrices. Lower detection and quantification limits might be obtained by processing large volume of samples using automated-online liquid membrane extraction methods if the sample volume is not a limiting factor. The optimized liquid membrane extraction method was selective towards all the triazines in all sample matrices considered in this study.

The method can be optimized and applied to other liquid food sample matrices for extraction of all other triazine herbicides and a wide variety of pesticides. By varying the SLM extraction parameters; mainly donor phase flow rate, donor phase pH, acceptor phase pH and membrane solvent composition, it could be possible to obtain even better selective enrichment and lower detection and quantification limits.

5.0. References

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