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Application of HPLC to Pharmaceutical and Natural Product Analysis

by

Gaurav Sharma

A thesis

submitted in partial fulfilment

of the requirement for the degree of

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Committee Approval

The members of the committee appointed to examine the thesis of Gaurav Sharma find it satisfactory and recommend that it be accepted.

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Gaurav Sharma

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List of Abbreviations

ACN	Acetonitrile
AG	Analytical Grade
BP	Boiling Point
CBZ	Carbamazepine
DAD	Diode Array Detector
DDD	Defined Daily Dosages
DDT	Dichlorodiphenyltrichloroethane
F	Flow Rate
FDA	Food & Drug Administration
FLU	Fluoxetine
GC	Gas Chromatography
HF- LPME	Hollow Fibre- Liquid Phase Microextraction
HPLC	High Performance Liquid Chromatography
HPLC-DAD	High Performance Liquid Chromatography coupled with DAD
i.d.	Internal Diameter
ICH	International Conference of Harmonization
L	Liter
LOD	Limit of Detection
Log- D	Distribution coefficient
LOQ	Limit of Quantification
LR	Laboratory Reagent
M	Molar
m/z	Mass- to- charge ratio
mg	Milligram
Min	Minute
ml	Milliliter

MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Mass Spectrometry /Mass Spectrometry
MW	Molecular weight
ng	Nanogram
nm	Nanometer
POP	Persistent Organic Pollutants
PPCP	Pharmaceuticals and Personal Care Products
RI	Refractive Index
RPM	Rounds per Minute
RSD	Relative Standard Deviation
Rt	Retention time
S	Slope
S/N	Signal to noise ratio
SD	Standard Deviation
SIM	Single Ion Monitoring
SPE	Solid Phase Extraction
STP	Sewage Treatment Plant
UPLC	Ultra Performance Liquid Chromatography
USP	United States Pharmacopeia
UV	Ultraviolet
v/v	Volume by volume
VEN	Venlafaxine
w/v	Weight per volume
µg	Microgram
µl	Microliter

CHAPTER 1

Introduction to HPLC

1. Introduction

The branch of analytical chemistry, which deals with the separation, resolution, identification, determination, and purification of a given sample of a pharmaceutical or medicine, is called pharmaceutical analysis. It also includes the detection and estimation of impurities that may be present in the pharmaceuticals [1].

High Performance Liquid Chromatography (HPLC) is an analytical technique used to separate the components in a mixture. There are two types of HPLC separation, i.e. Normal phase and Reverse phase. In normal phase the column is filled with silica particles and the solvent used is non-polar whereas in reverse phase the silica particles are non-polar and solvent used is polar. Injection of the sample can be manual or automated. The sample passes through the columns and is detected by the detector. The time taken by a particular compound to pass through the column is called the retention time.

Various types of detectors are used for the analysis of compound using HPLC. Following are the various types of detectors used in HPLC: UV-VIS detector, photo diode array detector, fluorescence detector, mass spectroscopic detector, refractive index detector, light scattering detectors etc. Table 1 shows the flow chart showing the flow scheme of an HPLC.

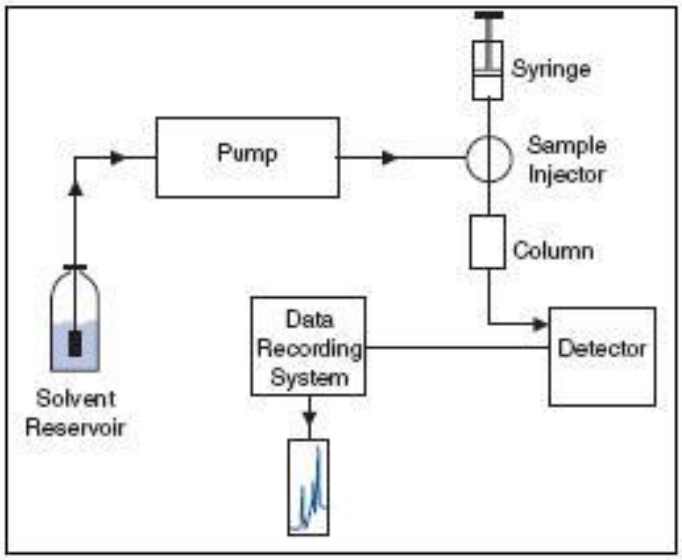

Figure 1.1 A schematic diagram of an HPLC.

2. ICH guidelines

The ICH guidelines are also known as International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. These guidelines are used to make recommendations for achieving harmonization in the interpretation and application of requirements for the registration of pharmaceutical products. The Q2 (R1) guidelines from ICH guidelines are used to validate an analytical procedure. Following are the validation characteristics which should be considered before doing the validation of an analytical method [2].

Accuracy: "it determines the closeness of agreement between the value which is a reference value or a conventional true value" [3].

Precision: "it determines the closeness of the agreement between a series of measurements, multiple sampling of the same homogenous sample under the prescribed conditions." Precision is divided into 2 characteristic i.e. repeatability (also known as intra-assay precision) and reproducibility (also known as inter-assay precision).

Specificity: it is the "ability to assess unequivocally the analyte in the presence of components which may be expected to be present."

Detection limit: it is the lowest amount of sample which can be detected in an analytical method.

Quantitation limit: it is the lowest amount of sample which can be quantitatively determined with precision and accuracy.

Linearity: it is the ability of an analytical technique to obtain the results directly proportional to the concentration of analyte in the sample.

Range: it is the interval between the upper and lower limit concentration of analyte in the sample.

Robustness: it is the capacity of an analytical method to remain unaffected by small variations like temperature, pH etc.

3. Reference

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3. Validation of analytical procedures: text and methodology Q2 (R1). (2005). *International Conference of Harmonization.* Retrieved From the ICH website: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf

CHAPTER 2: A Specific HPLC-UV and Fluorescence Method for the Detection of Three Anti-Depressant pharmaceuticals in Various Water Systems

Abstract

A new, fast and economical HPLC method was developed for the analysis of carbamazepine, venlafaxine and fluoxetine in water samples. A reverse-phase HPLC assay was used with UV and fluorescence detectors. Sample was passed through Gemini C18 110A (250 x 4.60 mm, 5 µm) column at a flow rate of 1.0 ml/min. A mixture of citric acid (100 mM) and EDTA (10 mM) was mixed (pH adjusted at 4.5 pH by 0.1 M NaOH) in water and was used as a solvent A. Three HPLC runs were carried out at an injection volume of 100 µl. From spiking experiments, limit of detection (LODs) and limit of quantification (LOQs) for carbamazepine were 10 ng/l and 100 ng/l, for venlafaxine were 1 µg/l and 1 ng/l, and for fluoxetine were 100 ng/l and 1 µg/l, respectively. HPLC can be used to detect the trace amount of pharmaceuticals in water. The technique requires no derivatization steps, requires less time and is more cost-effective.

Keywords: HPLC, solid phase extraction, fluoxetine, venlafaxine, carbamazepine.

1. Background

Pharmaceuticals in water are considered as a major emerging pollutant because of their ubiquity in the aquatic environment and their negative health effects. Pharmaceuticals and their metabolites have been found in many environmental systems such as water, waste water, sludge and sediments. The common sources of contamination of pharmaceuticals are household waste, waste water treatment plants, industrial units, hospitals and animal breeding farms. Surprisingly, pharmaceuticals, like antibiotics, sex hormones and antidepressants, have been found in 41 million American's drinking water supplies [1]. As the concentration of the pharmaceuticals are very low (generally parts per trillion), not much special attention is given to the problem. However, the above shown facts could be alarming especially in the worst-case scenario like areas close to a pharmaceutical industry or over consumption of a particular pharmaceutical in an area. Apart from human health it also causes some major negative effects to the fishes and aquatic wildlife. Some of the biological impacts on aquatic wildlife are 1. masculization of female fish or feminization of male fish, 2. delayed sexual development in fish, 3. delayed metamorphosis in frog, 4. embryo motility, 5. abnormal hormonal levels, 6. structural and neurological damage and 7. impaired reproductive and immune systems [1]. Depending on their hydrophilicity, pharmaceuticals can enter the aquatic environment or remain absorbed in aquatic environment [Figure 2.1].

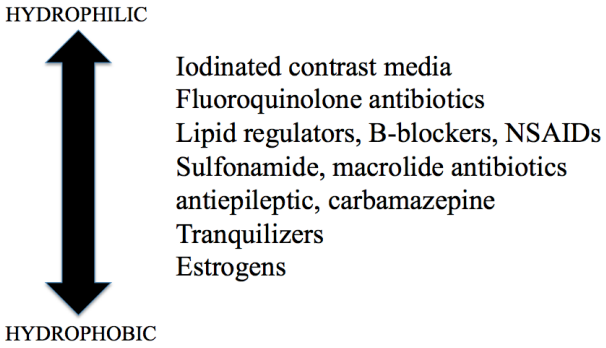


Figure 2.1 Level of hydrophobicity and hydrophilicity of pharmaceutical compounds [1].

LIPID REGULATORS	Bezafibrate, Gemfibrozil, Clofibric acid, Fenofibrate
BETA-BLOCKERS	Metoprolol, Propranolol, Nadolol, Atenolol, Sotalo, Betaxolol
ANTIINFLAMMATORY DRUGS/ANALGESICS	Acetylsalicylic acid (Aspirin), Diclofenac, Ibuprofen, Acetaminophen, Metamizol, Codeine, Indometacine, Naproxen, Phenazone
ANTIBIOTICS	Erytromicyn, Ofloxacin, Chlortetracycline, Oxytetracycline, Streptomycin, Flumequine, Ciprofloxacin, Trimetoprim, Sulfamethoxazole, Lincomycin, Penicillin, Lincomycin, Amoxycillin
STEROIDS AND RELATED HORMONES	17-β-estradiol, Estrone, 17-α-ethinyl estradiol, Diethylstilbestrol, Diethyalstilbestrol acetate
CANCER THERAPEUTICS	Cyclophosphamide, Ifosphamide
DIURETICS	Furosemide

ANTIEPILEPTICS	Carbamazepine
ANTIDEPRESSANTS	Mianserin
TRANQUILIZERS	Diazepam

Table 2.1 Some common pharmaceuticals present in the environment [2-5].

The most common human pharmaceuticals present in the environment are shown in Figure 2.1 [2-5]. Caffeine is the most common pharmaceutical among them [6-8] whereas drugs like diclofenac [9], acetaminophen, clofibric acid, aspirin, ibuprofen, artorvastatin, carbamazepine, fluoxetine, gemfibrozil, 17 β-thynylestradiol [10] have been found in the wastewater and surface water [11, 12].

1.1 Source of pharmaceutical occurrence in the environment

Pharmaceuticals like anti-inflammatory and antibiotics are commonly used in veterinary medicine in most of the European countries. Countries like Germany, England and Austria, more than 100 tons/year of pharmaceutical products are used [13]. Some of the most commonly used pharmaceuticals are the oral antidiabetic metformin, non-steroidal anti-inflammatory drugs (NSAIDS) including paracetamol, acetyl salicylic acid or aspirin, naproxen, ibuprofen and diclofenac and the antiepileptic carbamazepine [14]. Nikolaou et al. illustrated the sources and fate of the pharmaceuticals compounds in the environment Figure 2.2 [1].

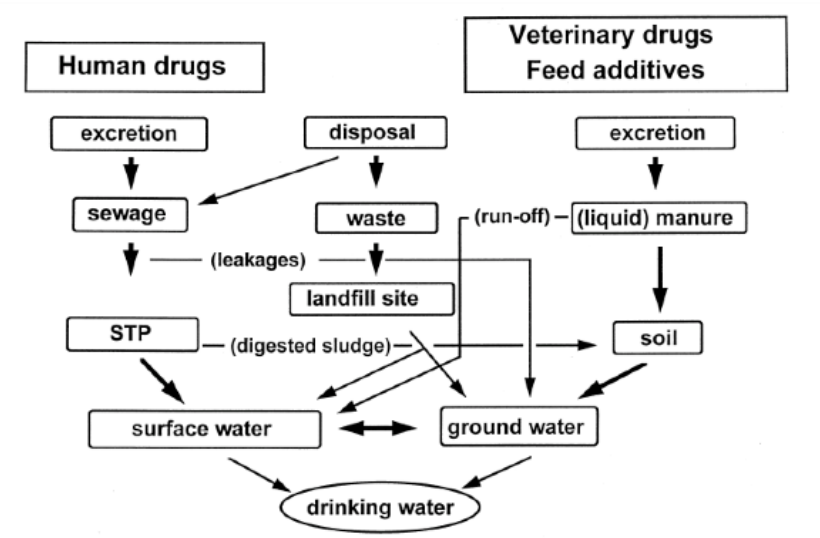


Figure 2.2 Source and fate of pharmaceuticals in the environment (STP: sewage treatment plant) [56].

Because of incomplete elimination of pharmaceuticals from the wastewater treatment plants, have been reported in surface water [15, 16]. Hospital and municipal wastewaters are the most important sources of pharmaceutical contamination [17]. Contributions are also known to be from wastewater bodies, pharmaceutical manufactures, landfill leachates, and disposal of unused medicines into the environment. Application to fields and subsequent runoff is the main cause of veterinary pharmaceuticals being in the environment [18, 19]. Recent advances of new analytical techniques have allowed the determination of a broader range of compounds, such as pharmaceuticals, and have therefore permitted more comprehensive assessment of environmental contaminants. The techniques for the detection include gas chromatography (GC-MS), tandem gas chromatography (GC-MS-MS), liquid chromatography (LC-MS), and tandem liquid chromatography (LC-MS-MS).

LC-MS-MS is becoming more common for the detection of pharmaceuticals as it has the ability to confirm compounds and has high sensitive as compared to the

fluorimetricdetection or ultra-violet (UV) detection. Compounds having the same molecular mass but different product ions can be detected by using LC-MS-MS. Hence, tandem mass spectrometry is preferred over the other analytical techniques [20]. Before conducting the GC-MS analysis, derivatization of polar pharmaceuticals is necessary. This step includes the use of highly toxic and carcinogenic diazomethane, benzyl halides, acid anhydrides and alkylchloroformates. Moreover, the derivatization steps may also affect the accuracy of the method [21]. Hence, generally the LC-MS method is preferred to the GC-MS method. Ternes et al. compared GC-MS with LC-electrospray ionization LC-ESI-MS-MS and found that LC-ESI-MS-MS could be used for the separation of extreme polar compounds (such as β-blocker, sotalol and atenolol) [61]. This was because of the incomplete derivatization of the functional group. Moreover, the relative standard deviation (RSD) was found to be lower for the LC-ESI-MS-MS techniques. However, the detection capacity of ESI method decreases when highly contaminated water (like sewage) is used as a sample. So to obtain accurate and reproducible data, the samples should be cleaned prior to detection.

Farre et al. compared the LC-ESI-MS and GC-MS for the analysis of acidic and polar analgesics (such as ketoprofen, diclofenac, gemfibrozil, salicylic acid, ibuprofen and naproxen) in wastewater and surface water samples [22]. They found a good correlation between the LC and GC methodologies. The limit of detection for LC-MS-MS is slightly higher than those obtained with GC-MS method [20]; however, LC-MS gives the advantage of easy sample preparation (no derivatization required) and is more versatile.

1.2 Sample preparation

Acidification of the water sample is required for the detection of pharmaceuticals containing acidic group in their functional group (exist largely in ionized form at neutral pH) [23]. Organic matter containing in the sample may disrupt the analytical process and decrease its efficiency. Generally, the water sample is filtered through the 0.2 µm or 0.45 µm glass fibre filters. Various techniques are used for the samples preparation such as solid phase extraction (SPE), solid phase micro extraction (SPME), liquid phase microextraction (LPME) and lyophilization [24-26]. Sorbent (e.g., Oasis HLB, Lichrolut C18, ENV+, Strata-X and Lichrolut EN) used in the SPE techniques is for the precondensation and clean-up of the samples. The following sorbents are used as they provide better recovery of both polar and non-polar compounds and have greater capacity than alkyl-bonded silica. Mostly SPE cartridges are made up of octadecylsilica, polymeric or hydrophilic-lipophilic balanced (HLB) and used at low pH (typically, pH=2) for the extraction of pharmaceuticals in water [27].

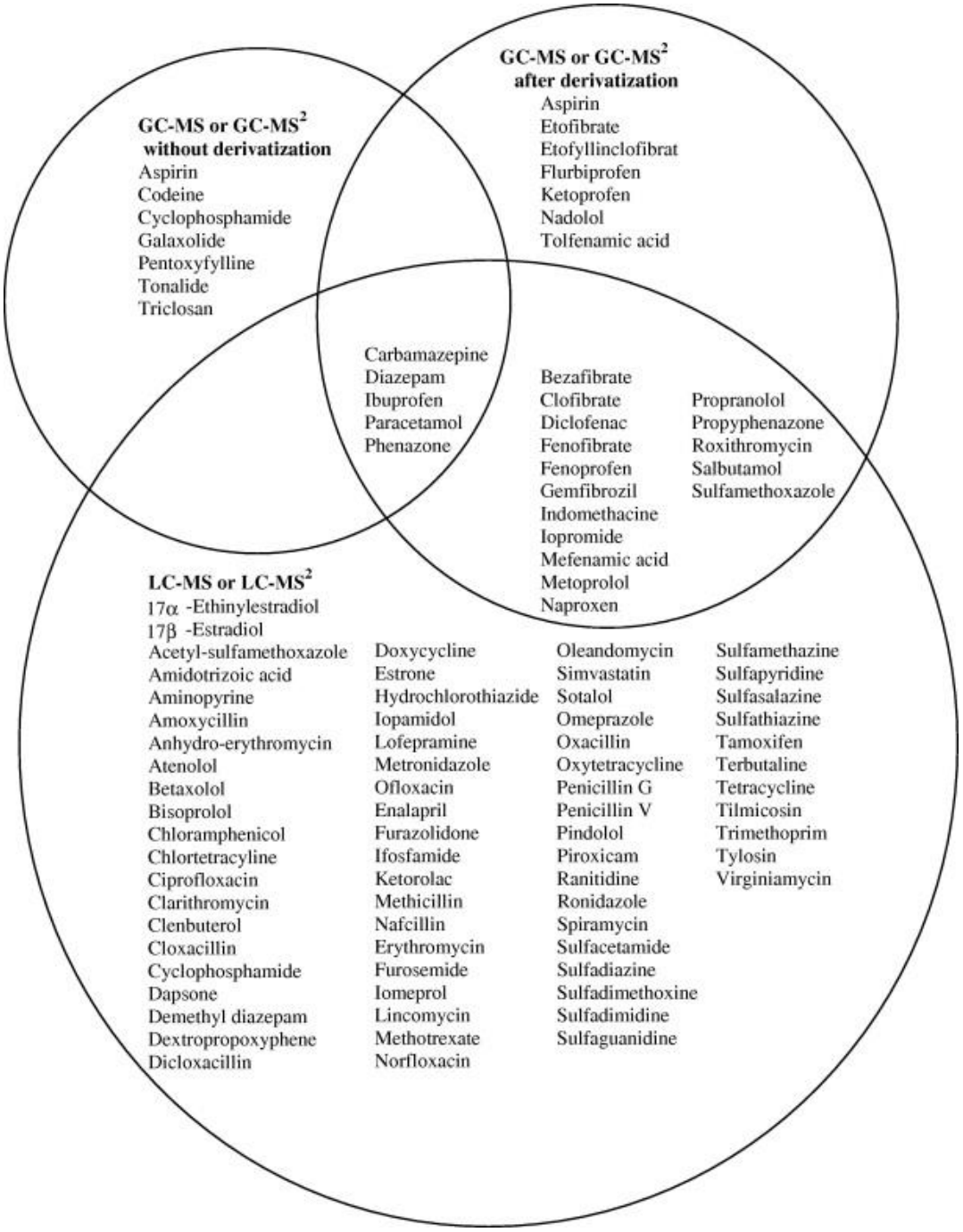


Figure 2.3 Drugs detected by GC-MS and LC-MS techniques [10].

1.3 Extraction of pharmaceuticals from aqueous environmental samples [Figure 2.3]

1.3.1 GC-MS in detection

The first GC-MS determination of PPCPs in the environment was done in 1976 [28]. Still GC-MS and LC-MS-MS are the most widely used techniques because of their wide applicability in environmental laboratories their well-established electron impact (EI) MS libraries, and their superior sensitivity. With appropriate derivatization steps GC-MS and GC-MS-MS are sensitive, cost effective techniques for the detection of PPCPs in the environment. Moreover, GC-MS and GC-MS-MS observe less matrix effect than commonly observed in ESI based LC-MS or LC-MS-MS analysis [29]. Usually the column dimensions are 30 mX 0.25 mmX 0.25 μm columns are used but longer and chiral separation columns are also used for better separation [30]. Helium is used as a career gas at a sample injection volume of 1-3 μL.

1.3.2 LC-MS in detection

LC separation is an important part of the MS separation method. Mostly reversed phase (C8 and C18) analytical columns are used in the separation of PPCPs. Usually the inner diameter of the analytical column is 2 mm in the LC-MS or LC-MS-MS detection. The pH of the aqueous mobile phase is normally adjusted with acetic acid, formic acid, ammonium formate, ammonium acetate and ammonium hydroxide, whereas the organic mobile phase employs acetonitrile, methanol or a combination of these two solvents. Sometimes, buffer solution is also used as a mobile phase. Ouintana et al. used the tri-n-butylamine (TrBA) as an ion-pairing agent with a phenyl-hexyl adsorbent column, in order to separate the acidic drugs and triclosan [31]. Peru et al. reported the application of hydrophilic interaction chromatography (HILIC) for the separation of some very polar compounds such as lincomycin and spectinomycin [32]. Petrovic et al. used the ultra-performance liquid chromatography (UPLC) in the determination of pharmaceuticals. The column used in UPLC gives

higher column efficiency, shorter analytical time, narrower peaks and improved separations. Unfortunately, it also results in higher backpressure, requiring special solvent delivery systems thereby limiting its routine use in analysis [33]. Lopez de Alda et al. used LC-MS for the detection of estrogen and progesterone in river sediments using the deprotonated molecular ion $[M-H]^-$ and sodium adduts $[M+Na]^+$ [34]. LC-MS has an ability to acquire high sensitivity, full scan mass spectra using MS and has been used for the analysis of drugs like sulphonamide [35] caffeine, sulphonamide and tetracycline [36]. Yang et al. used the above technique for the determination of sulphonamide and tetracyclines in surface water using positive-ionization LC-IT-MS [37]. QqQ LC-MS2 can be used for the detection of native pharmaceuticals as well as their multi-residue in the environmental samples [38]. Detection of highly suspicious pharmaceuticals was made possible with the advent of a new hybrid quadrupole/linear IT instruments [39]. Stolker et al. compared the QqQ and qTOF-based mass detector for LC screening and confirmation of pharmaceutical residue in water [40].

1.3.3 HPLC detection

Gas chromatography coupled to electron ionization (EI) with MS is very sensitive and selective for the determination of drugs like sulfonamide. However, derivatization of thermally labile and non-volatile pharmaceuticals are required before their analysis [9]. This leads to an increase in the analysis time and it may also cause errors to the analytical technique. When analysing highly contaminated samples such as wastewater, suppression of ESI is most likely to occur. Use of an improved sample clean up method and quantification by internal standard or standard addition method can bring a solution of the problem above [41, 42]. Use of DAD with HPLC has

proved to be a powerful method for the identification and determination of compounds as it allows the on-line acquisition of their UV spectra.

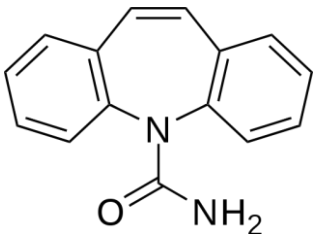
An analytical method was developed (using SPE-HPLC-DAD) for the simultaneous determination of veterinary medicines such as a fluoroquinolone (enrofloxacine), sulfonamides (sulfadiazine, sulfamethazine, sulfaguanidine), a tetracycline (oxytetracyclin), a sulphonamide synergist (trimethoprim) and β-lactam (penicillin G/procaine) in a highly complex wastewater sample. Foran et al used SPE for the sample pre-treatment followed by HPLC coupled with DAD. The method was used for the determination of above pharmaceuticals in wastewater from pharmaceutical industry [43]. HPLC-DAD is an inexpensive analytical method compared to HPLC-MS and GC-MS for the routine analytical of pharmaceuticals in wastewater [43]. An accurate, sensitive and inexpensive HPLC-post-column photochemically induced fluorimetry method (alternated to HPLC-MS) was developed for the routine determination of pharmaceuticals in water system. The method was used for the determination of both acidic and neutral pharmaceutical by active compounds [44].

1.4 Drug profile [Table 2.2]

1.4.1 Carbamazepine (CBZ)

Molecular formula: $C_{15}H_{12}N_2O$

Structure:



IUPAC name: 5H-dibenzo [b,f]azepine-5-carboxamide

Molecular weight: 236.269 g/mol

Melting point: 190.2 °C

Category: Anticonvulsant

Description: It is an anticonvulsant and mood-stabilizing drug used primarily in the treatment of epilepsy and bipolar disorder, as well as trigeminal neuralgia. It is also used in attention deficit hyperactivity disorder, schizophrenia, phantom limb syndrome, paroxysmal extreme pain disorder, neuromyotonia etc.

Water solubility: 17.7 mg/L

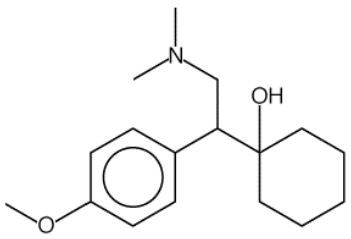
Bioavailability: 89%

Half-life: Initial half-life values range from 25-65 hours, decreasing to 12-17 hours on repeated doses.

1.4.2 Venlafaxine (VEN)

Molecular formula: $C_{17}H_{27}NO_2$

Structure:



IUPAC name: 1-[2-(dimethylamino)-1-(4-methoxyphenyl) ethyl]cyclohexanol

Molecular weight: 277.401 g/mol

Melting point: 215-217 °C

Category: Antidepressant

Description: It is an antidepressant of the serotonin-norepinephrine reuptake inhibitor class. It is used for the treatment of major depressive disorder and as a treatment for generalized anxiety disorders with depression. The major active metabolite is O-desmethylvenlafaxine, a dimethyl form of the parent compound.

Solubility: 572 mg/ml

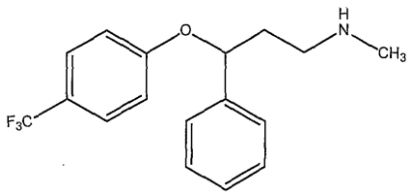
Bioavailability: 45%

Half-life: 5 days

1.4.3 Fluoxetine (FLU)

Molecular formula: $C_{17}H_{18}F_3NO$

Structure:



Chemical name: N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine

Molecular weight: 309.33 g/mol

Melting point: 179°C

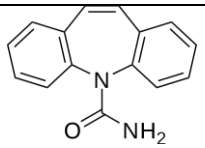
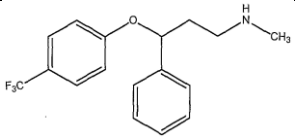
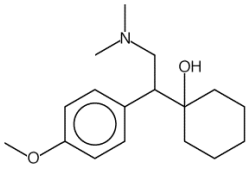
Category: Antidepressant

Description: It is an antidepressant of the selective serotonin reuptake inhibitor class. Bioavailability of fluoxetine is 72% and peak plasma concentrations are reached in 6-8 hours. It is mainly metabolized in liver by isoenzymes of cytochrome P450 system, including CYP2D6. Norfluoxetine is the only metabolite, which is biologically active.

Solubility: 14 mg/mL

Bioavailability: 72%

Half-life: There is a variation in the elimination rate of the drug; its elimination half-life changes from 1-3 days, after single dose, to 4-6 days, after long-term use.

Compound	**Carbamazepine**	**Fluoxetine**	**Venlafaxine**
Abbreviation	CBZ	FLU	VEN
Chemical structure			
Water solubility (g/L) (25 ^{0}C)	0.018	0.014	0.270
Half-life	36 hours (single dose), 16-24 hours (repeated dosing)	1–3 days (acute) 4–6 days (chronic)	5±2 hours (parent compound for immediate release preparations), 15±6 hours (parent compound for extended release

			preparations), 11±2 hours (active metabolite)
n-Octane/water partition coefficient (log K_{ow})	1.624	2.080	3.280
Henry's law constant at 25^0C (atm m^{-3} mol^{-1})	1.08 X 10^{-10}	8.90 X 10^{-8}	2.04 X 10^{-11}
Excretion in urine (% of the dose)	Urine (72%), faeces (28%)	Urine (80%), faeces (15%)	Ren (87%; 5% as unchanged drug; 29% as desvenlafaxine and 53% as other metabolites)

Table 2.2 Physical properties of carbamazepine, fluoxetine and venlafaxine [44].

1.5 Adverse effects of pharmaceuticals in water

As pharmaceuticals are manufactured with the intent of causing biological effects, they might induce more adverse effects than other environmental contaminants even at low concentrations. Moreover, they are made in such a way to prevent their degradation before exerting their therapeutic effects [45]. Jorgensen et al. commented that the potential adverse effects of the pharmaceuticals are largely

unknown [46]. However, 10-15 % of common pharmaceuticals found in surface water are intrinsically toxic to the body [47]. John et al. reported that the top 25 pharmaceuticals in UK exceed the environmental risk assessment trigger value in surface waters, and may cause acute and chronic effects in the aquatic environment [48]. Sanderson et al. reported the risk assessment of four pharmaceuticals classes, i.e., antibiotic, antineoplastic, cardiovascular, and sex hormones. They concluded that the antibiotics resistance represents the most significant human health hazard. Moreover, the largest non-target organism hazards are sex hormones such as endocrine modulators [49].

Adverse effects caused by pharmaceuticals are not readily detectable but have the potential to lead to ecological changes. Recent studies show the potential widespread occurrence of low levels of pharmaceuticals and their metabolites in the aquatic environment [50]. Some PPCPs (e.g., nitro and amino-nitro) have shown high acute aquatic toxicity. Others pharmaceuticals such as SSRIs also have some negative effects across numerous species [51]. Moreover, the persistent presence of pharmaceuticals in the environment might cause an increase in the chances of the development of resistant genes in bacteria, rendering a particular antibiotic useless. The cause of resistance may be related to transposons or conjugative plasmids as mobile genetic elements and those elements can transfer the resistance genes from one bacterium to another through horizontal gene transfer [52]. Stanford et al. reported the resistant genes for the tetracycline in lagoons and groundwater from two swine-production facilities [53].

Moreover, the Eco-toxicity caused by doxycycline have been reported by using a multi-species soil system and the tolerance of the soil microbial communities affected by sulfachloropyridazine [54, 55]. According to Ternes et al. the chemicals

released into the environment may have endocrine-disrupting effects in living organisms. There are indications that changes in the reproductive health of humans such as declining male fertility, breast and testicular cancer, birth defects, could be linked to the exposure to endocrine-disrupting chemicals (EDCs). It would be useful to test the toxicity of the drugs in low doses, as chronic toxicity may exert long-term effects on aquatic species. According to the study of Ternes et al. the eco-toxicological data are available only for <1 % of the currently used pharmaceuticals [56].

1.6 Factors affecting the drugs concentration in water

The factors include the following.

1. Chemical structure: Complex chemical structures are not easily degraded. Moreover, highly branched side chains are generally less amenable to biodegradation than unbranched compounds with shorter side chains [4]. Unsaturated aliphatic compounds are generally more accessible to biodegradation than saturated analogs or aromatic compounds with complex aromatic ring structures and sulphate or halogen groups [4].
2. Water solubility (g/L): As solubility in the water increases the chances of increasing amounts to the drug present in water increase.
3. n-octane/water partition coefficient (low K_{ow}) [5].
4. Henry's law constant at 25° C: the lower the Henry's law coefficient, the lesser the volatilization from the water phase into the air.
5. Excretion in urine (% of the dose): the greater the amount of unchanged drug excreted, the greater the amount of drug reaching to the environment.

6. Sales in the country (kg/year): More drug sales result into more drug levels in the environment.

1.7 Degradation of the drugs

1. **Biodegradation:** Potential degradation by aerobic or anaerobic microorganisms leads to the reduction of the parent compounds and their metabolites in WWTPs. Some of the degradation occurs during in-pipe transport to the STPs, but mostly the degradation occurs in the secondary stage of treatment when the water is exposed to a higher number of microorganisms [57].
2. **Deconjugation:** Mostly pharmaceuticals are metabolized in the liver and result in the excretion of glucoronide and sulphate conjugates of the parent pharmaceuticals [58]. Enzymes such as b-glucuronidase produced by *Escherichia coli* causes the deconjugation of the organic compounds such as steroid hormone and result in the increase in the concentration of the parent compounds [59].
3. **Partitioning**: There is a very strong relation between pharmaceuticals and the octanol/water partition coefficient K_{ow}. Pharmaceuticals with high log K_{ow} values will get absorb by sludge. On the other hand, lower log K_{ow} will stay in the aquatic phase (depending on the individual compounds) [60].
4. **Removal during sludge treatment:** Most of the pharmaceuticals are degraded during composting due to heat (as well as chemical and biodegradation) [61]. For instance, probenecid concentration was decreased from 5100 mg/kg to <10 mg/kg within 20 weeks during mesophilic treatments [62].

5. **Photodegradation:** Many pharmaceutically active compounds have been shown to degrade in the presence of sunlight [63]. Analgesic/anti-inflammatory drug diclofinac [63] and topical antimycotic drugs naftifine, sulbentine, cloxiquin, tolnaftate, and chlorphenes in are light-sensitive and are degraded by UV light [64]. However, the light level in the WWTPs will be lowest, which can decrease the degradation of the pharmaceuticals [65].

1.8 Objective, innovation and significance

Objective of this study: To develop a novel, sensitive, accurate and cost-effective alternative method to GC-MS and LC-MS in the detection of three pharmaceuticals (carbamazepine, fluoxetine and venlafaxine) in water system.

Innovation: Introduction of 200 μL of sample will increase the peak area and hence the LOD of the pharmaceuticals. Finally, the use of 4L of water sample will increase the amount of pharmaceutical extracted in the SPE process and will help in increasing the LOD and LOQ of the pharmaceuticals.

Significance: The major significance of the developed analytical method is that it can be used for the routine analysis of wastewater in waste water treatment plants (WWTPs). The method is more cost-effective than LC-MS and GC-MS; hence it can become more widely adopted. Moreover, the current method will help in determining the potential dosage of pharmaceuticals consumed by humans through the drinking water. Finally, the project will help in determining the relation between psychoactive pharmaceuticals i.e., (carbamazepine, fluoxetine and venlafaxine) and development of autism.

2. Materials

2.1 Chemical and reagents

The following chemical and reagents were used for the experiments:

1. Carbamazepine (Sigma Aldrich, St. Louis, MO)
2. Venlafaxine (TCI, St. Portland, OR)
3. Fluoxetine (TCI, St. Portland, OR)
4. HPLC grade acetonitrile (Fisher Scientific, Fair Lawn, NJ)
5. HPLC grade methanol (Fisher Scientific, Fair Lawn, NJ)
6. Citric acid monohydrate (Sigma Aldrich, St. Louis, MO)
7. Sodium hydroxide (Mallinckrodt, St. Louis, MO)
8. Ethylenediaminetetraacetic acid (EDTA) (Calbiochem, La Jolla, CA)
9. Ultrapure water from Barnstead International purification system (Barnstead International, Dubuque, IA)
10. Solid phase extraction cartridge (Waters Corporation, Milford, MA)

2.2 Instrumentation

Pump:	SP 8000 ternary HPLC Pump, (Spectra Physics, San Jose, CA)
HPLC column:	Gemini C18 110A (250 x 4.60 mm, 5 μm) Column (Phenomenex)
Detector:	SP 8450 UV/Vis Detector (Spectra Physics, San Jose, CA)
	HP 1046 A (Hewlett Packard) florescent detector
pH meter:	Beckman Instruments INC (Irvine, CA)
Analytical balance:	Mettler AM 100 (Highstown, NJ)

Autopippette: 100-1000 µL Eppendorf (Brinkmann Instruments, INC. Westbury, NY)

Nylon membrane filter: Whatman Int. (Maidstone, England)

3. Method

3.1 Preparation of reagents and solutions

1: Preparation of mobile phase: A mixture of citric acid (100 mM) and EDTA (10 mM) was mixed (pH adjusted at 4.5 by using 0.1 M NaOH) in water and was used as solvent A. Mobile phase was made from the solvent A and methanol (20:80, v/v). It was filtered by a 0.22 µm nylon membrane filters and was degassed with helium prior to use.

2: Preparation of stock solution: Stock solution of carbamazepine was prepared in acetonitrile (10 mg/50 ml). Stock solution of fluoxetine and venlafaxine was prepared in water (10 mg/50 ml). Stock solution was used for the calibration standards and quality control of the method. Working aqueous solutions were prepared daily.

3: Preparation of sample solution: Sample solution were prepared by diluting all three stock solutions in water to a concentration of 100, 50, 25, 20, 12.5, 6.25, 3.125, 2, 0.2, 0.02 and 0.002 µg/ml. Concentration of the sample solution was calculated from the chromatogram of the standard solution. All the stock solutions (50 ml) and sample solutions (1.5 ml) were stored in aliquots at 4^0 C.

Isolation of pharmaceuticals from the water samples was done by using SPE cartridge (Oasis HLB, 30 µm) on a VacElut apparatus. First, the cartridge was activated by passing 5 ml of methanol. Subsequently 4 L of water sample containing each of the three drugs was passed through a Teflon tube at a flow rate of 3 ml/min. by applying vacuum. The loaded cartridge was eluted by passing 1 ml of methanol (three 1 ml aliquots) at a flow rate of 3 ml/min. The combined aliquots were evaporated to dryness by a stream of nitrogen. The residue left was dissolved in 600 µl of methanol so that 200 µl of 3 injections can be done in HPLC.

According to the International Conference on Harmonization guidelines (ICH, 2005), method validation was done by evaluating linearity, specificity, LOD and LOQ, accuracy, repeatability and reproducibility, robustness and system suitability. Linear regression of peak area of standards solutions against the respective concentrations was used to prepare the calibration curve. System suitability test was performed to evaluate the chromatographic parameters (capacity factor, number of theoretical plates, asymmetry of the peaks and resolution between two consecutive peaks) before each validation run. The system suitability criterion is resolution between the three pharmaceuticals and standard (caffeine) and peaks. The estimation of the LOD and LOQ was done by injecting standard solution serially diluted until the signal-to-noise ratio for LOD was 10:1 and for LOQ was 3:1.

Evaluation of the method precision was done by intra- and inter-day repeatability method. For the intraday repeatability, three replicates of spiked water samples using same equipment and same analytical procedure in 1 day was done.

3.2 Chromatographic method development

Following are the conditions used for the development of the analytical method.

Mobile Phase: Citric acid (100 mM)/EDTA (10 mM) and methanol (pH=4.5)

Column: Gemini C18 110A (250 x 4.60 mm, 5 μm) column (Phenomenex)

Type: Isocratic elution

Flow rate: 1.0 ml/min.

Detection: 285 nm

3.2.1 Carbamazepine UV-Vis detection

Sample concentration: 20 μg/L

Retention Time:

Elution number	Retention time (min.)	Area
1	3.75	305668
2	3.80	140683
3	3.79	7264

Table 2.3 Retention time of carbamazepine in UV detection at 20 µg/L sample concentration.

Conclusion: Carbamazepine was detected in 4 L of water sample with a retention time of 3.78 min.

3.2.2 Carbamazepine UV-Vis detection

Sample concentration: 2 µg/L

Retention Time:

Elution number	Retention time (min.)	Area
1	3.76	50260
2	3.66	29674
3	-	-

Table 2.4 Retention time of carbamazepine in UV detection at 2 µg/L sample concentration.

Conclusion: Carbamazepine was detected in 4 L of water sample with a retention time of 3.71 min.

3.2.3 Carbamazepine UV-Vis detection

Sample concentration: 0.02 µg/L

Retention Time:

Elution number	Retention time (min.)	Area

1	3.80	10036
2	3.79	10984
3	3.76	15020

Table 2.5 Retention time of carbamazepine in UV detection at 0.02 µg/L sample concentration.

Conclusion: Carbamazepine was detected in 4 L of water sample with a retention time of 3.78 min.

3.2.4 Fluoxetine fluorescence detection

Sample concentration: 2 µg/L

Retention Time:

Elution number	**Retention time (min.)**	**Area**
1	2.96	54253
2	3.1	21864
3	-	-

Table 2.6 Retention time of fluoxetine in florescence detection at 2 µg/L sample concentration.

Conclusion: Fluoxetine was detected in 4 L of water sample with a retention time of 3.03 min.

3.2.5 Fluoxetine fluorescence detection

Sample concentration: 0.2 µg/L

Retention Time:

Elution number	**Retention time (min.)**	**Area**
1	2.95	8219
2	2.5	3690

3	-	-

Table 2.7 Retention time of fluoxetine in florescence detection at 0.2 µg/L sample concentration.

Conclusion: Fluoxetine was detected in 4 L of water sample with a retention time of 2.73 min.

3.2.6 Venlafaxine fluorescence detection

Sample concentration: 2 µg/L

Retention Time:

Elution number	**Retention time (min.)**	**Area**
1	2.9	16878
2	3.03	12176
3	-	-

Table 2.8 Retention time of fluoxetine in florescence detection at 2 µg/L sample concentration.

Conclusion: Venlafaxine was detected in 4 L of water sample with a retention time of 2.97 min.

3.2.7 Venlafaxine fluorescence detection

Sample concentration: 0.2 µg/L

Retention Time:

Elution number	**Retention time (min.)**	**Area**
1	2.95	4595
2	-	-
3	-	-

Table 2.9 Retention time of venlafaxine in florescence detection at 0.2 µg/L sample concentration.

Conclusion: Venlafaxine was detected in 4 L of water sample with a retention time of 2.95 min.

3.2.8 Fluoxetine and venlafaxine simultaneous fluorescence detection

Sample concentration: 2 µg/L

Retention Time:

Elution number	**Fluoxetine retention time (min.)**	**Area**	**Venlafaxine retention time (min.)**	**Area**
1	3.72	2137	3.01	91275
2	3.64	4794	3.09	28449
3	-	-	3.05	1642

Table 2.10 Retention time of fluoxetine in florescence detection 2 µg/L sample concentration.

Conclusion: Fluoxetine and venlafaxine were simultaneously detected in 4 L of water sample with a retention time of 3.7 and 3.05 min. respectively.

Venlafaxine, fluoxetine were detected by florescence detector whereas carbamazepine was detected by UV/Vis detector. The performance of the SPE-HPLC was characterized by validation procedure with spiked water samples. Detection of carbamazepine was done at 1 µg/L, 100 ng/L and 10 ng/L concentration with a retention time of 3.76, 3.79 and 3.71 sec. respectively [Figure 2.4.1:a, b, c]. The analysis was validated by performing three-sample analysis of each concentration. Simultaneous detection of fluoxetine and venlafaxine was done at 1 µg/L concentration with a retention time of 3.01 and 3.72 sec. respectively [Figure 2.4.2:a].

Detection of fluoxetine was done at 1 µg/L and 100 ng/L concentration with a retention time of 2.96 and 2.95 sec. respectively [Figure 2.4.3:a,b]. Finally, detection of venlafaxine was done at 1 µg/L concentration with a retention time of 2.90 sec. [Figure 2.4.4:a]. Limit of detection (LODs) and limit of quantification (LOQs) for carbamazepine were 10 ng/l and 100 ng/l, for venlafaxine were 1 µg/l and 1 ng/l, and for fluoxetine were 100 ng/l and 1 µg/l, respectively. The retention time and LOQ is shown in the [Table 2.11].

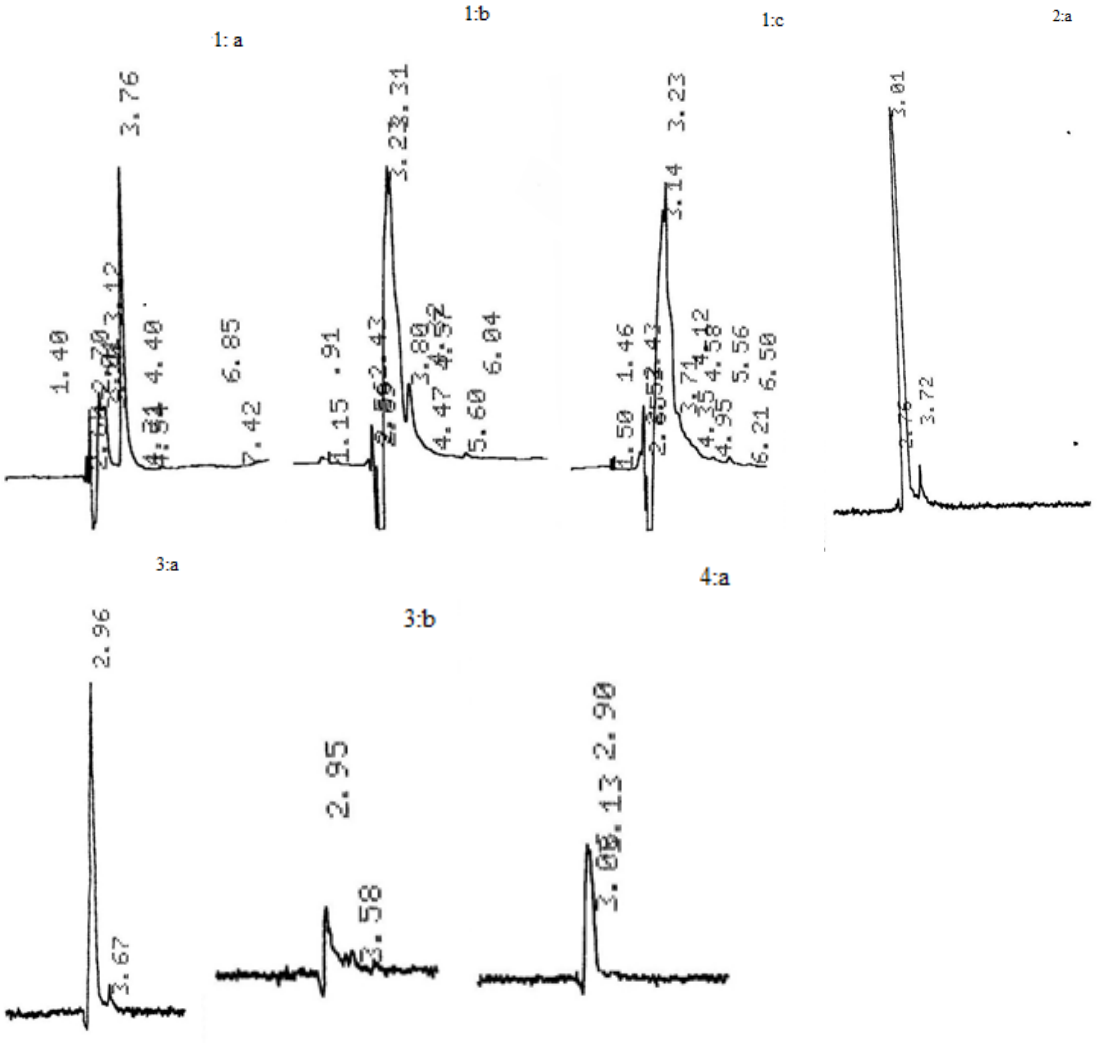


Figure 2.4 Pharmaceticals chromatogram: 1:a,b,c- chromatogram of carbamazepine, 2:a- venlafexine and fluroxetine, 3:a,b-fluroxetine and 4:a- venlafexine.

S.No.	Pharmaceutical	Retention Time (min.)	Min. concentration (LOQ)
1	Carbamazepine	3.76	1 µg/L
2	Carbamazepine	3.80	100 ng/L
3	Carbamazepine	3.71	10 ng/L
4	Venlafaxine and fluoxetine	3.01 and 3.72	1 µg/L
5	Fluoxetine	2.96	1 µg/L
6	Fluoxetine	2.95	100 ng/L
7	Venlafaxine	2.90	1 µg/L

Table 2.11 Pharmaceuticals their retention time and the minimum measurable concentration.

3.3 Chromatographic conditions standardized for the analysis of water samples

HPLC was carried with an isocratic elution (20:80) of mobile phase comprising of citric acid (100 mM), EDTA (10 mM) and methanol adjusted to the pH of 4.5 and with a flow rate of 1mL/min. Gemini C18 110A (250 x 4.60 mm, 5 µm particles) column was used and was equilibrated for 30-40 min. with mobile phase before making an injection. The injection volume was set up to 200 µL, column temperature was maintained at 25^0 C and a post-run equilibrium time of 3 min. was used. Carbamazepine was detected by using UV/ Vis detector at a set wavelength of 285 nm. Fluoxetine and venlafaxine was detected by using fluorescence detector, with an excitation and emission wavelength of 230 and 300 nm. Pharmaceuticals were

identified by comparing the retention time of the peaks with that of standard solutions. UV-spectra of the peaks in the standard solution and sample solution chromatogram were used to confirm the pharmaceuticals [Table 2.12].

Stationary Phase	Gemini C18 110A (250 x 4.60 mm, 5 µm) column (Phenomenex)
Elution type	Isocratic elution
Column	Gemini C18 110A (250 x 4.60 mm, 5 µm) column (Phenomenex)
Mobile phase	A mixture of citric acid (100mM) and EDTA (10mM) was mixed (pH adjusted at 4.5 by using 0.1 M NaOH) in water and was used as Solvent A. Mobile phase was made from the Solvent A and methanol (20:80, v/v)
Flow rate	1.0 ml/min.
Injection volume	200 µl
Wavelength	285 nm
Temperature	25 ^{0}C
Runtime	10 min.

Table 2.12 Chromatographic conditions of HPLC.

4. Results and Discussions

Three public Parks Peachwood/Heldane, Rings point and 628 West Craig 3 Road Park were selected as a sampling site. Carbamazepine was detected in none of the samples from public parks. Venlafaxine was detected in the samples from Rings Point Park and 628 West Craig 3rd Road Park but it was not detected in the samples from Peachwood/Heldane Park. However, very low concentration of fluoxetine was detected in all the sample of public parks. Moreover, none of the pharmaceuticals were detected when the tap water was taken as a sample. The reason for the negative detection of pharmaceuticals may be that the pharmaceuticals were not present in the water or their concentration was lower than the detection limit of the method [Table 2.14, 2.15, 2.16, 2.17]. Detection of pharmaceutical in wastewater samples from WWTP of Pocatello city, ID was also tried. However, the detection of the pharmaceuticals in the wastewater was not possible due to high matrix effect.

4.1 Field sample 1: Peachwood/Heldane Park.

Retention Time:

Pharmaceutical	Retention Time (min.)	Area	Concentration
Carbamazepine	-	-	-
Fluoxetine	5.80	3389	0.5071
Venlafaxine	-	-	-

Table 2.13 Detection of three pharmaceuticals in field sample 1: Peachwood/Heldane Park.

4.2 Field sample 2: Rings Point Park.

Retention Time:

Pharmaceutical	Retention Time (min.)	Area	Concentration

Carbamazepine	-	-	-
Fluoxetine	5.79	4623	0.6271
Venlafaxine	3.88	2952	1.254

Table 2.14 Detection of three pharmaceuticals in field sample 2: Rings point park.

4.3 Field samples 3: 628 West Craig 3rd Road Park.

Retention Time:

Pharmaceutical	**Retention Time (min.)**	**Area**	**Concentration**
Carbamazepine	-	-	-
Fluoxetine	5.79	3764	0.5436
Venlafaxine	3.89	9357	1.538

Table 2.15 Detection of three pharmaceuticals in field samples 3: 628 West Craig 3rd Road Park.

4.4 Tap water samples

Retention Time:

Pharmaceutical	**Retention Time (min.)**	**Area**	**Concentration**
Carbamazepine	-	-	-
Fluoxetine	-	-	-
Venlafaxine	-	-	-

Table 2.16 Detection of three pharmaceuticals in tap water samples.

Few studies are reported where HPLC has been used for the detection of pharmaceuticals in water system [66, 67]. In the current method SPE-HPLC was used for the determination of three psychoactive pharmaceuticals such as carbamazepine, fluoxetine and venlafaxine. Further, few modifications were made to increase the detection limit of UV-Vis and florescence detector which gives the LOD of 10-1000

ng/l. First, instead of using 1 L of water sample 4 L water sample was used. This increased the amount of pharmaceutical extracted in SPE process and enhanced the LOD. However, using 4 L of water sample increased the matrix effect in the detection and hence wastewater sample analysis could not be possible. Second, we used 200 µL injection volumes instead of 25 µL injection volume. This increased the amount of pharmaceuticals in the sample and enhanced the LOD.

Babic et al. have used the SPE-HPLC-DAD method for the detection of sulfadiazine, sulfamethazine, sulfaguanidine, oxytetracycline, trimethoprim, enrofloxacine and penicillin G/procaine in the wastewater matrix. Here they have obtained the LOQ of 1.5-100 µg/L [66]. Santos et al. have used HPLC with DAD and fluorescence detector for the determination of pharmaceutically active compounds in wastewater samples. The method has been used for the determination of pharmaceuticals such as diclofenac, ketoprofen, acetaminophen, carbamazepine, caffeine (by DAD) and naproxen, and ibuprofen (by florescence detection). They have obtained the LOQ in the range of 6.2-319.8 and 3.0-160.0 ng/ml for the influent and effluent wastewater samples respectively [67]. The obtained LOQ by Babic et al. and Santos et al. was lower than our LOQ because water samples in our study were from clean water source and did not show any matrix effect.

Most of the pharmaceuticals get degraded in the environment. However, the degradation of the three pharmaceuticals is very slow because of their complex structures. Moreover, the presence of a double bond makes them harder to get degraded. Drugs like carbamazepine are metabolized to carbamazepine-10,11-epoxied (pharmacologically active) and then hydrolyzed into carbamazepine-10, 11-trans-dihydrodiol (pharmacologically inactive) and excreted in the urine. Unfortunately, the

glucuronide conjugates of carbamazepine are cleaved during wastewater treatment process and enter in the water system [20].

There is a need for more advanced water treatments technology such as ozone oxidation as the conventional techniques (flocculation, sedimentation, flocculation and filtration) are unable to serve the purpose efficiently [20]. Unfortunately, advanced treatment plants incur a high expense and needs constant maintenance. Hence, an effective way of tackling the problem is to make people aware of the proper disposal methods of the pharmaceuticals. Finally, as prevention is better than cure, awareness should be created regarding the proper disposal of the pharmaceuticals in the environment.

5. Conclusion

A novel, fast, sensitive, accurate and cost-effective HPLC-UV method was developed. The method was used for the determination of three psychoactive pharmaceuticals: carbamazepine, fluoxetine and venlafaxine. Use of only SPE and HPLC made it a cost-effective and hence an alternate to GC-MS and LC-MS methods. With the current method we were able to obtain the LOQ of 100 ng/l, 1000 ng/l, 10ng/l respectively and LOD of 10 ng/L, 100 ng/L, 1 ng/L respectively for carbamazepine, fluoxetine and venlafaxine. The current method can perform the routine analysis of the pharmaceuticals discharged from the WWTPs and can be used to evaluate the performance of the WWTP. Further work is needed to develop a cost-effective HPLC methods for the determination of pharmaceuticals and their metabolites in the environment such as surface water, groundwater, and drinking water.

6. Prospects of future studies

- In order to detect the pharmaceuticals present in the environment more HPLC analytical methods need to be developed.
- Better sample purification techniques with reduce the matrix effect (especially in waste water sample) needs to be development.
- Need to implement analytical techniques like pre- and post-column derivatization, especially for analysing less florescent pharmaceuticals.
- Use of monolithic column in the detection of pharmaceuticals would give a better performance to the HPLC and hence better output.
- The knowledge of the effects of low-level pharmaceuticals in water on humans is non-existent and requires more in-depth study.
- Study not only on the healthy individuals but also on the more susceptible individuals (such as patients, pregnant and fetus) needs to be done.
- Pregnant women and fetuses are more susceptible to these pharmaceuticals and more research emphasis should be directed toward them.
- As prevention is better than cure, awareness should be created regarding the proper disposal of the pharmaceuticals in the environment.

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CHAPTER 3: HPLC Examination of Potential Blueberry Polyphenols Metabolites

Abstract

Polyphenols present in blueberries exert beneficial effects on cardiovascular health. Characterizing the role of intestinal microbes in producing bioactive compounds from polyphenols is important in understanding why humans respond differently to polyphenol-rich diets. In the present study attempts were made to determine the metabolism of blueberry (*Vaccinium corymbosum*) polyphenols performed by *Lactococcus lactis*. An isocratic reverse-phase HPLC-DAD was used to detect the changes in the polyphenols metabolized by *L. lactis*. Sample was passed through a Gemini C18 110A (250 x 4.60 mm, 5 µm, Phenomenex) column at a flow-rate of 1.0 ml/min. The Test samples containing *L. lactis*, blueberry and media supernatant, major changes in the peaks were observed at 9 hours, 18.5 hours and 24 hours. The result showed that *L. lactis* metabolizes the blueberry constituents, especially polyphenols. The developed method was used for the analysis of polyphenolic metabolism by *L. lactis.* It was found that *L. lactis* can and does metabolize polyphenols present in blueberry.

Keywords: blueberry, polyphenols, *Lactococcus lactis*, HPLC.

1. Background

The microorganisms that provide health benefits to humans and animals when consumed are called probiotics [1]. The term is the composite of the Latin preposition *pro* (for) and the Greek adjective *biotic* (life). The first written information about the use of probiotics for good health can be found in the Persian Bible in which Genesis noted that Abraham owned his longevity to the daily consumption of fermented milk products. The concept was introduced by the Nobel Prize recipient Eli Metchnikoff, who suggested that "the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" [2]. He also developed the theory that toxic bacteria in the gut cause aging and the presence of lactic acid in the gut could prolong life. His theory inspired the Japanese scientist Minoru Shirota to investigate the relation between the good intestinal health and bacteria, which eventually led to the production of probiotics and to the development of the probiotic drink (Yakult) containing *Lactobacillus casei* strain *shirota* [3].

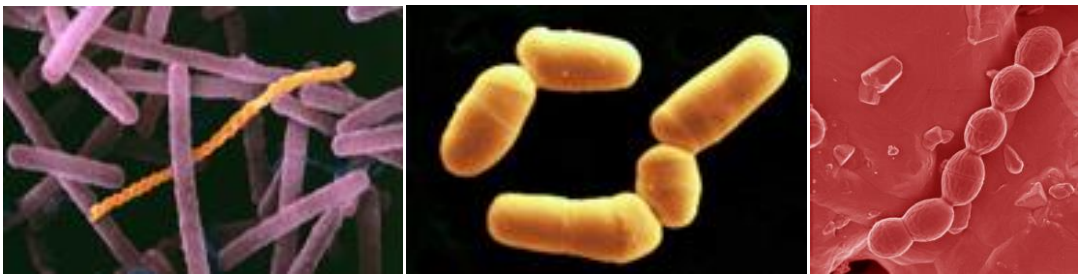
1.1 Some common definitions

Probiotics: A probiotics are a live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host [4].

Prebiotics: A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health [5]. In short, they increase the number of probiotic bacteria and their activity in the colon, and provide energy to some beneficial bacteria like *bifidobacteria* and *lactobacilli*.

Synbiotics: the term is used when a product contains both prebiotics and probiotics. For instance, a product containing *bifidobacteria* as probiotic and oligofructose as prebiotic come under the category of synbiotic [6].

Some common examples of probiotic bacteria are: *Lactobacillus (L.) acidophilus, L. casei, L. bulgaricus, L. plantarum, L. salivarius, L. rhamnosus, L. reuteri, Bifidobacterium (B.) bifidum, B. longum, B. infantis and B. thermophilus*. *Lactobacilli* are gram-positive, non-spore forming rods or *coccobacilli* and are found mainly in habitats where rich carbohydrate containing substrates are available such as in human mucosal membranes [Figure 3.4.a] [4]. *Bifidobacteria* are gram positive rods and are mainly found in the normal intestinal microflora in humans [Figure 3.4.b] [4]. *Lactococcus lactis* used in the current study is a Gram-positive bacterium and is very commonly used in the production of buttermilk and cheese [Figure 3.4.c] [8].



(a) (b) (c)

Figure 3.1 Scanning electron micrograph of a: *Lactobacillus acidophilus,* b: *Bifidobacterium bifidum* c: *Lactococcus lacti* [10].

According to broad application, probiotics can be placed in several FDA regulatory categories such as food, food ingredient, medical food, dietary supplement, drug and biological product [7]. Rijkers et al. has mentioned some applications of probiotics in humans [9].

1.2 Mechanism of action of probiotics

On the basis of site of action, the following three different levels of action of probiotics have been proposed [Figure 3.2] [9].

1. Within the gut lumen: Probiotics interact with the complex ecosystem of the gut microbiota. (Level 1)
2. Within the gut mucus: Probiotics interact with the gut mucus and the epithelium, including barrier effects, mucosal immune system, digestive processes and enteric nervous system. (Level 2)
3. Beyond the gut: Probiotics interact through signalling to the host beyond the gut to the systemic immune system, liver and brain. (Level 3)

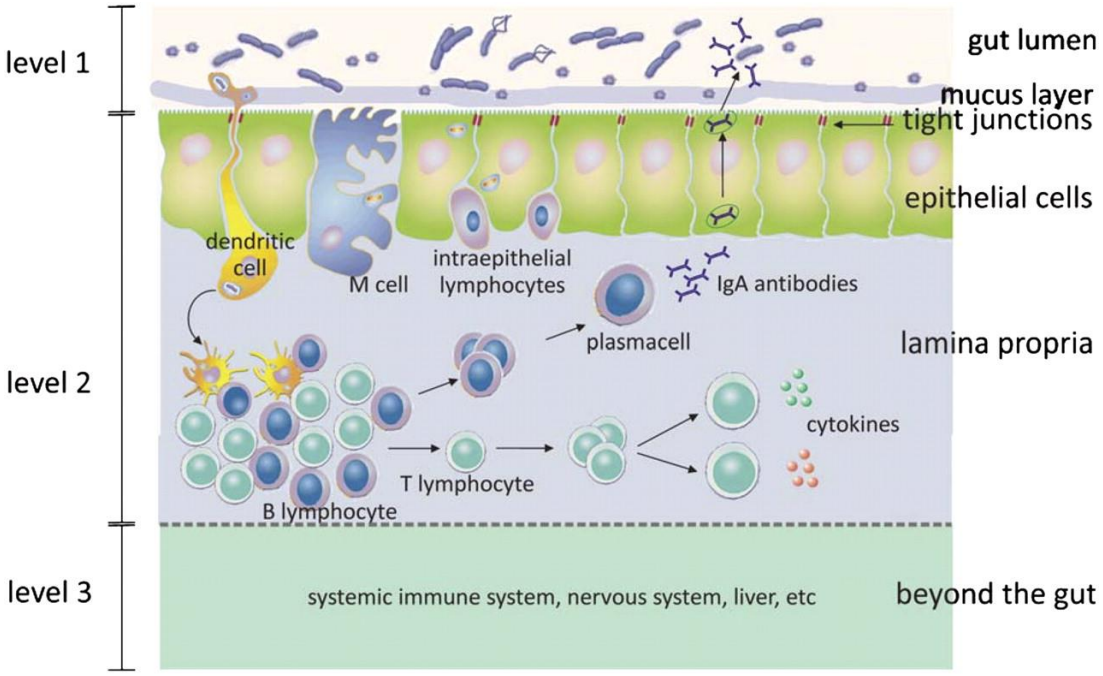


Figure 3.2 Three levels of action of a probiotic. Level 1: Probiotic bacteria inhibit the growth and survival of pathogenic microorganisms present in the gut lumen. Level 2: Probiotic bacteria strengthen the mucosal immune system and mucosal barrier function. Level 3: Probiotic bacteria improve the immune system and other cell and organ system [9].

1.3 Safety of probiotics

Lactococcus have been used in food for a long time and are considered as safe for human consumption [11]. Some other common bacteria used in probiotics are *Streptococci*, *Bifidobacterium*, *Enterococcus*, *Bacillus* and yeast. Generally probiotics are considered as safe, but there is also some theoretical risk related to them such as 1. they might have a negative effect on the gastro-intestinal (GIT) physiology and function; 2. they might cause adverse effect on localized and generalized immune system; and 3. they may also transfer the antibiotic resistance within the GIT flora [12]. Besides some potential risk of probiotics, Syndman et al. have reported the following suggestions to monitor the safety of probiotics, such as: [12]

1. Population-based surveillance should be conducted on all the stains used in clinical trials.
2. Complete knowledge of the susceptibility profile for any strain should be obtained.
3. Attention should be given to special medical conditions such as weak immune system, premature infant short bowel syndrome, patients with central venous catheters, elderly patients, and patients with cardiac valve disease.
4. A proper study-by-study evaluation should be performed which includes an appropriate involvement of a human investigation review committee.
5. An accurate benefits-to-risk study should be done, which will determine both the therapeutic promise and peril of probiotics.

1.4 Probiotics in United States

The current market of probiotics in the USA has many small distributors with a limited number of fermentation facilities. Unfortunately, many of the probiotic

distributors' outrageous claims are based on little or no objective data. In USA, probiotics are mainly provided in the form of sachets and capsules [13]. Very few probiotic products such as yogurt in the form of food are available. In 2005 the US sale of probiotics was estimated to be $764 million [14].

Three important things to probiotic bacteria are 1. they should be able to get administered in an active form. 2. they should be able to colonize in the gut lumen, and 3. they should be able to attach and adhere to the lining of the GIT [15]. Following are the examples of some of the common probiotics bacteria used in United States:

1. Viral diarrhea: *L. acidophilus, Lactobacillus* GG*, and Lactobacillus reuter* [16].
2. Antibiotic-associated diarrhea: *Lactobacillus* GG [17].
3. *C. difficile*-associated diarrhea: *Lactobacillus* GG, the yeast *Saccharomycesboulardii* [18].
4. Traveler's diarrhea: *Lactobacillus* GG [19].
5. Atopic dermatitis [20].
6. Pouchitis: mixture of different species of *lactobacilli* [21].
7. Irritable bowel syndrome [22].

Various case reports and preliminary data exist for the use of probiotics for the following conditions: 1. rheumatoid arthritis [23], 2. crohn's disease and/or ulcerative colitis [24], 3. small-bowel bacterial overgrowth [25], 4. dental caries [26], 5. infantile allergies and/or asthma (prevention) [27], 6. lactose intolerance [28], and 7. colon cancer (reduction) [29] and high cholesterol [30].

1.5 Review of literature

1.5.1 Blueberry (*Vaccinium corymbosum*): It is a perennial flowering plant with indigo-colored berries from the section *Cyanococcus* within the genus *Vaccinium* [31]. The plants are native to North America [32]. The smaller species are known as "low bush blueberries" and the larger species are known as "high-bush blueberries". Blueberries contain polyphenols, which are secondary plant metabolites [31] and may influence several metabolic or signalling pathways involved in cardiovascular, gut, and bone health and carcinogenesis [32-34]. These phytochemicals consist of a wide variety of molecules, which ranges from highly polymerized proanthocyanidins to low-molecular-weight phenolic acids, which are largely found in the fruits like apples, grapes, pears, berries, and cherries at concentration up to 200-300 mg polyphenols per 100 gram weight. Polyphenols are also found in coffee, tea, red wine, dry legumes, cereals and chocolate [35, 36].

1.5.2 Structure of Polyphenols: Currently, more than 8000 polyphenolic compounds have been identified in various plant species. Almost all plant phenolic compounds arise from a common precursor, shikimic acid or a close intermediate, phenylalanine. They are present either in conjugated forms (with one or more sugar residues linked to hydroxyl groups) or directly linked to the sugar (monosaccharide or polysaccharide) of an aromatic carbon [Figure 3.3] [37].

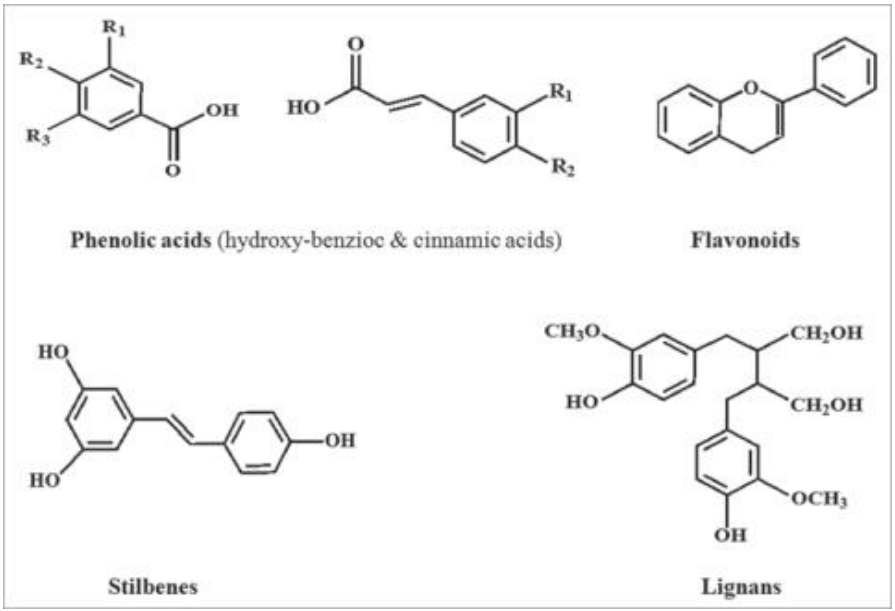


Figure 3.3 Chemical structure of the different classes of polyphenols.

1.5.3 Classification of polyphenols: Polyphenols are classified on the basis of number of phenol rings they have and the structural element that binds these rings. On this basis they are mainly divided into four classes:

1. Phenolic acids: They are further divided into:

   A. hydroxyl benzoic acids

   B. hydroxyl cinnamic acids

e.g.: caffeic acids, gallic acid, ferulic acid, p-coumaric acid and sinapic acids.

2. Flavonoids: They consist of two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle. These are divided into six subclasses: flavones, flavonols, flavanols, flavanones, isoflavones and anthocyanins.

3. Stilbenes: They contain two phenyl moieties connected by a two-carbon methylene bridge. They are mainly found in grapes and red wine.

4. Lignans: They are diphenolic compounds containing 2, 3-dibenzylbutane structures formed by the dimerization of two cinnamic acids. They are mainly found in linseed.

1.5.4 Metabolism of polyphenols: Metabolism of the polyphenols starts with the hydrolysis of glycosylated, polymeric and esterified compounds by brush border and microbial enzymes: this step is required for the absorption and bioactivity [38]. For instance, when the humans consume citrus fruits, the rhamnose part of hesperidin (hesperetin-7-O-rutinoside) is hydrolyzed by colonic microbiota and produces hesperetin-7-glucoside and hesperidin. A four-week hesperidin intake decreases the diastolic blood pressure and improves postprandial microvascular endothelial reactivity only when measured at the peak hesperidin plasma concentration [39].

1.5.5 Applications of polyphenols: Polyphenolic antioxidants provide protection against unstable molecules or free radicals and hence provide resistance against cell damage leading to chronic and degenerative disease [40]. Kalt et al. stated that almost all the dark colored fruits have a high antioxidative capacity [41, 42]. Moreover, cranberries have been used for the prevention and treatment of urinary tract infection (UTI) [43]. Yatao et al. and Howell et al. stated that the proanthocyanidines present in the cranberries prevent the adhesion of bacteria to cell walls [44, 45] and helps in decreasing the risk of bacterial infection [46]. There is a positive link between the above proposed mechanism and prevention of UTI [47], ability to inhibit gum disease, dental caries [50, 51] and stomach ulcer caused by bacteria [48, 49]. Blueberries and cranberries helps in maintaining a good cardiovascular health by decreasing the cholesterol and low-density lipoprotein (LDL) level in the blood [52-55].

As stated above, the polyphenol rich diet provides protection against cardiovascular disease. These studies have also been supported by clinical trials [56]

Williamson et al. have shown that the polyphenols in olive oil decrease cardiovascular disease risk factors. However, a variation among individuals in physiologic response to polyphenols has been observed. The major reason of this variation may be due to the cooperation between human enzymes and intestinal microbes in metabolizing polyphenols to their bioactive products [57]. Hence, understanding the role of the intestinal microbes in producing bioactive compounds from polyphenols is important to understanding why humans respond differently to polyphenol-rich diets. Microbiota populations vary between individuals, resulting in differences in metabolite profiles and their downstream effects [58]. One of the ways of reducing the variability among individuals is by stabilizing microbiota population among through probiotic feeding. Consumption of microbiota in adequate amount can help the beneficial bacteria colonize the intestine, often combating pathogenic bacteria [59]. Thus, in a probiotic-fed population with a relatively homogenous microbiota community, the profile and concentration of metabolites of dietary polyphenols can reach greater conformity and produce a more reproducible pattern of biologic effects.

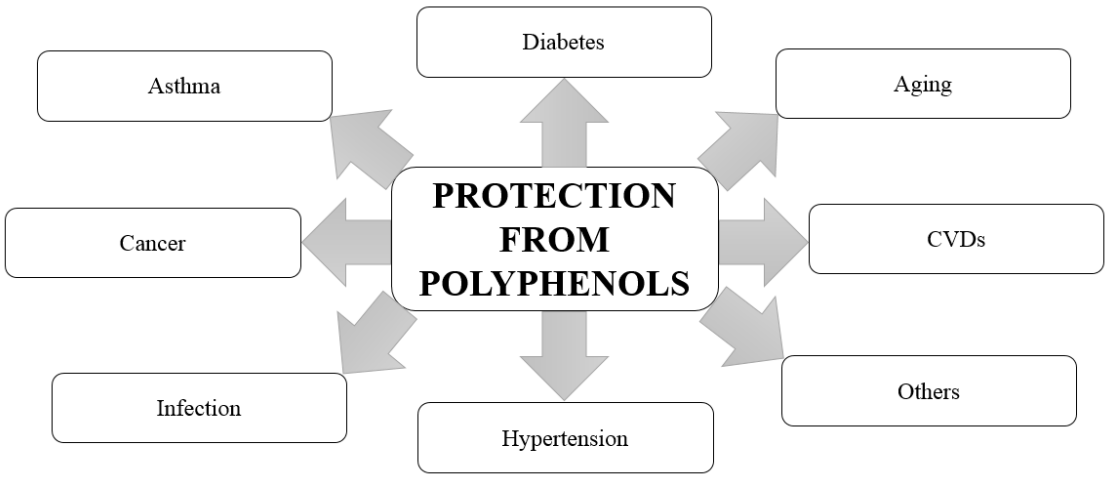


Figure 3.4 Health benefits of polyphenols [1].

1.5.6 Mechanism of action of polyphenols:

There are several potential mechanisms of blood pressure reduction [60] such as:

1. Decrease in oxidative stress.
2. Interference with renin-angiotensin-aldosterone stress.
3. Improving vascular function in an endothelium-dependent or -independent manner.

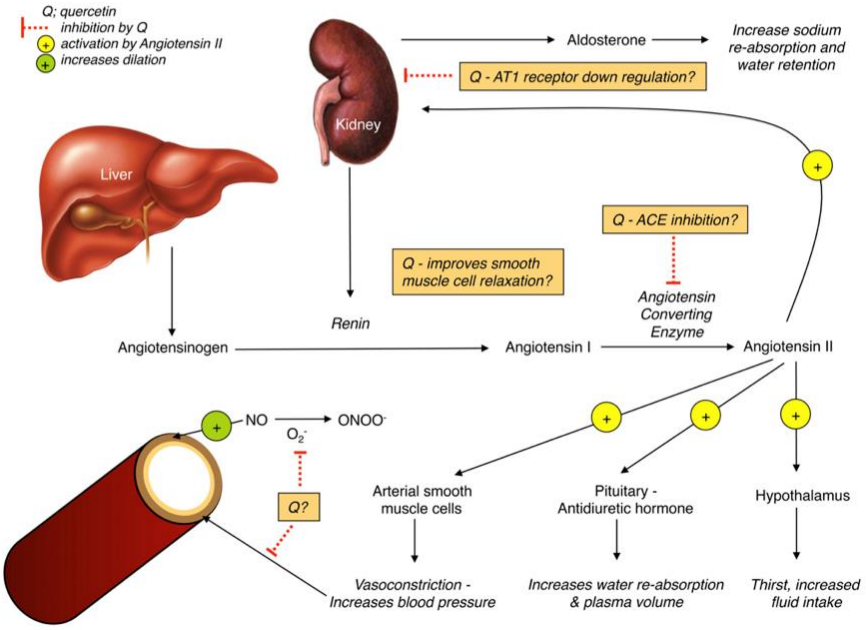


Figure 3.5 Potential mechanisms of blood pressure reduction.

1.6 Chromatography fingerprints

Chromatographic fingerprinting is performed to measure the similarities and differences in a chromatographic pattern of a sample investigated [61]. Hence, chromatography fingerprinting can be used for the identification and authentication of the samples (‘integrity’) even if the concentrations of the characteristic constituents are slightly different from the same sample (‘fuzziness’). In plant extracts the numbers of unknown components are observed with some variability [61]. Therefore, obtaining a chromatography fingerprint representing the desired and chemical characteristics is not very simple [61]. Factors affecting chromatography fingerprint

are plant origins, harvest seasons, possible contamination, drying processes, and presence of heavy metals and chemical toxins [61]. Chromatography fingerprinting involves following steps:

1. Plant extract is prepared and its characteristics are determined by analytical methods.

2. Quantitative and qualitative profiles of all the constituents is obtained by using a hyphenated technique with high sensitivity and efficiency such as HPLC-DAD, GC-MS or HPLC-MS. These hyphenated techniques can be used to obtain the chromatographic fingerprints and further combined with a chemometric approach to create a perfect tool for the quality assurance and quality control of the sample [61].

The fingerprint spectra analysis is divided into two major aspects:

1. Spectroscopy fingerprint spectra: Use ultraviolet spectroscopy and infrared spectroscopy for the analysis. The method is quick, accurate, easy and mostly used for qualitative analysis.
2. HPLC fingerprint spectra: Use HPLC which is more complex, but can be used to determine the partial component in the sample and their concentration quantitatively.

Hua-Bin et al. have discussed the methods and application of HPLC in herbal quality control [62]. The common methods of chromatographic fingerprinting are 1. comparing methods, 2. clustering analysis, 3. principle component analysis, 4. similarity analysis and 5. fingerprint spectra invariableness analysis [62].

1.7 Hypothesis, objectives, innovation and significance

Hypothesis: Probiotic bacteria strains demonstrate differential growth rates and metabolic activity toward polyphenols when cultured in medium containing blueberry powder.

Objective: Quantify the disappearance of blueberry polyphenols and appearance of blueberry polyphenol metabolites in the probiotic culture medium.

Innovation: This project represents a novel approach in directing gut bacteria toward the production of bioactive metabolites from polyphenols. By manipulating gut bacteria populations across humans, the response to polyphenol consumption might become more reproducible.

Significance: The major significance of the project is that by controlling the probiotic bacteria in polyphenol metabolism the blood pressure of the cardiovascular disease patient may be controlled.

2. Material

2.1 Chemical and reagents

The following chemical and reagents were used for the experiments:

HPLC grade methanol (Fisher Scientific, Fair Lawn, NJ).

Ultrapure water (Barnstead International purification system, Dubuque, IA).

2.2 Instrumentation

Pump:	SP 8000 ternary HPLC pump, (Spectra Physics, San Jose, CA)
HPLC column:	Gemini C18 110A (250 x 4.60 mm, 5 µm) column (Phenomenex, Torrance, CA)
Detector:	DAD, (GenTech Scientific, Inc. San Francisco, CA)
pH meter:	Beckman Instruments INC. (Irvine, CA),
Analytical balance:	Mettler AM 100, (Highstown, NJ)
Auto pipette:	100-1000 µL Eppendorf Brinkmann Instruments, INC. (Westbury, NY).

Nylon membrane filter: Whatman Int. (Maidstone, England).

3. Methods

3.1 Selection of UV wavelength

Selection of DAD wavelength depends on the wavelength, which gives good response for the drug to be detected. The UV spectra 254 nm was selected as the wavelength for study. The λ max was found to be 254 nm.

3.2 Chromatographic conditions

High performance liquid chromatography (HPLC) was carried with an isocratic elution with the water: methanol (70:30) of mobile phase. Gemini C18 110A (250 x 4.60 mm, 5 µm particles) column was used and was equilibrated for 30-40 min. with mobile phase before making an injection. The injection volume was set up to 5 µL, column temperature was maintained at 40^0 C and a post-run equilibrium time of 3 min. was used. The sample was detected by using DAD detector at a set wavelength of 254 nm. Different chromatographic condition were tried to optimize the HPLC method as detailed in [Table 3.1]:

Elution type	Isocratic elution
Column	Gemini C18 110A (250 x 4.60 mm, 5 µm) column (Phenomenex)
Mobile phase	Water: Methanol (70:30)
Flow rate	1.0 ml/min.
Injection volume	5 µl
Wavelength	254 nm
Temperature	25° C
Runtime	20 min.

Table 3.1 Chromatographic condition of HPLC.

3.3 Preparation of reagents and solutions

1. Preparation of mobile phase: Water and methanol (70:30) was used as a mobile phase and pass through the C18 column at a flow rate of 1 ml/min. It was filtered by a 0.22 μm nylon membrane filter and was degassed with helium prior to use.

2. Preparation of samples: One test sample (test sample D) and 4 control samples (control sample A, B, C, E) were obtained from Dr. Cynthia Blanton, Division of Health Sciences – Dietetics, Idaho State University. Test sample D contained *L. lactis*, blueberry and supernatant from incubated media. Control samples A contained DMSO and supernatant form incubated media. Control samples B contained blueberry and supernatant from incubated media. Control samples C contained *L. lacti* and supernatant from incubated media. Control samples E contained *L. lacti*, DMSO and supernatant from incubated media. All samples contained supernatant from media incubated for 6, 9, 18.5 and 24 hours as detailed in [Table 3.2]. Medium sample was also used as blank control. The samples were stored at -80^{0}C and were analysed by HPLC within one month. Before making an HPLC injection the samples were gradually thawed to 4^{0}C, diluted 3 times (sample: water; 1:3) and the 5 μl of sample was injected into the HPLC. The HPLC analysis was done within 3 days. Each sample was run in triplicate for HPLC analysis and identical chromatograms were found for each sample.

Samples	**Incubation period**			
	6 hrs.	**9 hrs.**	**18.5 hrs.**	**24 hrs.**
Control sample A	DMSO Supernatant	DMSO Supernatant	DMSO Supernatant	DMSO Supernatant

Control sample B	Blueberry Supernatant	Blueberry Supernatant	Blueberry Supernatant	Blueberry Supernatant
Control sample C	*L. lactis* Supernatant	*L.lactis* Supernatant	*L. lactis* Supernatant	*L. lactis* Supernatant
Test sample D	*L. lactis* Blueberry Supernatant	*L. lactis* Blueberry Supernatant	*L. lactis* Blueberry Supernatant	*L. lactis* Blueberry Supernatant
Control sample E	*L. lactis* DMSO Supernatant	*L. lactis* DMSO Supernatant	*L. lactis* DMSO Supernatant	*L. lactis* DMSO Supernatant

Table 3.2 Details of the samples used in HPLC analysis.

4. Result and Discussion

In the control samples A, containing DMSO and supernatant, no changes in peaks was observed till 9 hours. Changes in the peaks were observed at 18.5 and 24 hours of incubation. The result showed that DMSO and supernatant does not show any major changes in the chromatogram of the control samples A. However, minor changes at 18.5 and 24 hours might have occurred due to chemical degradation of the constituents of the medium [Figure 3.6.1-4].

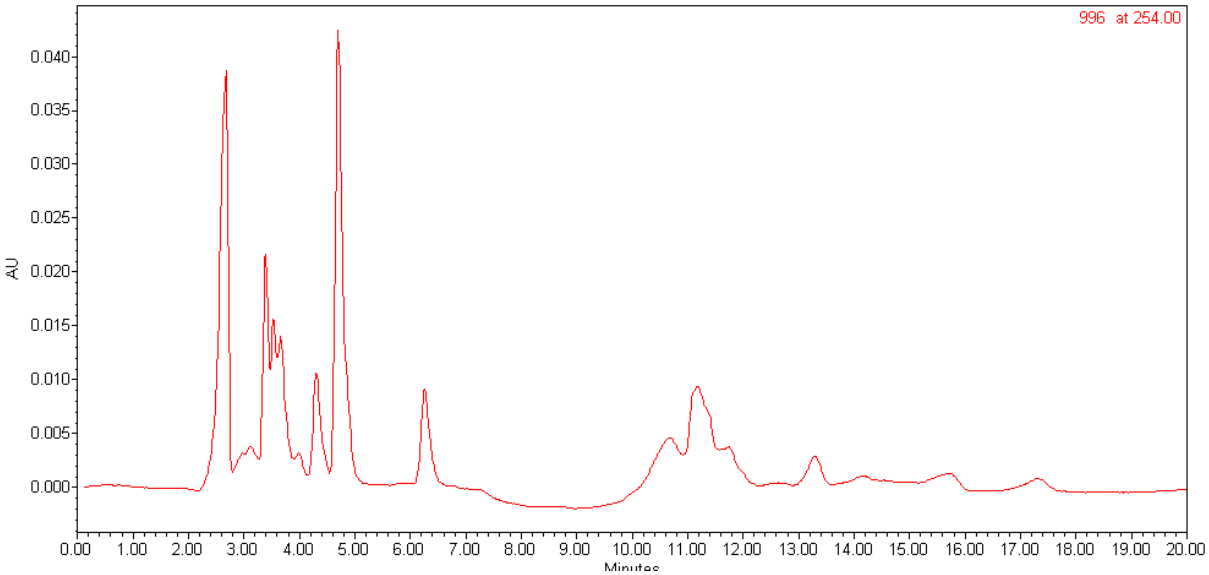


Figure 3.6.1 Chromatogram of control sample A containing DMSO and supernatant after 6 hours of incubation.

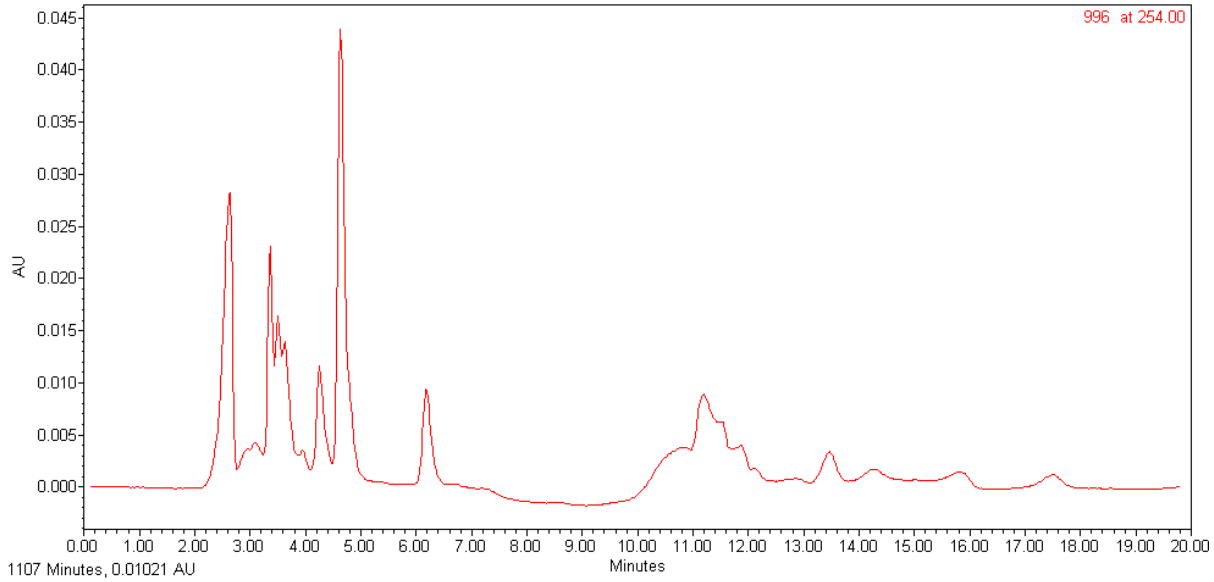


Figure 3.6.2 Chromatogram of control sample A containing DMSO and supernatant after 9 hours of incubation.

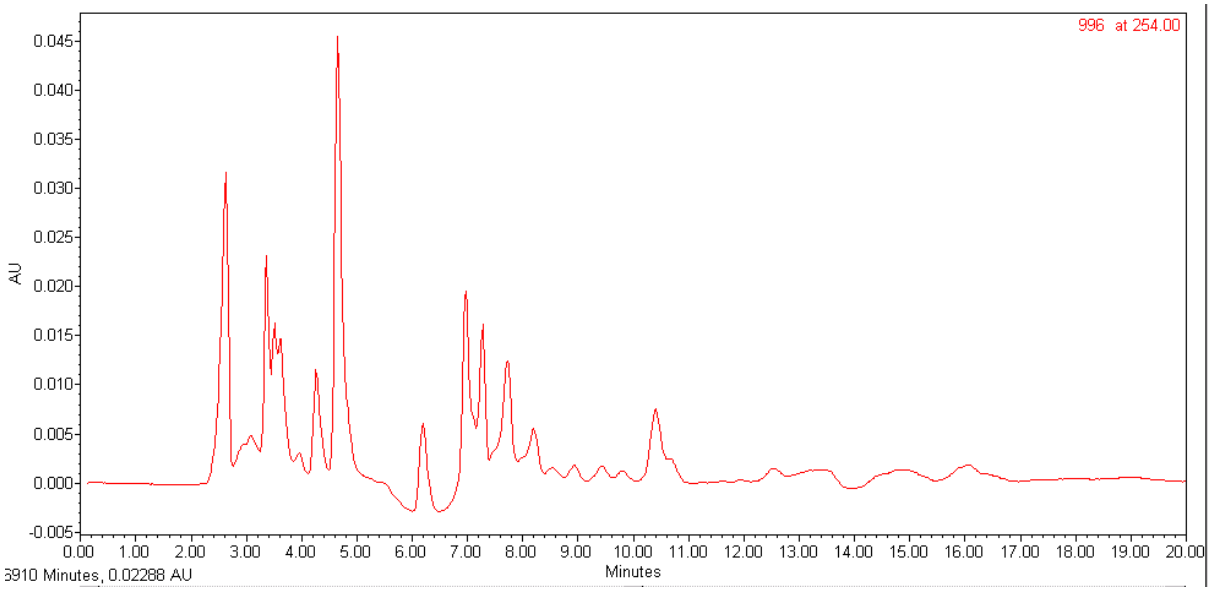


Figure 3.6.3 Chromatogram of control sample A containing DMSO and supernatant after 18.5 hours of incubation.

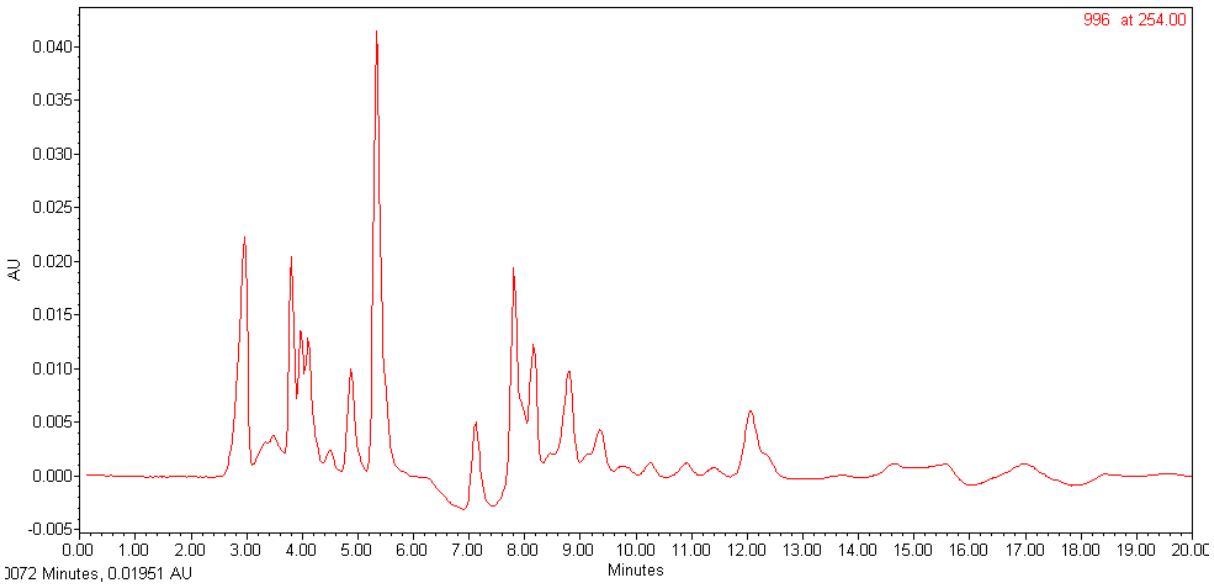


Figure 3.6.4 Chromatogram of control sample A containing DMSO and supernatant after 24 hours of incubation.

Similar observations were found in the Control B samples containing blueberry and supernatant [Figure 3.7.1-4].

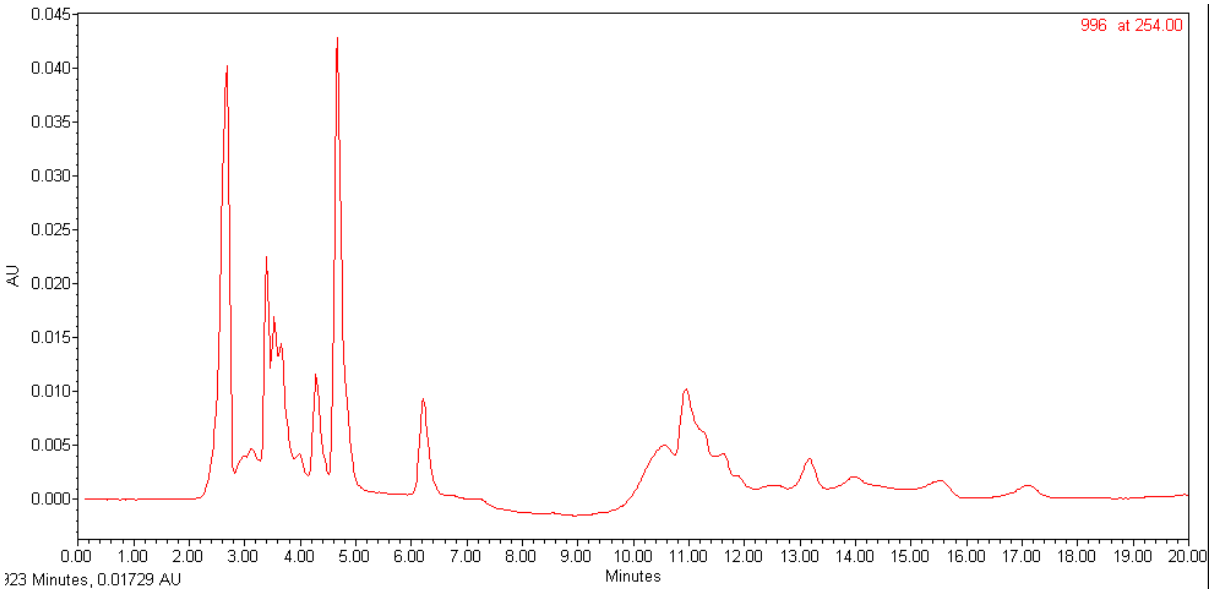


Figure 3.7.1 Chromatogram of control sample B containing blueberry and supernatant after 6 hours of incubation.

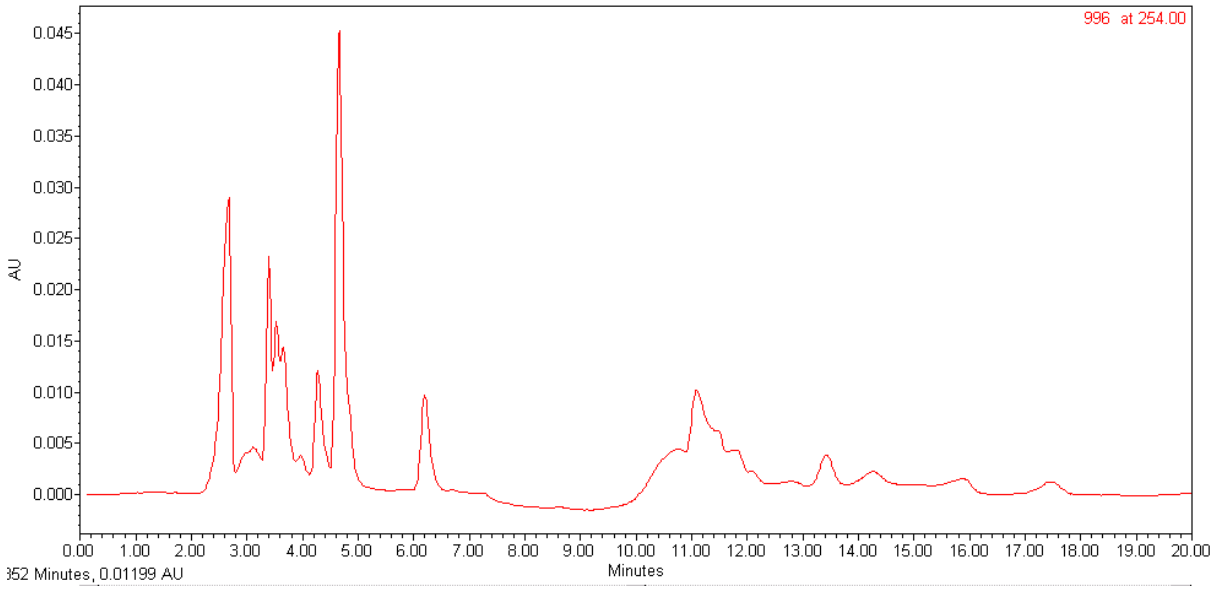


Figure 3.7.2 Chromatogram of control sample B containing blueberry and supernatant after 9 hours of incubation.

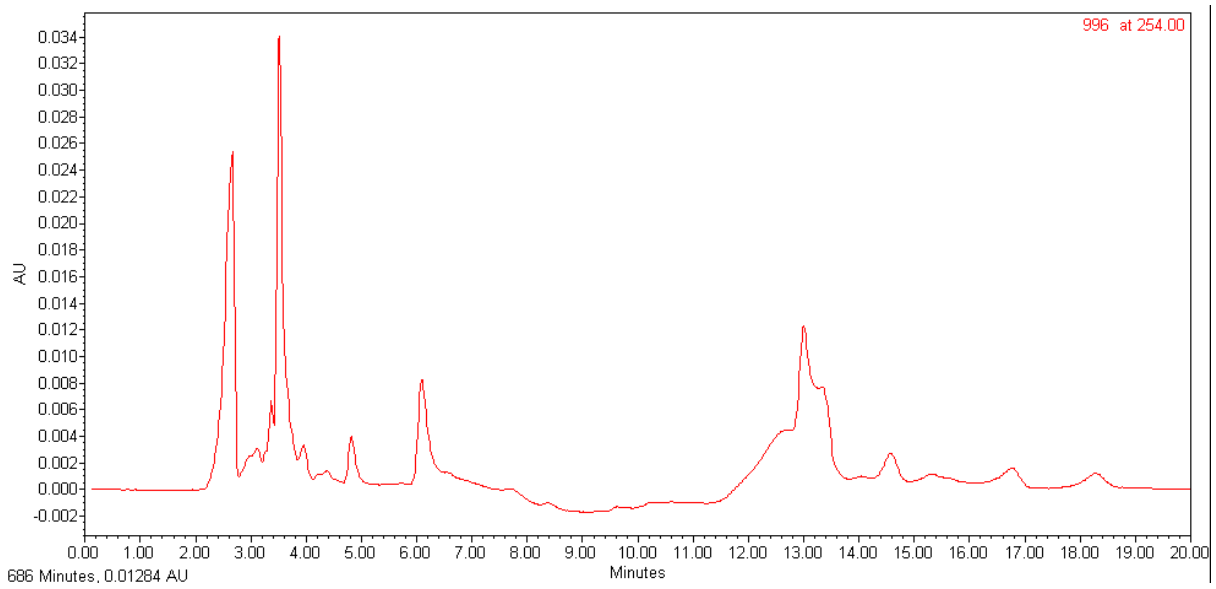


Figure 3.7.3 Chromatogram of control sample B containing blueberry and supernatant after 18.5 hours of incubation.

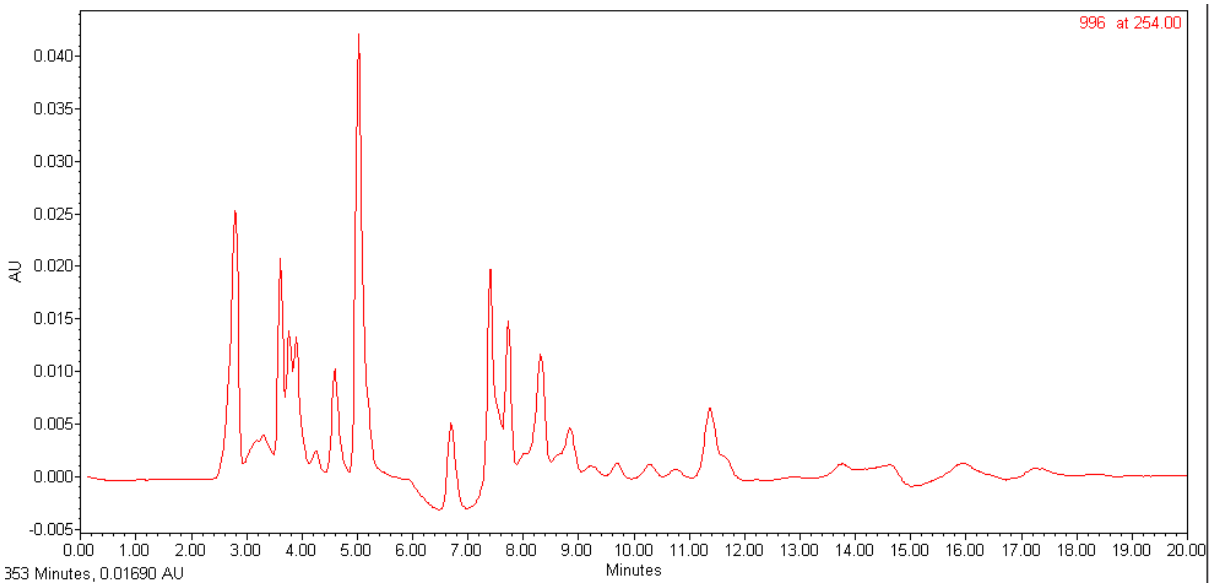


Figure 3.7.4 Chromatogram of control sample B containing blueberry and supernatant after 24 hours of incubation.

In the control samples C, containing *L. lactis* and supernatant, changes in the peaks were observed at 9 hours, 18.5 hours and 24 hours. The result showed that *L. lactis* does cause the biological degradation of the constituent of the medium [Figure 3.8.1-4].

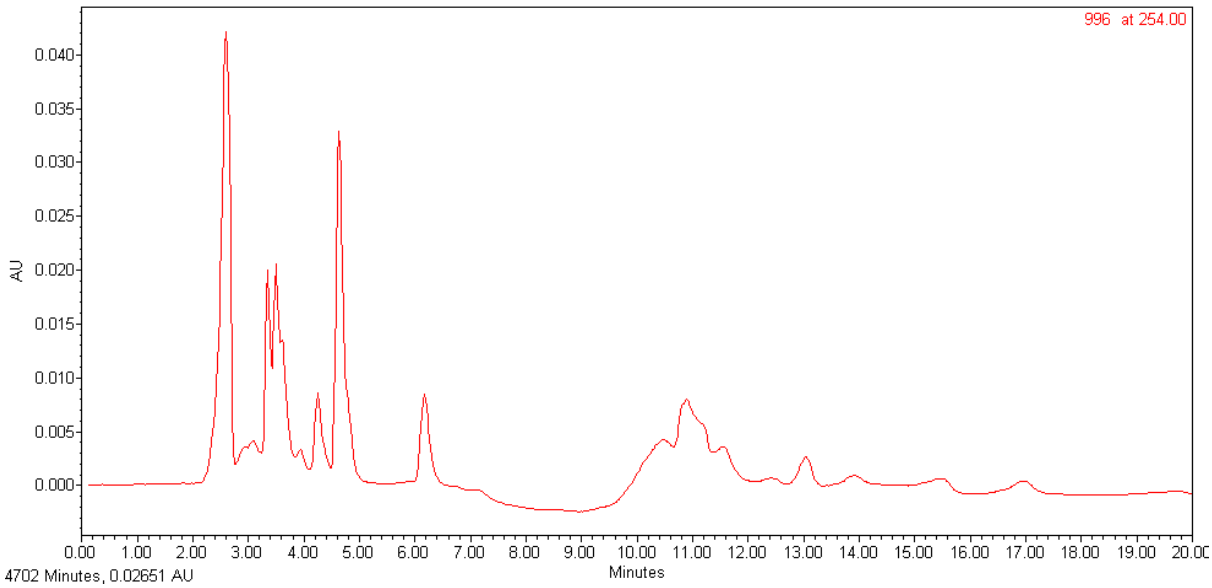


Figure 3.8.1 Chromatogram of control sample C containing *L. lacti* and supernatant after 6 hours of incubation.

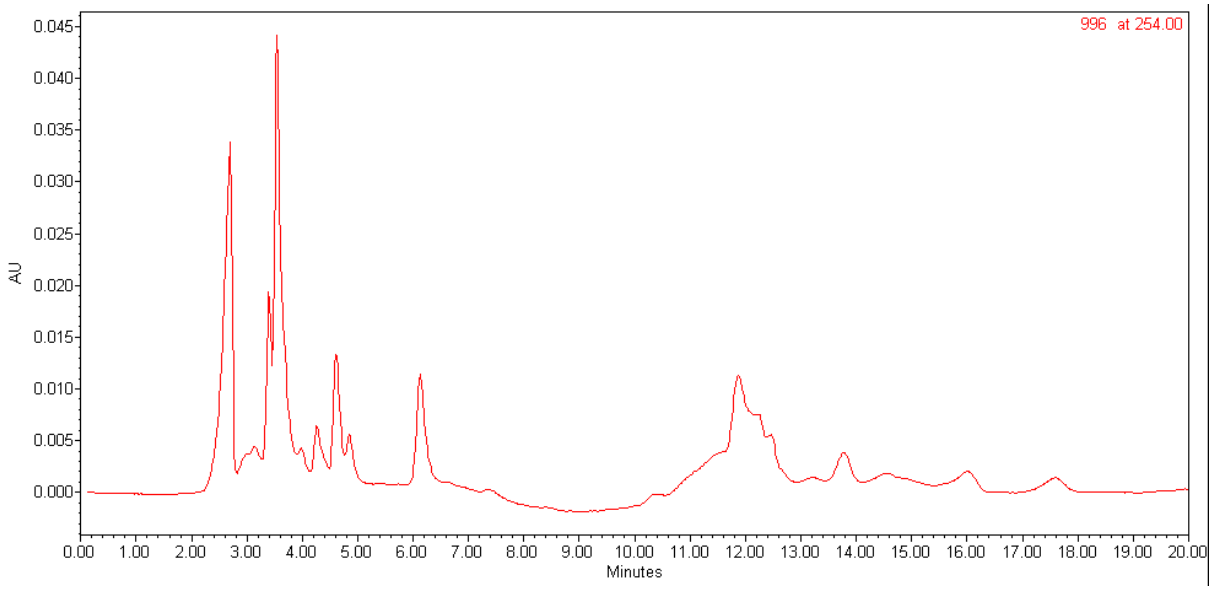


Figure 3.8.2 Chromatogram of control sample C containing *L. lacti* and supernatant after 9 hours of incubation.

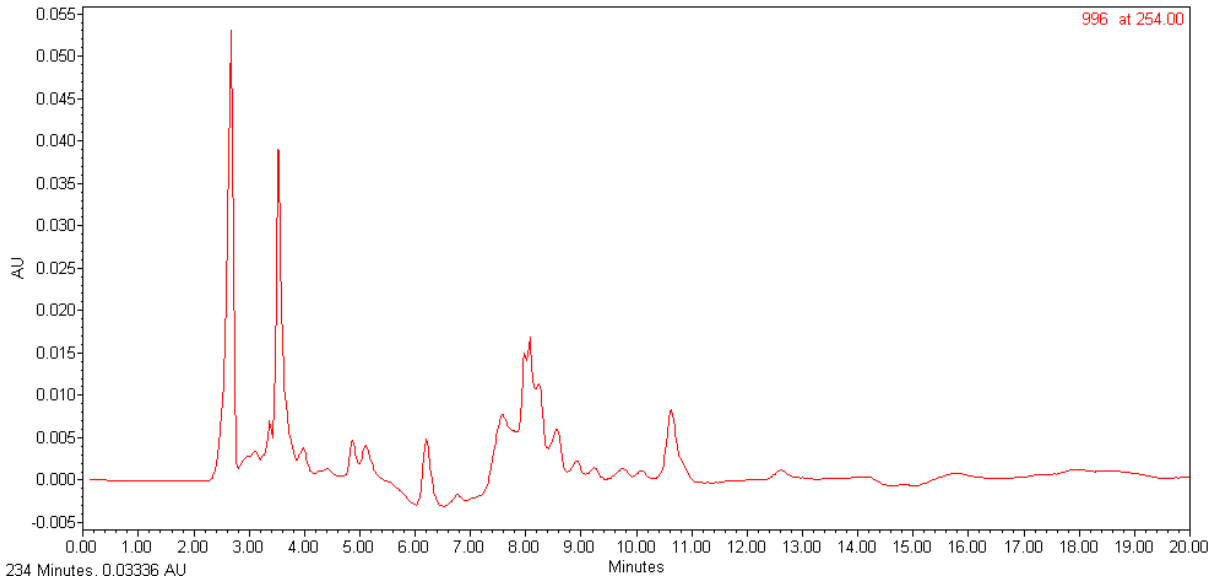


Figure 3.8.3 Chromatogram of control sample C containing *L. lacti* and supernatant after 18.5 hours of incubation.

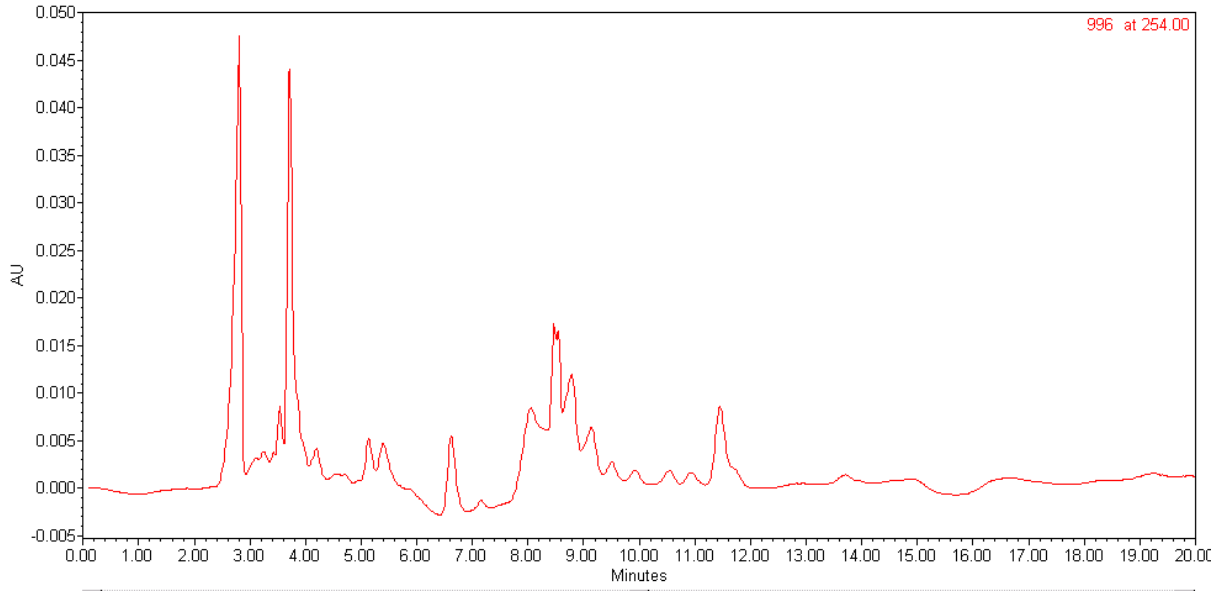


Figure 3.8.4 Chromatogram of control sample C containing *L. lacti* and supernatant after 24 hours of incubation.

In the test sample D, containing *L. lactis*, blueberry and supernatant, major changes in the peaks were observed at 9 hours, 18.5 hours and 24 hours. The result showed that *L. lactis* metabolize the blueberry constituents specially polyphenols [3.9.1-4].

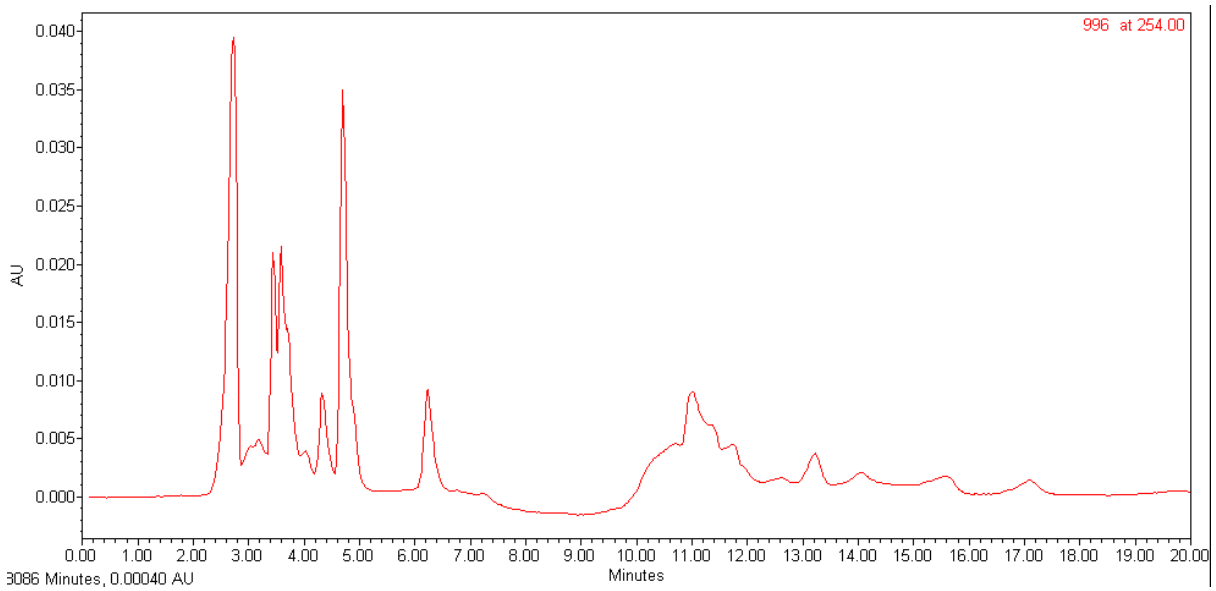


Figure 3.9.1 Chromatogram of test sample D containing *L. lacti*, blueberry and supernatant after 6 hours of incubation.

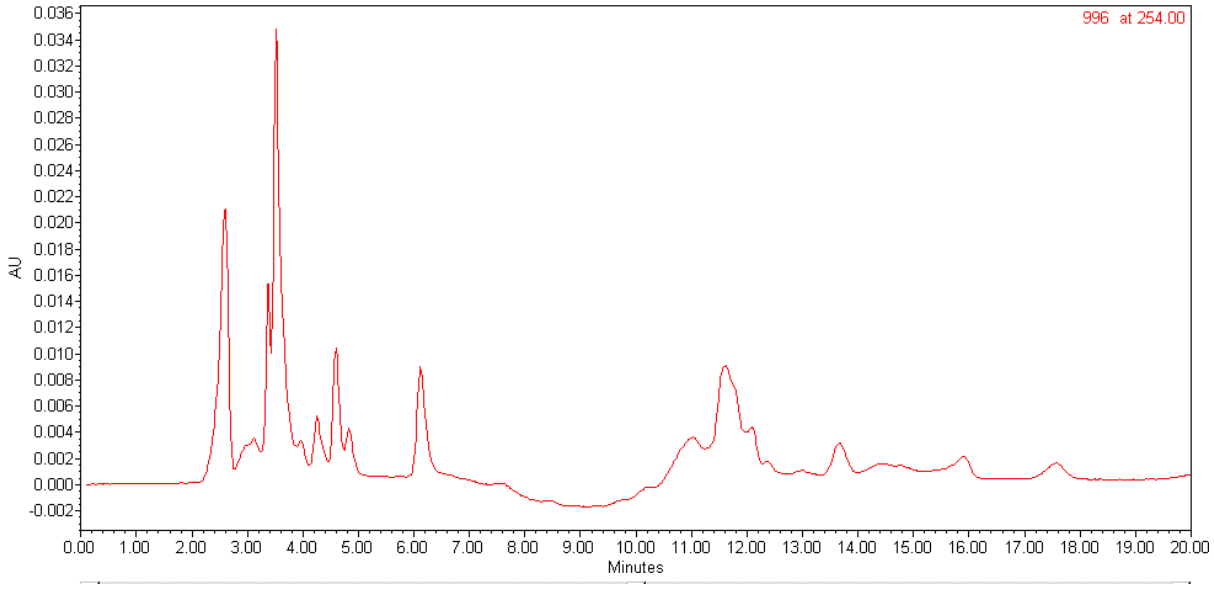


Figure 3.9.2 Chromatogram of test sample D containing *L. lacti*, blueberry and supernatant after 9 hours of incubation.

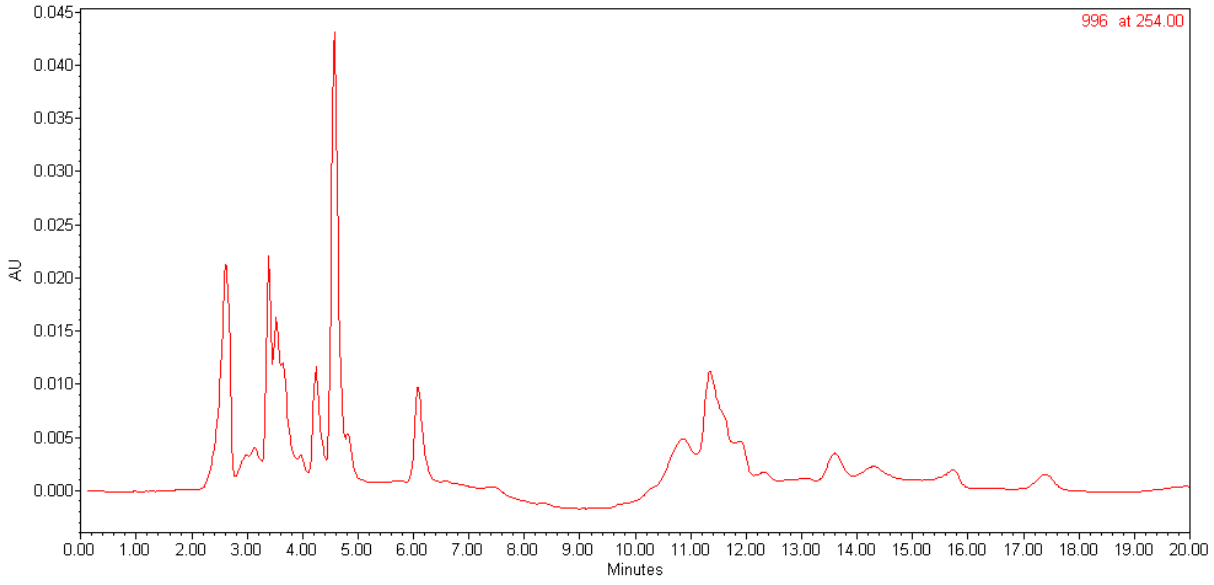


Figure 3.9.3 Chromatogram of test sample D containing *L. lacti*, blueberry and supernatant after 18.5 hours of incubation.

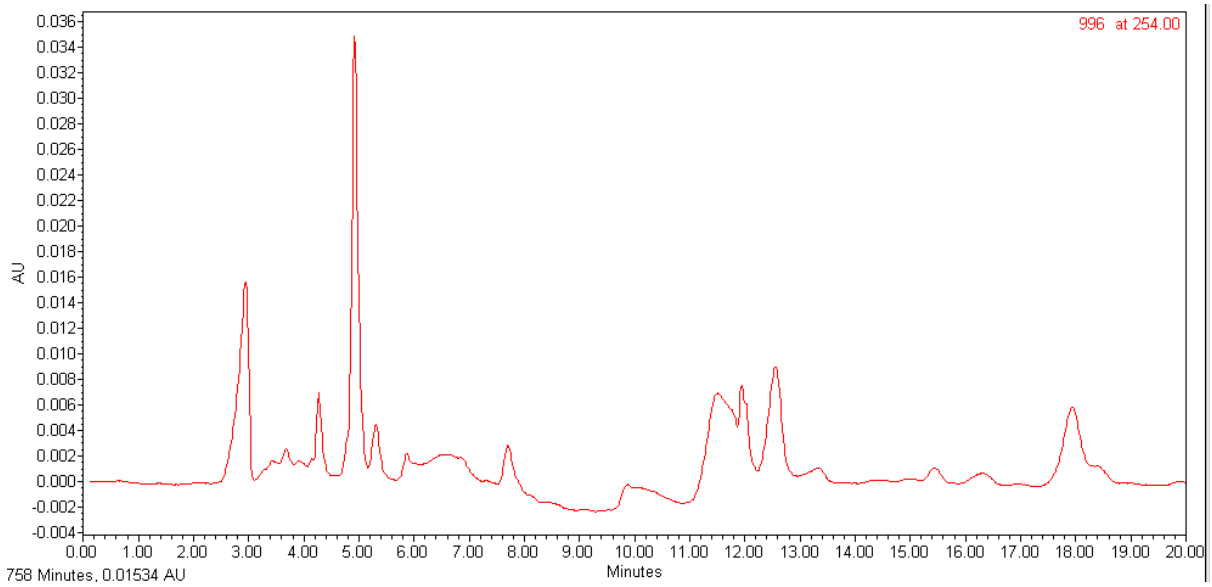


Figure 3.9.4 Chromatogram of test sample D containing *L. lacti*, blueberry and supernatant after 24 hours of incubation.

In the control samples E, containing *L. lactis*, DMSO and supernatant changes in the peaks were observed at 9 hours, 18.5 hours and 24 hours however these changes were less as compared to the test sample D. The result showed that *L. lactis* metabolize the blueberry constituents more than DMSO [3.10.1-4].

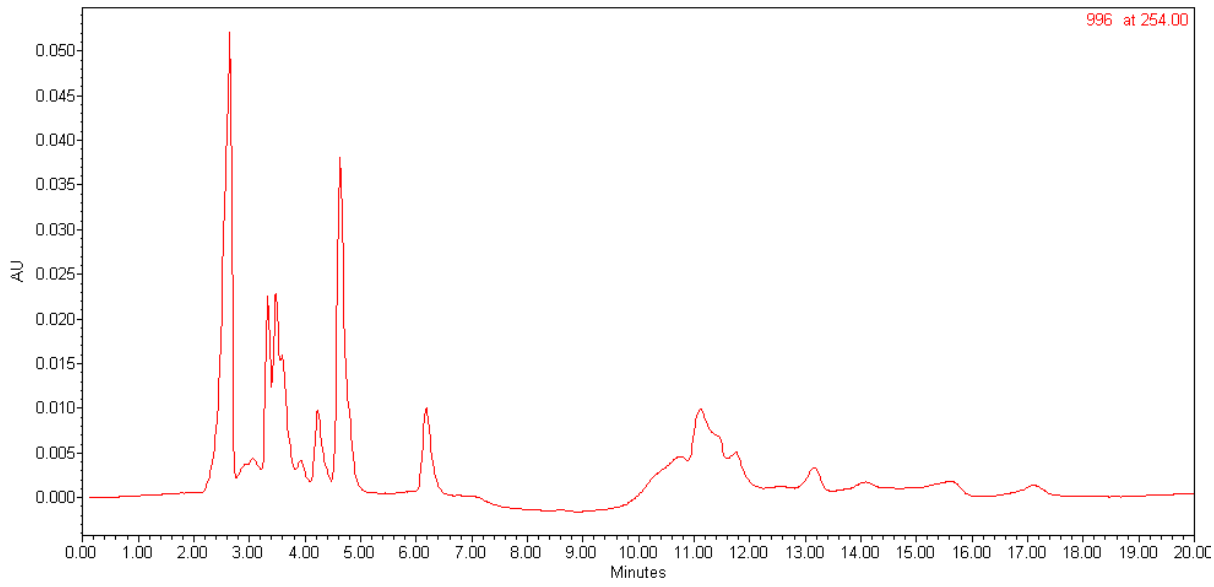


Figure 3.10.1 Chromatogram of control sample E containing *L. lacti*, DMSO,blueberry and supernatant after 6 hours of incubation.

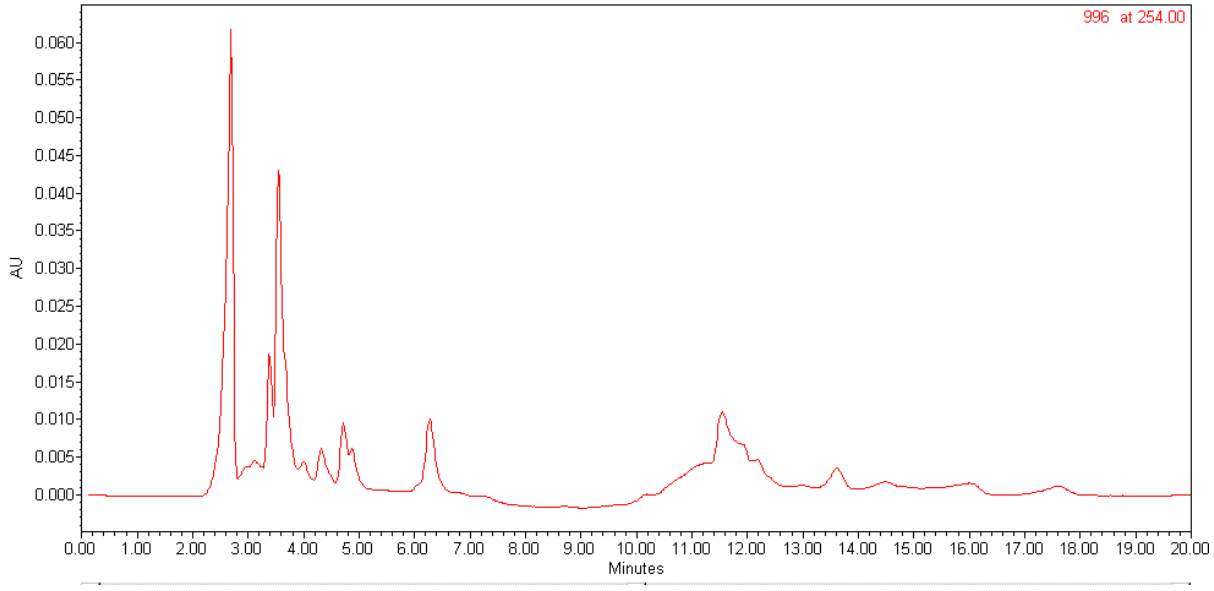


Figure 3.10.2 Chromatogram of control sample E containing *L. lacti*, DMSO,blueberry and supernatant after 9 hours of incubation.

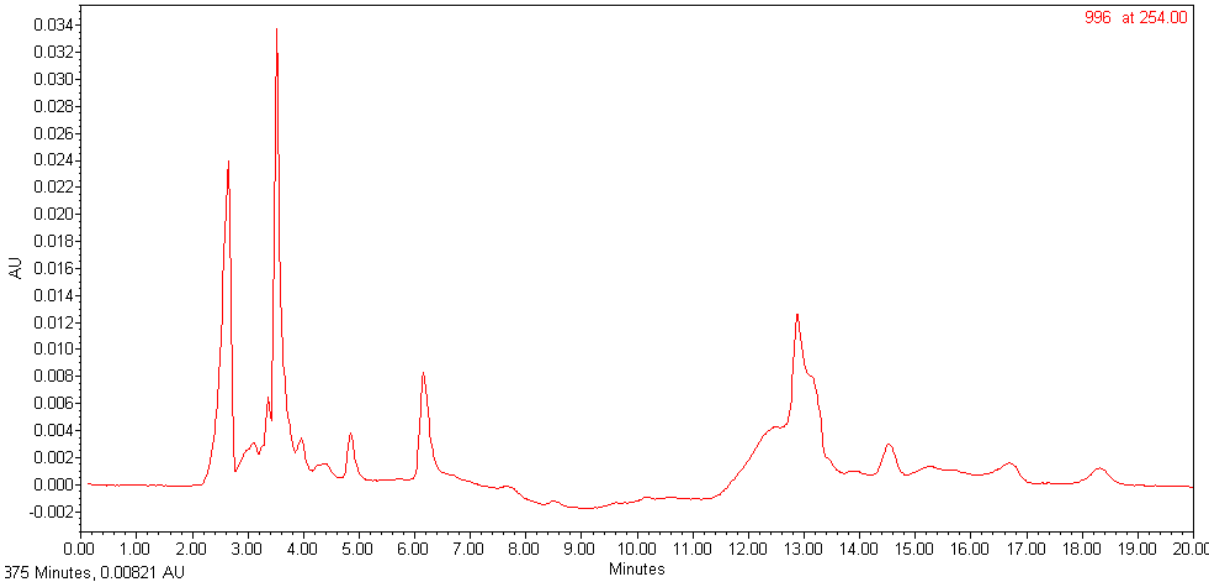


Figure 3.10.3 Chromatogram of control sample E containing *L. lacti*, DMSO,blueberry and supernatant after 18.5 hours of incubation.

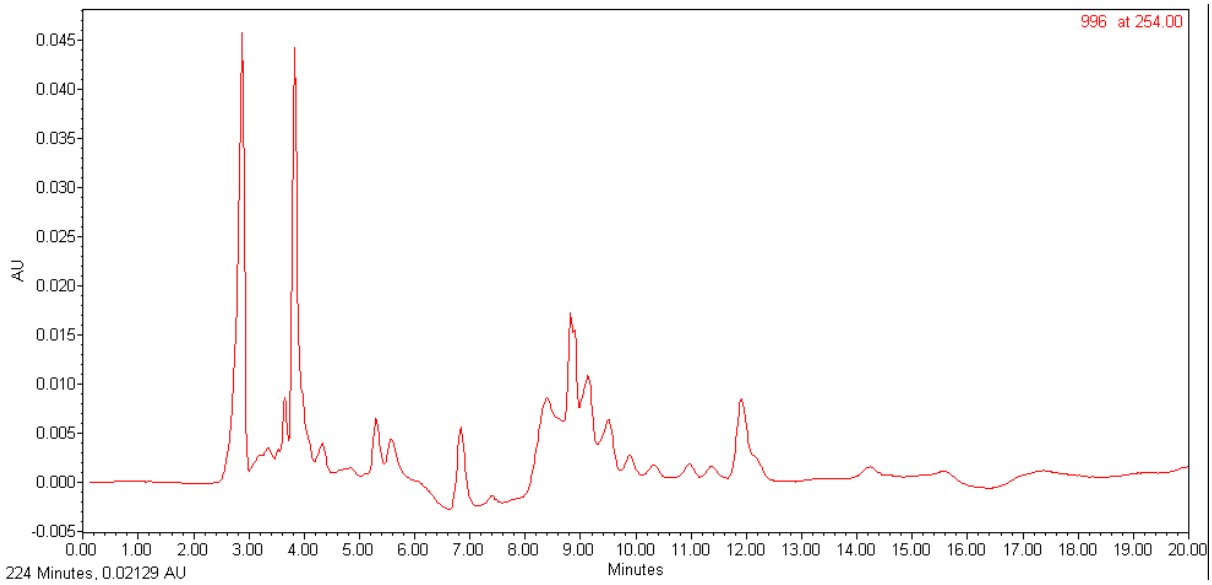


Figure 3.10.4 Chromatogram of control sample E containing *L. lacti*, DMSO,blueberry and supernatant after 18.5 hours of incubation.

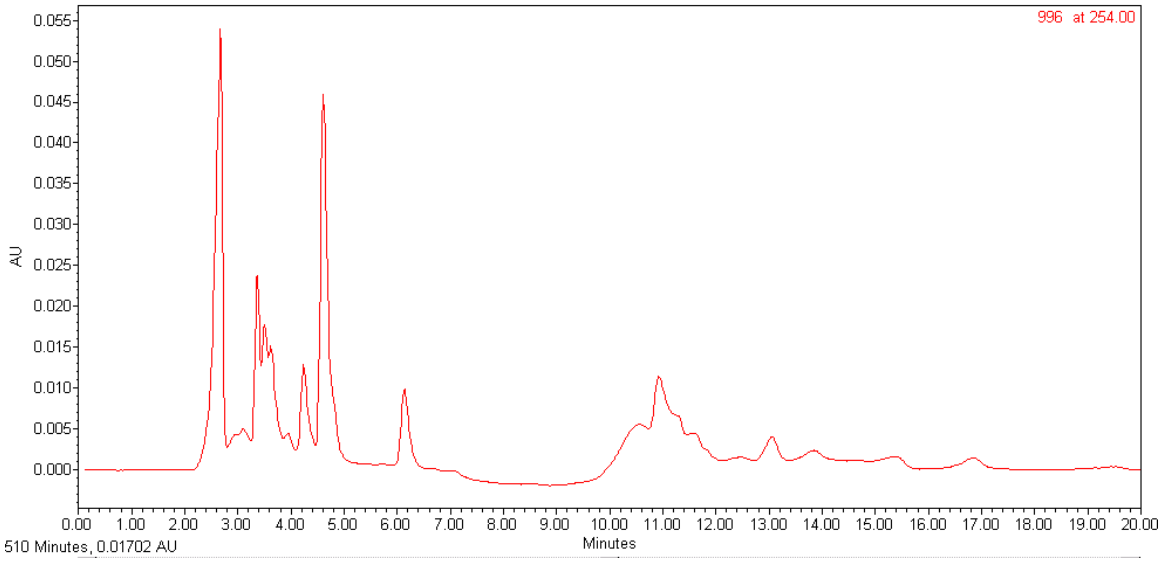


Figure 3.11 Chromatogram of blank sample.

Probiotic bacterial strains are known to metabolize polyphenols. The present study was focused on the use of *L. lactis* to metabolize blueberry constituents, specifically polyphenols. This was accomplished by quantifying the disappearance of blueberry polyphenols and appearance of polyphenol metabolites in medium by

HPLC generated fingerprints. Blueberry powder is a form of this common, popular fruit and is a suitable source of polyphenols, therefore it can be used to plan appropriate food intervention trials in humans. Intestinal *Bifidobacteria* and *Lactobacillus* numbers have been reported to increase significantly in both humans consuming a drink containing blueberry powder [63] and laboratory rats fed blueberry extract [64]. Blueberries have been found to exert beneficial effects on cardiovascular diseases, by reducing inflammation. This has been found useful in redcing oxidative stress as well as vasoconstriction. The variability has been found to be present across individuals in physiologic response to polyphenols [65]. Microbiota populations are known to vary across individuals, resulting in variability in metabolite production [66].

5. Conclusion

A HPLC-DAD method was successfully developed for the examination of major blueberry polyphenolic compounds metabolized by probiotic bacteria. The current chromatographic fingerprint method was rapid, reliable, effective and was suitable for quantitative evaluation and quantitative determination of potential blueberry polyphenols metabolites. By manipulating gut bacteria populations across humans, the response to polyphenol consumption might become more reproducible.

6. Prospects for future studies

The following are aims for future studies.

- To develop and validate a LC-MS and GC-MS method and to determine the structures of the polyphenolic compounds present in the blueberry sample.
- To carry out similar chromatographic fingerprinting studies similar to this study on other probiotic bacteria.
- To determine the dose regimen and dose response of the probiotics.
- To characterize the microbiota phylotypes associated with polyphenol metabolite production.

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