STUDIES ON SELECTIVE EXTRACTION AND PURIFICATION OF BIOACTIVE COMPOUNDS FROM KOKUM (*Garcinia indica*) FRUITS USING ALCOHOL BASED AQUEOUS TWO-PHASE SYSTEMS

Thesis

Submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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D E C L A R A T I O N

I hereby *declare* that the Research Thesis entitled **"Studies on selective extraction and purification of bioactive compounds from kokum (*Garcinia indica*) fruits using alcohol based aqueous two-phase systems"** which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfilment of the requirements for the award of the Degree of **Doctor of Philosophy** in the Department of Chemical Engineering, is a *bonafide report of the research work carried out by me*. The material contained in this Thesis has not been submitted to any University or Institution for the award of any degree.

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CERTIFICATE

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DEDICATED TO MY

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ABSTRACT

The bioactive compounds namely natural pigments and antioxidants are gaining high demand in the global market because of the increasing public awareness on the positive health benefits of these compounds due to their greater functional and bioactive properties and their potential applications in food, nutraceuticals, and cosmetic industries. The difficulty in the recovery from the complex natural sources and the instability due to the denaturation during the extraction and purification were inhibiting the application of these molecules in various industries. The substantial effort has been made to develop new technologies to extract and purify these bioactive compounds by retaining their native characteristics and stability. Kokum fruits (Garcinia indica) contain the important bioactive compounds like anthocyanin (ACNs), hydroxycitric acid (HCA), garcinol (GL), and isogarcinol (IGL) at a significant quantity. The simultaneous extraction of all these bioactive components in a crude extract is achieved using the aqueous mixtures of 1-propanol and ethanol as solvents. The aqueous two-phase extraction (ATPE) is explored for the first time to simultaneously partitioning the four bioactive compounds from the crude extract of kokum rinds. Alcohol-based aqueous two-phase systems (ATPSs) were proved to be suitable for the simultaneous partitioning during screening studies. The ethanol-ammonium sulphate and 1-propanol-ammonium sulphate/magnesium sulphate ATPSs are proved to be better for differential partitioning of GL and IGL into alcohol rich top phase and ACNs and HCA into the salt rich bottom phase. The effect of phase compositions and Tie Line Length (TLL) on the differential partitioning was investigated in terms of partitioning coefficient and extraction efficiency. The Response Surface Methodology (RSM) was adopted to optimize the process variables through desirability based multi-response optimization by considering the partitioning coefficients (K) and extraction efficiency (EE) of all the four bioactive compounds as responses. The ATPS consisting of 15.202 % (w/w) 1-propanol, 10.242 % (w/w) ammonium sulphate having the TLL of 28.505 % (w/w) at a crude load of 25 % (w/w) able to partition 97.39 % GL (K=370.770) and 92.38 % IGL (K=120.581) in the top phase and 99.19% ACNs (K=0.080) and 99.83% HCA (K=0.016) in the bottom phase with a purity higher than 99% by implementing the second stage ATPS. An attempt was further made to extract the bioactive compounds in the continuous extractor, Rotating Disc Contactor (RDC) with 1-propanol-

ammonium sulphate ATPS. The efficacy of RDC column was analysed by studying the dispersed phase holdup, mass transfer coefficient, and recovery and separation efficiency of the bioactive compounds at different operating conditions.

Keywords: anthocyanin, hydroxycitric acid garcinol, isogarcinol, aqueous two-phase extraction, simultaneous partitioning, rotating disc contactor

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LIST OF ABBREVIATIONS

a, b, c, d, e	Co-efficient of the equations
ACNs	Anthocyanins
ADI	Acceptable Daily Intake
ATPE	Aqueous Two-Phase Extraction
ATPS	Aqueous Two-Phase System
BSA	Bovine Serum Albumin
BHT	Butylated Hydroxytoluene
bw	body weight
CCD	Central Composite Design
EE	Extraction Efficiency
EFSA	European Food and Safety Authority
FDA	Food and Drug Administration
GI	*Garcinia Indica*
GL	Garcinol
HCA	Hydroxycitric acid
IGL	Isogarcinol
K	Partition coefficient
LLE	Liquid Liquid Extraction
mg	milligrams
mL	millilitre
P	Purity
PEG	Poly Ethylene Glycol
PI	Phase Inversion
R	Recovery
RDC	Rotating Disc Contactor

RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
RPM	Rotation Per Minute
RSM	Response Surface Methodology
SE	Separation Efficiency
SSE	Sum of Square Error
SLE	Solid-liquid extraction
STL	Slope of tie line
TFA	Tri-Fluoro Aceticacid
TLL	Tie Line Length

Symbols

ρ	Density (kgm^{-3})
μ	Viscosity of the liquid (mPas)
σ	Surface tension (mN/m)
EE_{HCA}	Extraction efficiency of hydroxycitric acid
EE_{ACN}	Extraction efficiency of anthocyanin
EE_{GL}	Extraction efficiency of garcinol
EE_{IGL}	Extraction efficiency of isogarcinol
H	Hold up
K_{HCA}	Partition coefficient hydroxycitric acid
K_{ACN}	Partition coefficient of anthocyanin
K_{GL}	Partition coefficient of garcinol
K_{IGL}	Partition coefficient of isogarcinol
K_Da	Volumetric Mass Transfer Coefficient
m^t/ m_t	Mass of top phase
m^b/ m_b	Mass of bottom phase
R^{bot}	Recovery in continuous phase/bottom phase
R^{top}	Recovery in dispersed phase/bottom phase
R_{Total}	Total Recovery
V_B	Volume of bottom phase

V_C	Continuous phase velocity
V_D	Dispersed phase velocity
V_T	Volume of top phase

Scripts

t	Top phase
b	Bottom phase

CHAPTER 1

1. INTRODUCTION

Since time immemorial to till the 1850s, natural products such as isoprenoids (carotenoids), alkaloids and flavonoids from plants, animals, and mineral sources have been used by humans as colourants, flavours, and fragrances (Patil 2007). Due to the scarce availability and high price of natural compounds, synthetic compounds came into the market and gained more importance in the food and pharmacy industries for the last few decades. These compounds are well established for reasons like purity, stability, low cost, and higher quality than natural compounds. With the growing demand in the market, numerous synthetic food additives like colourants were developed to be added to improve the quality and organoleptic characteristics of food products. The synthetic additives most commonly used are studied in terms of security, side effects, toxicity at short, medium, and long terms, as well as health impact. Their application in food products is currently allowed by Food and Drug Administration (FDA) and European Food and Safety Authority (EFSA), with already established acceptable daily intake (ADI) doses (Martins et al. 2016).

Despite the huge advantages of synthetic additives, some of them were banned over the period due to the evident side effects, toxicity, health impairment abilities, and carcinogenic effects (Carocho et al. 2014). Some of the artificial colourants gained a bad reputation due to the public awareness regarding the unreasonable usage, negative impacts on health (Rodriguez-Amaya 2016) due to the potential side-effect of the chemicals used in the synthesis and increased addition of hazards to the environment (Carocho et al. 2015). FD&C Red No. 2 (amaranth) a synthetic red colourant for foods has been banned in the United States since 1976 as a suspected carcinogen (FDA), 2012). The synthetic red food colourants like FD&C Red No. 3 (erythrosine B) and FD&C Red No. 40 (allura red) have also been avoided by the consumers (Kang et al. 2014). In 2007, the researchers from Southampton University and others reported the increased hyperactive behaviour of children due to the effect of certain artificial food colours when it is combined with the food preservative,

sodium benzoate (Arnold et al. 2012; McCann et al. 2007; Weiss 2008). Further, (EFSA) in the United Kingdom declared the ban on the azo-dyes, known by 'Southampton 6' colours including Sunset yellow FCF (E110), Quinoline yellow (E104), Carmoisine (E122), Allura red (E129), Tartrazine (E102), Ponceau 4R (E124) for the application in food products (Chapman 2011; EFSA 2009; Galaffu et al. 2015).

Although synthetic compounds have been ruled or favoured the industries, naturally derived products are in demand these days due to the huge side effects of the synthetic colourants and additive compounds to mankind. The consumer expectations were changed and the preference of addition of natural additives to foods and other stuff has increased in place of synthetic compounds (Martins et al. 2016). The natural compounds having the colouring properties with antioxidant and biological properties are gaining importance and popularity to replace the synthetic colourants. Accordingly, the food industries shifted their priority towards the usage of natural additives or colours in recent days, due to the reasons like (a) Increasing demand and consumer awareness for natural food in comparison to synthetic one; (b) Health-promoting properties of natural compounds; (c) Increasing demand for the low-fat content food formulations by replacing fats with natural thickeners or food additives. The food industries and scientists have been looking for natural molecules as food additives, which can deliver the colours, flavours, and fragrances along with health benefits and food preservative properties. However, most of the natural additives under research are antioxidants, antimicrobials, colourings, and sweeteners which were well reported (Carocho et al. 2015; Rodriguez-Amaya 2016; Solymosi et al. 2015). The global natural food colours market size was about 1.32 billion USD in 2015 and is likely to witness rapid growth about 10 to 15 % (6.22%, by revenue) over the period owing to high demand in food applications (Institute of Food Technologists 2016) mainly in soft drinks, confectionery and bakery goods, dairy and frozen products (Cortez et al. 2017). Besides, stringent regulations towards the use of synthetic and identical colours will also act as a major driver for this natural sector. Hence, the natural extracts which can able to fulfil the current requirement are

explored and new strategies for the extraction and purification from a variety of natural sources are in great demand.

1.1 BIOLOGICALLY ACTIVE COMPOUNDS FROM PLANTS

Plants are the valuable sources of a variety of chemical compounds that are traditionally used as the main source for the development of new drugs (e.g. steroids and alkaloids), functional foods (e.g. plant sterols and stanols as cholesterol-lowering ingredients in functional foods), and as food additives (e.g. natural flavour, aroma, and colour) (Azmir et al. 2013). These compounds are referred to as bioactive compounds due to their pharmacological or toxicological effects in humans and animals. The natural bioactive compounds are mainly classified as natural antioxidants, natural antimicrobials, natural colourants, and natural sweeteners (Carocho et al. 2015). These typical bioactive compounds are generally considered as secondary metabolites because they are produced in plants through different biological pathways in secondary metabolism processes (Bernhoft 2010). The main role of these compounds is to protect plants from unfavourable conditions during biotic or abiotic stress (Bubalo et al. 2018). These compounds can be categorised depending upon chemical groups, chemical classes, and their biochemical pathways which are mentioned as follows (Bernhoft 2010; Bubalo et al. 2018):

a) Glycosides (cardiac glycosides, cyanogenic glycosides, glucosinolates, saponins and anthraquinone glycosides- example: garcinol)
b) Phenolic compounds (phenolic and hydroxycinnamic acids, stilbenes, flavonoids, and anthocyanins)
c) Tannins (condensed tannins and hydrolysable tannins)
d) Terpenoids, phenylpropanoids, lignans, alkaloids, furocoumarins, and naphthodianthrones
e) Proteins and peptides

The bioactive compounds are playing an important and significant role in developing novel therapeutical agents and as food additives which have a profound effect on human health by both direct and indirect means and also possess immense medicinal properties (Karasawa and Mohan 2018).

1.1.1 Natural colourants

Natural pigments/colourants are the dyes obtained from living organisms such as plants, animals, fungi, and microorganisms, which are capable of colouring foods, cosmetics, drugs, etc. These natural colourants can be recovered from leaves, seeds, flowers, fruits, vegetables, and algae, part of animals, animal secretions, and microorganisms. Several pigments from natural sources were identified and accepted as convenient and safe substitutes for synthetic colourants. The fruits and vegetables are the potential sources of natural pigments as they contain four main types of pigments namely; the green chlorophylls, the yellow-orange-red carotenoids, the red-blue-purple anthocyanins and the red betalains (Rodriguez-Amaya 2016). Now a day's natural food colourants are considered to be as effective as those derived from chemical synthesis. They have the advantages and benefits of being safer, providing health benefits besides offering organoleptic features, exerting two or more benefits as food ingredients. Several of these food additives exerting colourant effects along with act as antioxidants, even as preservatives and contributing functional properties to food products. These properties of natural colourants motivated the modern health concern consumers likely to choose functional foods for their disease-preventing properties and risk-reducing or appearance-enhancing properties (Carocho et al. 2014; Rodriguez-Amaya 2016). In this sense, the natural food pigments/colourants, as well as other natural additives, are considered not only as organoleptic improvement agents but also enhancers of nutritional status and health promoters.

Further, proper regulatory practices and guidelines are still being developed and improved towards legislation to provide consumer's safety and better life quality for the usage of natural pigments. Currently, some of the natural pigments were approved and the E codes and ADI were established as; caramel, E150 (160-200 mg/kg b. w.); brilliant black, E151 (1 mg/kg b. w.); vegetable carbon, E153; brown, FK E154 and brown, HT E155 (0.15 mg/kg b. w.) (Martins et al. 2016). Similarly, the yeast-derived natural pigments like monascin - a secondary yellow natural pigment produced by the genus *Monascus* spp. are under spot-light to be used as food additives having several biological activities, such as anti-cancer, anti-inflammatory, anti-diabetic, and anticholesterolemic effects have been reported (Wang et al. 2015).

However, the majority of naturally-occurring food colourants added to the foods namely chlorophylls and chlorophyll derivatives (E140 and E141), carotenoids (annatto and α-, β-, and γ- carotene; E160, E161), anthocyanins (E163) and beet colourants- betalains (E162), which are considered as four main classes of plant pigments, have been studied (Cortez et al. 2017; FDA 2020; Solymosi et al. 2015). The other phenolic compounds, carminic acid, and some curcuminoids, particularly curcumins were also being studied as food additives.

Anthocyanins (ACNs) are the most widely studied natural food colourants, being obtained from flowers, fruits, leaves, and even whole plants. Commercial ACNs, namely cyanidin 3-glucoside, pelargonidin 3-glucoside, and peonidin 3-glucoside have been used as food colourants and studied their effectiveness. The ACNs colour may vary from red to purple and blue because of their structure and the influence of pH and even the other factors like temperature, light, oxygen, the presence of other flavonoids, stress conditions, and even storage conditions (Castañeda-Ovando et al. 2009; Rodriguez-Amaya 2016). The utilization of ACNs as food colourants and functional ingredients is improving day by day. The ACNs present in the grape colour extract and grape skin extract has been listed in the list of FDA's colour additives (FDA 2020). The lower stability and interaction with other compounds in the food matrix should be addressed to improve their utilization in the food formulation/application.

1.1.2 Natural antioxidants

Antioxidants are one among the bioactive compounds which can prevent or inhibit oxidation processes, even at relatively small concentration and thus have a diverse physiological role in food products and the human body. The prevention of oxidation of molecules is achieved by donating a hydrogen atom or an electron by the reduction of antioxidant and becomes the radical form. But contrary to other radicals, antioxidants are stable when they are in the radical form and do not allow further reactions to take place (Carocho et al. 2014). These antioxidants act as radical scavengers or chain-breaking antioxidants, which help in converting the radicals to less reactive species; chelators, that bind to metals and prevent them from initiating

radical formation; quenchers, which deactivate high-energy oxidant species; oxygen scavengers, that remove oxygen from systems, avoiding their destabilization; and the antioxidant regenerators, that regenerate other antioxidants when these become radicalized (Carocho et al. 2015). The natural antioxidants are present in almost all the edible plant parts and products.

The antioxidants are used for extending the shelf life of foods by reducing the common types of oxidation like lipid peroxidation and rancidification, which are known to destroy foods. The antioxidant supplementation is a generally accepted method to prolong the stability and storage life of food products mainly those containing fats. Some commonly used antioxidants are ascorbic acid (E300), sodium ascorbate (E301), calcium ascorbate (E302), fatty acid esters of ascorbic acid (E304), tocopherols (E306), α-tocopherol (E307), γ-tocopherol (E308), δ-tocopherol(E309), lecithins (E322), sodium lactate (E325), potassium lactacte (E326), calcium lactate (E327), citric acid (E330), sodium citrate (E331), potassium citrate (E 332), calcium citrate (E333), tartaric acid (E334), sodium tartrate (E335), potassium tartrate (E336), sodium potassium tartrate (E337), sodium malate (E350), potassium malate (E351), calcium malate (E 352), calcium tartrate (E354), and tri-ammonium citrate (E380). However, the artificial compounds with antioxidant properties, like butylated hydroxyanisole (BHA, E320; ADI 0.5 mg/kg bw) and butylated hydroxytoluene (BHT, E321; ADI 0.05 mg/kg bw), propyl gallate, (PG, E310; ADI 1.4 mg/kg bw) (BHT) have been used for limited food applications due to their potential carcinogenicity (Carocho et al. 2014; Carocho and Ferreira 2013).

The growing demand for natural antioxidants was observed in the food and cosmetic industries, which forces the search for new sources of these compounds. Fruits and vegetables and their by-products and wastes are very rich in bioactive components, which are considered to have a beneficial effect on human health. The spread of free radicals which are eventually led to a stroke, heart attack, arthritis, vision problems, Parkinson’s disease, Alzheimer’s disease, and various types of cancer may be scavenged with the intake of fruits and vegetables which are rich in bioactive compounds, specifically antioxidants. The protective effect of fruits and vegetables has been attributed to their bioactive antioxidant constituents, including

vitamins, carotenoids, polyphenols, flavonoids, benzophenones, and lactones. The most important activities of these bioactive compounds include antidiabetic, anticancer, antihypertensive, neuroprotective, anti-inflammatory, antioxidant, antimicrobial, antiviral, stimulation of the immune system, cell detoxification, cholesterol synthesis, anticonvulsant and their ability to lower blood pressure (Karasawa and Mohan 2018).

From the last decade, efforts have been made to effectively extract and purify the natural antioxidants and other bioactive compounds from the fruits and vegetables and their wastes, which may be effectively used in the food and pharmaceutical industries. The important antioxidants like vitamin C (ascorbic acid), vitamin E, carotenoids namely beta-carotene, lycopene, lutein; flavonoids like anthocyanins and quercetin, cysteine Selenium; polyphenol antioxidants like glutathione, peroxidase and benzophenones namely garcinol (GL) and iso-garcinol (IGL) are the focus of research these days (Karasawa and Mohan 2018; Schobert and Biersack 2019).

1.2 *GARCINIA INDICA* (GI)

Garcinia indica Choisy, popularly known as kokum, belongs to the family Guttiferae and slow-growing slender tree with drooping branches with a height of 16–18 m, are distributed throughout the tropical Asian, African and Polynesian countries and have tremendous potential as a colourant or spice with medicinal value (Chandran 1996). It is found in India in the tropical humid evergreen rainforests of the Western Ghats of south India as well as in the north-eastern states of India. The tree grows extensively in the Konkan region of Ratnagiri district of Maharashtra, Goa, Uttara Kannada, coastal areas of Karnataka in Udupi and Dakshina Kannada Districts and Kasaragod area of Kerala, evergreen forests of Assam, Khasi, Janita hills, West Bengal and Gujarat (Swami et al. 2014). It is known by various names across India including Bindin, Biran, Bhirand, Bhinda, Kokum, Katambi, Panarpuli, Ratamba, or Amsol. It is the highly underutilized tree that is found to grow in forest lands, riversides, wasteland, and grown in home gardens. But, it also gets cultivated on a limited scale as a rain fed crop, usually mixed with other fruit trees in the Western Ghats region. Within India, 108 ha to 1200 ha was used to grow the kokum trees in

Maharashtra/Goa/Karnataka region, which produces about 10,200 metric tonnes with a productivity of 8.50 tons/ha (Ghosh 2015; Nayak et al. 2010a; Swami et al. 2014).

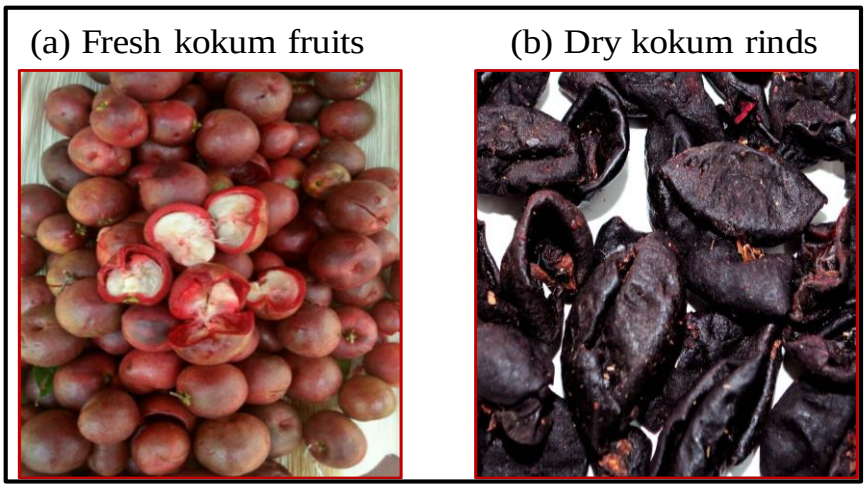


Figure 1.1 The figure showing (a) Kokum fresh fruits, (b) Kokum dried rinds

The ripen kokum fruit is in the colour of either dark purple or red-tinged with yellow. It contains 3–8 large seeds embedded in a red acid pulp, in a regular pattern like orange segments, in the white pulpy material (Figure 1.1). The shape of fruit varies from round to oval and it weighs around 21-85g. The expected shelf life of this fruit is about one week. Sun drying is a commonly used method to preserve the fruit and it takes around 6-8 days for complete drying. The rind and seed of fruit kokum have many applications such as culinary, foods, fruit drinks, and pharmaceuticals. It is also called an Indian spice with a pleasant acceptable flavour and has a sweet acidic (sour) taste which makes it a popular food- additive. It is traditionally used as an acidulant in many Indian dishes. The kokum rinds are commercially used to prepare concentrated syrups which on appropriate dilution gives the ready to use cool health drinks, especially during the off-season periods. The kokum fruit extract with high intense colour is directly used as an additive in food industries. The fruits are used in making health beverages, fruit extracts, seed oil, kokum butter or squash, and jellies like products that have the number of food, pharmaceutical and cosmetic applications (Nayak et al. 2010b). This crop is gaining importance these days as its fruits have various utilities ranging from the pharmaceutical uses to high-quality beverages. Kokum has been reported for the treatment of dysentery, heart complaints, stomach

acidity, and liver disorders (Bhaskaran and Mehta 2007; Krishnamurthy et al. 1982; Mishra et al. 2006).

1.3 BIOACTIVE COMPOUNDS IN KOKUM FRUIT

This fruit consists of three important bio-active components like 'Anthocyanin (ACN)', which has a great potential as a natural colourant and antioxidant, 'Hydroxycitric acid (HCA)' - major acid and used as an anti-obesity ingredient in pharmaceutical products and 'Garcinol (GL) and Isogarcinol (IGL)' a fat-soluble yellow pigment, which is a potent antioxidant and has a chelating activity. The composition of kokum fruit is given in Table 1.1. Even though these bioactive compounds have huge application potential and market demand, the extraction and purification on large scale are very less exploited.

Table 1.1 Chemical Constituents of the fresh rind of kokum (Krishnamurthy et al. 1982; Nayak et al. 2010a)

Sr. No.	Character	Value
1	Moisture (g/100g)	70- 76
2	Protein (N X 6.25) %	1.00
3	Crude Fat (%)	1.40
4	Crude Fibre (%)	1.48
5	Total Ash %	2.6
6	Total sugars (%)	4.10
7	Starch (%)	1.00
8	Pigments (%)	2.40
9	Tannin (%)	1.70
10	Pectin (%)	0.90
11	Ascorbic Acid (%)	0.02
12	Acid (as Hydroxyl Citric acid) %	5.90
13	Garcinol (%)	1.5

Kokum is a rich source of ACNs which contains about 2 to 3 % of red colour pigment and was first reported by Krishnamurthy and co-workers (Krishnamurthy et al. 1982). Phytochemical studies have shown that the kokum rind contains the highest

concentration of ACNs (2.4 g/100 g of fresh kokum fruit) when compared with any other natural sources (Nayak et al. 2010a; b). Two types of ACNs namely, cyanidin-3-glucoside and cyanidin-3-sambubioside which are usually present in the ratio of 4:1 in kokum (Nayak et al. 2010b). HCA is another important bio-active compound found in the kokum rinds. The fruit rinds showed the presence of about 10.3-12.7 % (w/w) of HCA along with minor quantities of HCA lactone and citric acid (Jayaprakasha and Sakariah 2002; Nayak et al. 2010a). *Garcinia indica* contains two polyisoprenylated phenolics, garcinol ($C_{38}H_{50}O_6$) and its colourless isomer isogarcinol. GL is a major constituent of fruit rind of kokum which resembles the known antioxidant curcumin (Yamaguchi et al. 2000a). The quantity of these compounds present is about 1.5 wt. % (Nayak et al. 2010a).

1.3.1 Anthocyanins (ACNs)

In the group of flavonoids, the ACNs are the most important pigments which have potential uses and health benefits (Khoo et al. 2017). ACNs have been used as food and beverage colourants (Aguilera et al. 2016; Chung et al. 2016) mainly in preparations of cream cheese, fermented milk, milkshakes, low-pH beverages (Kitts and Tomiuk 2013; de Mejia et al. 2015) and also in solid food matrices such as pancakes and omelets (Pineda-Vadillo et al. 2017). ACNs are also well known for their antioxidant and antimicrobial potential (Barnes et al. 2009; Muniyandi et al. 2019) which aid in the stabilization of foodstuffs and increase their shelf life. Furthermore, they also provide some health benefits by preventing the oxidative damage and providing biological benefits in human nutrition, food and medical applications (Carvalho et al. 2015; Kong et al. 2003) as anti-inflammatory, antimutagenic, anti-tumoral, anti-microbial, antiulcer activities (Carvalho et al. 2015; Decendit et al. 2013; Muniyandi et al. 2019), improved platelet function (Alvarez-Suarez et al. 2014), stabilization of blood sugar levels (Kong et al. 2003; Törrönen et al. 2012), reduction of liver lesions (Chen et al. 2014a, 2015; Morrison et al. 2015), etc. Hence, ACN molecules were considered for the prevention of neuronal and cardiovascular illnesses, cancer treatment, and diabetes (Konczak and Zhang 2004). Recently, eleven anthocyanin-rich plant extracts including red and purple grape, purple sweet potato, purple carrot, black and purple bean, black lentil, black peanut,

sorghum, black rice, and blue wheat were tested for anti-proliferative effect on human colon cancer cells. These extracts decrease the expression of anti-apoptotic proteins (survivin, cIAP-2, XIAP), induced apoptosis, and arrested cells in GI (Mazewski et al. 2018). On the other hand, topical effects are discussed when unabsorbed molecules protect the gastrointestinal mucosa against oxidative injury thereby delaying the onset of stomach, colon, or rectal cancer (Stintzing and Carle 2004). These qualities make ACNs an attractive alternative to synthetic dyes.

1.3.2 Hydroxycitric acid (HCA)

Hydroxycitric acid (HCA) (1, 2 dihydroxypropane-1, 2, 3-tricarboxylic acid), a derivative of citric acid is used as an antiobesity ingredient in pharmaceutical industries. HCA, both a free acid and lactone forms are present in the fruit rind of GI (Jena et al. 2002b; Yamada et al. 2007). It is also the only known anorectic agent, found as a natural constituent of edible foods consumed by humans. Due to its presence in high amounts in Garcinia species, it is also called as garcinia acid. HCA has reported having an inhibitory effect on ATP-citrate lyase (EC 4.1.3.8 an important enzyme in Kreb's Cycle) which is needed for the conversion of carbohydrates into fat. HCA limits the availability of acetyl–CoA, which plays a critical role in energy storage as fat (Jena et al. 2002b; a). It enhances glycogen synthesis in the liver. HCA has been well studied for its unique regulatory effect on the fatty acid synthesis, lipogenesis, appetite, and weight loss. As it has been shown to significantly reduce the body weight and lower lipid accumulation (Yamada et al. 2007), HCA has been patented for the use as a hypocholesterolemic agent. HCA is also used as an ingredient in anti-obesity formulations and the derivatives of HCA have been incorporated into a wide range of pharmaceutical preparations for enhancing weight loss, cardioprotection, and endurance in exercise.

1.3.3 Garcinol (GL) and Isogarcinol (IGL)

GL is a lipid-soluble superoxide anion scavenger, having both chelating activity and free radical scavenging activity. GL has the equivalent antioxidant capacity to that of ascorbic acid and having the ability to protect from diseases associated with oxidative stress (Kolodziejczyk et al. 2009). IGL is the isomeric form

of GL found associated with it in kokum, which has similar biological properties as GL. The GL and IGL have a promising diverse range of pharmacological activities like anti-oxidative, antiglycation, anti-inflammatory, antibiotic, anti-cancer, anti-bacterial, anti-ulcer and free radical scavenging activities (Chatterjee et al. 2003; Liu et al. 2015; Oike et al. 2012; Sang et al. 2001; Tang et al. 2013; Tsai et al. 2014; Yamaguchi et al. 2000b). GL can play an important role in the treatment of gastric ulcers caused by the hydroxyl radicals or chronic infection with *Helicobacter pylori* and it can be a viable alternative to Clarithromycin for treating H. *pylori* infection (Chatterjee et al. 2003). GL has antibacterial activity comparable to that of the antibiotic vancomycin against Methicillin-resistant Staphylococcus aureus (Rukachaisirikul et al. 2005). It also effective in blocking the formation of azoxymethane-induced colonic aberrant crypt foci and induces apoptosis in human HL 60 cancer cells (Pan et al. 2001; Tanaka et al. 2000). Another study has further demonstrated that cigarette smoke extract-induced COX-2 expression is blocked by GL pre-treatment (Yang et al. 2009). Similarly, IGL was also claimed to be an anti-inflammatory, antitumor, a lipase inhibitor, an antiobesity, and an antiulcer agent (Sang et al. 2001).

1.4 EXTRACTION AND PURIFICATION OF BIOACTIVE COMPOUNDS FROM PLANT SOURCES

Bioactive compounds from plant materials generally extracted by various classical extraction techniques involving the application of heat and/or mixing and the efficiency of the extraction is based on the extracting power of different solvents used in the process. Soxhlet extraction, Maceration, and Hydrodistillation are the existing classical laboratory scale techniques employed to obtain bioactive compounds from plants (Azmir et al. 2013). Solvent extraction is the most common method for the extraction of diverse bioactive compounds found in leaves, fruits, and vegetables. The phenolic compounds have been extracted by grinding, drying or lyophilizing the fruits, or only by soaking fresh fruits in the solvent and subsequent extraction. Solid-liquid extraction (SLE) is used to recover most hydrophilic components from plant tissues as their polarity facilitates the dissolution into polar solvents like methanol, ethanol, acetone, and water. The acidified solutions of methanol, ethanol, acetone,

water, and a mixture of acetone/methanol/water are traditionally used to extract ACNs. Non-polar solvents like hexane, benzene, and surfactants are used to extract fat/lipid-soluble compounds. However, these classical methodologies of extraction provide a better yield; but have the disadvantages of being time-consuming and labour-intensive, a larger quantity of solvent consumption and wastage, lower product extractability, and selectivity (Garcia-Ayuso et al. 1998).

Some improved techniques namely ultrasound-assisted extraction, pulsed electric field, enzyme assisted extraction, microwave heating, ohmic heating, supercritical fluid extraction (Heffels et al. 2015; Ongkowijoyo et al. 2018) and pressure accelerated solvents were used to enhance the overall yield and selectivity of bioactive compounds from plant materials as non-conventional methods. Some of these techniques are also considered as ‘‘green techniques’’ (Azmir et al. 2013; Bubalo et al. 2018) and have advantages of more effective mixing, faster energy transfer, reduced thermal gradients and extraction temperature, selective extraction, reduced equipment size, increased production and eliminates process steps. Further, conventional separation and purification methods like centrifugation, precipitation, electrophoresis, coagulation, filtration, flocculation, and membrane separation are exploited to concentrate and subsequent purification of bioactive compounds from complex plant sources, which are often associated with the difficulties/ problems such as more number of operational stages, losing biological activity, minimal selectivity, difficulty in handling, high purification cost, lower feasibility for scale-up and many other intrinsic and extrinsic inhibiting properties (Nagaraja and Iyyaswami 2015; Rosa et al. 2009).

Conventional solvent extractions and their improved techniques have been employed to recover the various bioactive compounds from kokum rinds. Even the solvents are chosen to selectively extract the ACNs, considerable quantities of other flavonoids and biomolecules which have similar properties get extracted from the natural resources along with ACNs. The removal of impurities stands as an important step, as they remain detrimental for their usage in the food industries and even in the analytical methods (Castañeda-Ovando et al. 2009; He and Giusti 2011). The crude extracts with impurities were usually subjected to the standalone purification methods

or the methods integrated with the extraction processes to obtain pure ACNs. The tedious procedures for the recovery of biological products have a major impact on the final product cost as separation and purification accounts for a major fraction (70–80%) of the total bioprocess cost (Nadar et al. 2017). The stability of the ACNs during the food processing and nutraceutical applications was maintained by using the pure ACNs molecules obtained from the purification of ACNs by eliminating the other impurities from the crude extract (Jampani et al. 2014; Wen et al. 2016).

1.5 LIQUID-LIQUID EXTRACTION

Liquid-liquid extraction (LLE) is a useful method for the simultaneous extraction and isolation of chemical and biological components from complex sources. The selectivity and the efficiency of the LLE are improved by modifying the conventional LLE method. The modified or advanced extraction systems being studied the most today for the purification of biological components from complex sources are rewrapped packages of old principles (Raghavarao et al. 2003). The advanced liquid-liquid extraction methods including cloud point extraction (CPE), aqueous two-phase extraction (ATPE), micellar and reverse micellar extraction (RME), and extractions using thermo separating polymers are studied and applied for the separation and purification of biological components. The diverse physical and chemical properties of the multiple phases of the systems, ease in solubilizing the normally hydrophobic and insoluble species, non-requirement of the use of volatile organic compounds for phase formation and their reliance on the structuring properties of liquid water for forming the multiphase and also their higher solubilizing power for a wide variety of biological solutes are the few advantages associated with the advanced liquid-liquid extraction methods (Raghavarao et al. 2003, Daniel et al. 2013). The solubilizing power and anisotropy make them suitable for solvent extraction while the non-requirement of volatile organic compounds for forming phases makes them useful for environmentally benign techniques (Huddleston et al. 1999).

1.6 AQUEOUS TWO-PHASE EXTRACTION

Aqueous two-phase extraction (ATPE) is similar to and more than a traditional method of Liquid-Liquid Extraction (LLE) and known as one of the downstream processing techniques, which is highly exploited in biotechnology/ bioprocessing industry. It is an interesting purification alternative as the early processing steps can be combined into a single operation since the selective separation is a key for this process. The ATPE phenomenon was first observed by the Dutch microbiologist Martinus Willem Beijerinck in 1896, who initially, found the formation of aqueous two-phase polymeric systems composed of agar, gelatin, and water in a certain concentration range. Later it is well established by Albertsson in the 1950s, applied successfully for the isolation, separation, and partition of various biomolecules such as nucleic acids, animal and plant cell organelles (Albertsson 1958).

Aqueous Two-Phase Systems (ATPS) is formed by mixing the two incompatible polymers (dextran/polyethylene glycol) or a polymer/salt combination (polypropylene glycol/ $(NH_4)_2SO_4$, $MgSO_4$, KCl or KCH_3CO_2 (Zhao et al. 2011) above a threshold concentration, which is known as ‘‘traditional systems’’. This system helps to differentially partition the required/desired product and the contaminants/debris into opposite phases. ATPS is a unique unit operation that encloses many operations like clarification, concentration, and partial purification (Gupta et al. 2002). ATPE has been recognized as a superior and versatile technique among the various downstream processes for the purification of proteins, enzymes, metabolites, recombinant products, flavour compounds, natural pigments from the fermentation broth, biological extracts and industrial effluents (Goja et al. 2013). The ATPE has the potential to purify the valuable compounds in the pilot plant and also the industrial scale (Raghavarao et al. 1998). The schematic diagram of the aqueous two-phase system formed from polymer-polymer or polymer-salt and related phase diagram for separation of the desired product is shown in Figure 1.2.

PEG–dextran and PEG-salt ATPS has been widely reported until now. However, Polymer/salt systems are considered as an efficient and cost-effective system in comparison to polymer/polymer systems (Rosa et al. 2010). Further

polymer/ salt systems are more preferred than the polymer/ polymer system as they offer lower viscosity, low chemical cost, and high phase separation rate (Raghavarao et al. 1995, 1998) which is highly important to operate the ATPE in scaled-up units. Because of this PEG-salt, ATPSs were extensively used for separation and purification of various biomolecules including continuous studies in extraction columns. The difficulty in recycling the phase forming components after the recovery of the partitioned solutes and separating the targets from the polymer-rich phase is restricting the application of ATPE in the industrial scale (Goja et al. 2013).

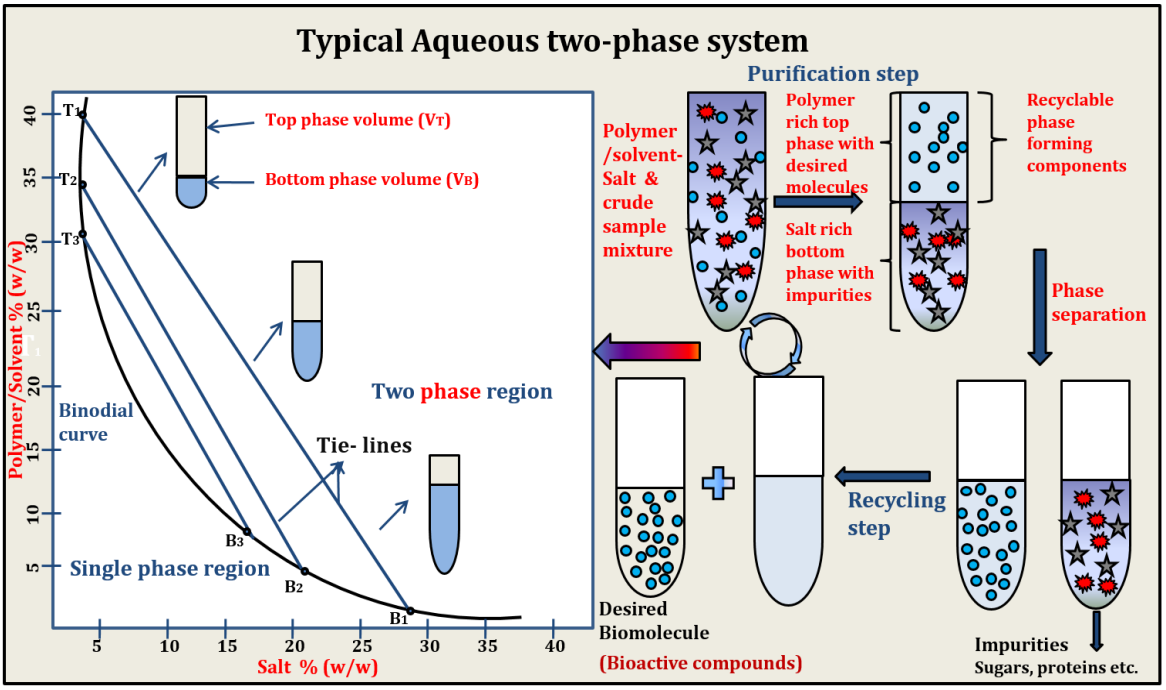


Figure 1.2 A graphical representation of a typical aqueous two-phase system

In recent times, other compounds have been successfully used as the replacement of traditional constituents such as the pairs alcohol–salt (Reis et al. 2014; Wang et al. 2011), ionic liquid–salt (McQueen and Lai 2019), ionic liquid–polymer (Pereira et al. 2013), and ionic liquid–carbohydrate (Freire et al. 2011) and polymer-starch to overcome the disadvantages of the traditional ATPE. Even ATPS based on acetonitrile-sugars and acetonitrile - polyols have been also reported (De Brito Cardoso et al. 2013, 2014). These are highly suitable for less concentrated samples and high-value compounds like recombinant products, pharmaceutical, aromatic compounds, and enzymes.

1.6.1 Alcohol based ATPS

As an alternative short-chain alcohol/inorganic salt systems have been explored these days as a suitable ATPS to purify the bioactive compounds (Jiang et al. 2009). Water-miscible alcohol–salt systems are suitable for the partitioning of hydrophilic and hydrophobic compounds because of their chemical and physical properties associated with their structure. Further, these alcohol-based ATPS are relatively cheaper than any other ATPS like PEG-salt, ionic liquid–salt, ionic liquid–polymer, and ionic liquid–carbohydrate. The short-chain alcohols like ethanol, 1-propanol, and 2-propanol could form stable ATPE system with inorganic phosphate and sulphate salts because of the salting-out effect and the low solubility of inorganic salt in alcohols (Peters 1987). The top phase will be rich in alcohol and the bottom phase in salts with 80% or more water content giving low interfacial tension (Liu et al. 2013a). Being a low-cost ATPS, these systems showed the advantages of giving good resolution with high yield and can solve major scale-up issues reported because of their low viscosity nature, high extraction efficiencies and allows easy recovery of the alcohol by evaporation or distillation (Liu et al. 2013a; Reis et al. 2014).

1.7 CONTINUOUS AQUEOUS TWO-PHASE EXTRACTION METHODS

The adaptability of ATPE for the recovery of various bioactive compounds and their increased demand in the biotechnology field made the researchers to develop the continuous extractors for the implementation of ATPE in the continuous extraction process. The aqueous two-phase extraction systems are similar to that of the conventional aqueous – organic extractors due to which liquid-liquid extraction (LLE) became a basis for the implementation of continuous extractors in ATPS. In the conventional LLE process, one of the phases is dispersed as small droplets within the other phase; column extractors are conventionally employed for the continuous mode of extraction. The column extractors, specifically the agitated contactors are exploited for the ATPE. The continuous ATPE process will render many advantages such as lower cost, high mass transfer, process automation, and continuous recycling of phase forming components (Iqbal et al. 2016; Nadar et al. 2017). The type of the extractor used for a specific application depends upon factors like the number of theoretical stages or transfer units required, residence time, production rate, tolerance properties

such as ease of cleaning, materials of column construction, as well as the capability to handle a range of physical properties of fluids (interfacial tension, density, and viscosity). The column geometry, operating parameters (rotor speed and phase flow rates), the relative capital and operating costs of the extractor unit are also parameters to be considered during fabrication or selecting the type of extractor.

The different types of conventional LLE equipment were utilised for use in ATPS (Espitia-Saloma et al. 2014), such as spray column (Jafarabad et al. 1992b), Packed bed column (Igarashi et al. 2004a), york-scheibel (Jafarabad et al. 1992a), Graesser raining bucket contactor (Giraldo-Zuniga et al. 2006), sieve plate column (Igarashi et al. 2004b), perforated rotating disc contactor (PRDC) (Cavalcanti et al. 2008; Porto et al. 2010; Sarubbo et al. 2003) and rotating disc contactor (RDC) (Kalaichelvi and Murugesan 1998; Kalaivani and Regupathi 2016) and others. PRDC and RDC have been experimented and documented as a more commonly used extractor for ATPE due to its greater efficiency and enhanced operational flexibility. RDC has several advantages over other conventional extractors, such as simplicity in construction, high throughput, and relatively low power consumption.

1.7.1 Rotating disc contactor (RDC)

The knowledge of phase formation and separation behaviour that includes droplet formation and drops size as a function of systems physical properties including the type of phase component and their composition, density, viscosity and interfacial tension and operational variables are required to develop the advanced continuous extractors for the betterment of industrial applications. This should overcome the economic barriers of ATPS towards the continuous extraction of bioactive compounds from different sources. The RDC is highly suitable for intermediate and high throughputs because of the economies they offer concerning agitation energy, floor space, and inventory. The rotating element of the RDC provides better contact between the two phases for efficient mass transfer. The performance of the RDC is improved by the action of rotating discs, which disturbs the drop motion and thereby increases the interfacial mass transfer by size reduction of the drops. Operational variables such as disperse and continuous phase flow rate

and rotor speed affect the hold-up capacity of the column and mass transfer coefficient which in turn defines the efficiency of the column. The RDC was used for the conventional LLE processes in the petrochemical industry like furfural extraction of lube oil, extraction of oxygen compounds from fruit juices with alcohol, propane deasphalting, phenol recovery, extraction of caprolactam and aromatics. However, it was implemented in the last decade for the ATPE of bovine serum albumin (Porto et al. 2000), proteins (Sarubbo et al. 2003), and α-lactalbumin from whey (Kalaivani and Regupathi 2016). The performance of RDC depends on column height to diameter ratio, rotor disc and stator ring diameter, compartment height, number of compartments, disc rotation speed, and phase flow rates. The fundamental hydrodynamic and mass transfer aspects like phase formation and separation and the extraction behaviours are mainly depending on the physical properties of the system (molecule size of PEG, composition, density, viscosity, and interfacial tension), and operational variables (agitation and phase flow rates) of the operation. Advances in these aspects may help to overcome the economic challenges of ATPS towards the application of RDC for the continuous extraction of bioactive compounds. Finally, the development of a suitable continuous ATPE will represent an attractive, alternative platform for the commercial adoption of continuous ATPE.

CHAPTER 2

2. LITERATURE REVIEW

2.1 ANTHOCYANINS (ACNs)

Nowadays, there is a sweeping demand for polyphenols and flavonoids due to their positive impacts on health by preventing cardiovascular, inflammatory, and neurological diseases (Silva et al. 2007). Anthocyanins are pigments, the name came from Greek i.e. (anthos = flower and cyan = blue), falls under the group of flavonoids that have been most widely studied for a long time (Valls et al. 2009). These pigments are found in many plant parts and fruits as they are responsible for the shiny orange, pink, red, violet, and blue colours of the leaves, petals, flowers, and fruits of some plants. Their presence is higher in fruits like red berries, cherries, pomegranates, plums, kokum, and also in vegetables like eggplants, red cabbage, purple corn or potatoes (Table 2.1). The ACNs are parts of the vascular plants. They are known to be harmless and can be easily mixed into the water which makes them interesting for their use as water-soluble colourants (Castañeda-Ovando et al. 2009; Pazmino-Duraan et al. 2001). They are consumed in diets for a long time and used by the food industry to produce juices, soft drinks, alcoholic beverages, and other products.

2.1.1 Physical and chemical properties of ACNs

ACNs belong to the flavonoids group and characterized by the presence of C_6-C_3-C_6 (phenyl 2-benzopyrilium) carbon skeleton consisting of two aromatic rings linked by an aliphatic three-carbon chain. Anthocyanins are made up of anthocyanidins as a basic structure which consists an aromatic ring called [A] bonded to a heterocyclic ring [C] that contains oxygen, which is also bonded by a carbon-carbon bond to a third aromatic ring [B] finally attached to sugar molecules (Figure 2.1a) (Konczak and Zhang 2004). Various types of ACN are found in nature depending upon the number of hydroxylated groups (-OH), methyl groups attached (-OCH_3), the nature and type of sugars attached, the aliphatic or aromatic carboxylates bonded to the sugar molecule and the position of these bonds (Kong et al. 2003). The

differences in functional groups at R_1 and R_2 position was noticed for different ACNs, which are also having different colours and wavelength.

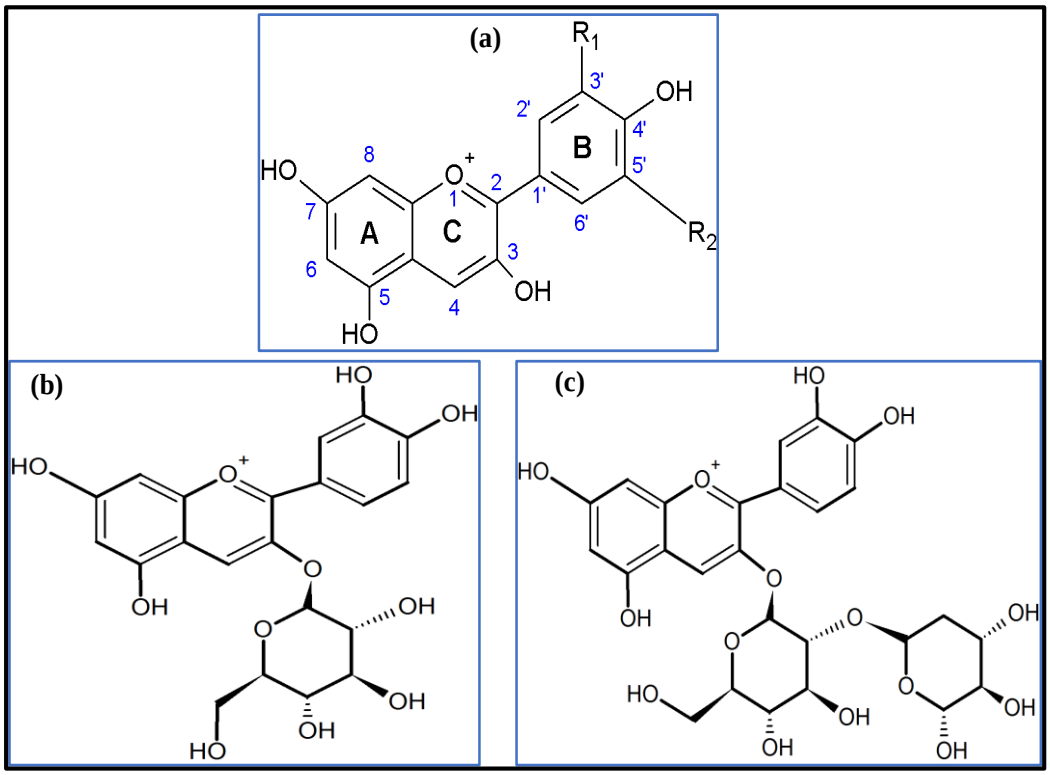


Figure 2.1 The chemical structures of ACNs, (a) general anthocyanin structure; (b) cyanidin-3-glucoside; and (c) cyanidin-3-sambubioside present in kokum rinds (*Garcinia indica*) (Castañeda-Ovando et al. 2009; Jamila et al. 2016; Nayak et al. 2010b)

The main six varieties of ACNs are the most common in vascular plants namely Pelargonidin (Pg), Peonidin (Pn), Cyanidin (Cy), Malvidin (Mv), Petunidin (Pt) and Delphinidin (Dp). The distribution of these ACNs in fruits and vegetables is Cy 50%, Dp 12%, Pg 12%, Pn 12%, Pt 7%, and Mv 7%. Further, different types of ACNs are formed depending upon attachment of monosaccharides, as a single (monosides) or in combination (biosides and triosides) mainly linked at C-3, C-5, C-7, C-3', C-4' and C-5' positions (example: 3-O-glycosides and 3, 5-di-O-glycosides) (Ignat et al. 2011; Li et al. 2012). Some of them are also found acylated by gallic, ferulic, caffeic, sinapic, p-hydroxybenzoic, p-coumaric acids, and also by aliphatic acids such as acetic, malonic, malic, oxalic, tartaric and succinic acids (Li et al. 2012). The three non-methylated ACNs (Cy, Dp and Pg) with sugar moieties are the most

found in plant parts in the ratio of 80% of pigmented leaves, 69% in fruits, and 50% in flowers (Castañeda-Ovando et al. 2009; Kong et al. 2003). As per the literature about more than 500 types of ACNs were identified by various researchers (Castañeda-Ovando et al. 2009; Kong et al. 2003). Among them, cyanidin-3-glucoside, Peonidin-3-glucoside, Delphinidin 3-glucoside, Malvidin 3-glucoside, Petunidin-3-glucoside, Cyanidin-3-rutinoside, Pelargonidin 3-rutinoside, Malvidin 3,5-diglucoside, Cyanidin 3,5-diglucoside are most common ACNs found in natural sources. The structure of ACNs influences their chemical properties, which have important implications for their stability, aqueous equilibrium, colour, co-pigmentation effects, reactivity and antioxidant and biological properties (Welch et al. 2009).

2.1.2 Sources and their composition of ACNs

ACN compositions differ from one source to another source and even the concentration may vary for each cultivar batch due to the cultivar condition or in a different part of plants such as flower, root, fruits, or leaves. Genotypic differences among different species and varieties will influence ACN synthesis and their accumulation including sugar accumulation and hormone levels in fruit maturation and development. Environmental factors and management practices like light, temperature, moisture, hormone treatments, and root restriction also affect ACN production. The overall composition and levels of produced anthocyanins depend on the interaction between the genotype, environment, and management practices (Zhang et al. 2016). Different sources and types of fruits, vegetables, flowers, grains were screened for ACN content, and concentrations were determined and well reported in the literature (Nayak et al. 2010a; Wu et al. 2006) which are shown in Table 2.1.

Table 2.1 List of anthocyanins/anthocyanidins quantified from different sources

Food	Total ACN (mg/100g of fresh weight)	Type of anthocyanins/anthocyanidins	References
Apple (*Malus pumila* P. Mill)	12-13	Cyanidin-3-glucoside Peonidin-3-glucoside	(Nayak et al. 2010a; Wu et al. 2006)

Rubus fruits *R. jamaicensis (blackberry), R. rosifolius (red raspberry), and R. racemosus and R acuminatus (black Raspberry and R. idaeus (red and yellow raspberry).*	68-2199	Cyanidin-3-glucosylrutinoside, Cyanidin-3-glucoside, Cyanidin-3-rutinoside, Pelargonidin-3-glucoside, Pelargonidin 3-rutinoside Cyanidin-3-glucosylmalonate	(Bowen-Forbes et al. 2010; McGhie et al. 2001)
Black raspberry (*Rubus occidentalis* L)	145-687	Cyanidin Pelargonidin	(McGhie et al. 2001; Nayak et al. 2010a; Wang and Lin 2000)
Berry Family Highbush blueberry, red huckleberry *(Vaccinium corymbosum* L, *Vaccinium angustifolium* Aiton, *Vaccinium virgatum* Aiton, *Vaccinium parvifolium* Smith,	34-515	Cyanidin-3-glucoside Peonidin-3-glucoside Delphinidin 3-monoglucoside Malvidin 3-monoglucoside Petunidin-3-monoglucoside	(Moyer et al. 2002; Prior et al. 1998)
Chagalapoli fruit *(Ardisia compressa K.)*	796.0 ± 2.3 mg	Malvidin-3-O-galactoside (35%) Delphinidin 3-O-galactoside (28%) Petunidin 3-Ogalactoside (19%)	(Joaquín-Cruz et al. 2015)
Bulgarian bilberry fruits *(Vaccinium myrtillus L.)*	162.4-217.0	Delphinidin 3-galactoside, Delphinidin 3–glucoside, Cyanidin 3–galactoside Delphinidin 3–arabinoside, Cyanidin 3–glucoside, Petunidin 3–galactoside + Cyanidin 3–arabinoside, Peonidin 3–galactoside, Petunidin 3–arabinoside, Peonidin 3–glucoside, Malvidin 3–galactoside, Malvidin 3–glucoside Malvidin 3–arabinoside.	(Georgieva et al. 2018)
Black Chokeberry (*Aronia melanocarpa*)	307-1480	Cyanidin-ACN Pelargonidin-ACN	(Liu et al. 2004; Nayak et al. 2010a; Strigl et al. 1995)

Cranberries (*Vaccinium macrocarpon* Aiton)	20-360	Cyanidin-ACN Delphinidin-ACN Peonidin-ACN Malvidin- ACN Pelargonidin-ACN	(Nayak et al. 2010a; Prior et al. 2001; Wu et al. 2004)
Black currant (*Ribes nigrum* L)	96-476	Cyanidin-ACN Delphinidin-ACN Peonidin-ACN Pelargonidin-ACN Petunidin-ACN	(Kampuse et al. 2001; Määttä-Riihinen et al. 2004)
Elderberries (*Sambucus nigra* L)	332-1374	Cyanidin-ACN Pelargonidin-ACN	(Määttä-Riihinen et al. 2004; Nayak et al. 2010a; Wu et al. 2004, 2006)
***Solanum nigrum* Fruits**	Peel-1800.215 Flesh-100.263 Between peel and flesh-300.954	Cyanidin- 3-rutinoside-5-glucoside Delphinidin-3-(p-coumaroyl)-rutinoside-5-glucoside Petunidin-3-O-rutinoside-(caffeoyl)-5-O-glucoside Petunidin-3-(cis-p-coumaroyl)-rutinoside-5-glucoside Petunidin-3-(trans-p-coumaroyl)-rutinoside-5-glucoside Petunidin-3-(feruloyl)-rutinoside-5-glucoside Petunidin-3-O-glucoside-5-O-glucoside Malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside	(Wang et al. 2017)
karanda fruit (*Carissa carandas* L.)	933 mg/100g DW	--	(Le et al. 2019)
Grapes and wine *(Vitis vinifera)*	72 to 1708 mg/L in red and concord grapes 26 mg/L in wines	Cyanidin 3-monoglucoside Delphinidin 3-monoglucoside Malvidin 3-monoglucoside Petunidin-3-monoglucoside Peonidin- 3-monoglucoside	Frankel et al., 1995, 1998 Wu X et al., 2006 Munoz-Espada et al. 2004
non-*Vitis vinifera* grapes (Marechal Foch, Norton, and Concord varieties)	258 for Foch, 888 for Norton, and 326 for Concord grapes	cyanidin aglycon peonidin aglycon delphinidin aglycon petunidin aglycon malvidin aglycon Cyanidin monoglucoside	(Muñoz-Espada et al. 2004)

Grape varieties (*Vitis vinifera, Vitis labrusca* and *Vitis hybrida*)	181.2 -716.4	malvidin-3-glucoside, delphinidin-3-glucoside, petunidin-3-glucoside, cyanidin-3 glucoside, and peonidin-3-glucoside	(Nile et al. 2015)
Strawberries (*Fragaria ananassa* Duch.)	13-315	Pelargonidin 3-glucoside, Cyanidin 3-glucoside Pelargonidin 3-rutinoside Delphinidin-3-glucoside, Malvidin-3-glucoside	(Cordenunsi et al. 2002; Hannum 2004; Karaaslan and Yaman 2017; Zhang et al. 2013)
Kokum fruits (*Garcina indica*)	1000-2400	Cyanidin 3-glucoside Cyanidin 3-sambubioside.	(Nayak et al. 2010b; a)
Sweet Cherries	122 ± 21.3	Cyanidin-ACN Pelargonidin-ACN Peonidin-ACN	(Wu et al. 2006)
Hungarian sour cherry varieties (*Prunus cerasus* L.) (C59, VN1, A, Db. Kj)	21–295	Cyanidin-3-O-rutinoside Cyanidin-3-O-glucoside Cyanidin-glucosyl-rutinoside Malvidin-3,5-O-diglycoside	(Homoki et al. 2016)
Black plum	124.5 ± 21.6	Cyanidin-ACN	(Wu et al. 2006)
Black bean	44.5	Delphinidin 3-glucoside Pentunidin 3-glucoside Malvidin 3-glucoside	(Takeoka et al. 1997; Wu et al. 2006)
Eggplant	87.5	Delphinidin-ACN	(Wu et al. 2006)
Red radish	11-100	Pelargonidin-ACN	(Giusti and Wrolstad 1996; Wu et al. 2006)
Purple corn (*Zea mays* L)	290-1642	Cyanidin-3-glucoside	(Jing and Giusti 2005; Lieberman 2007)
Purple corn bran	3.625 ± 0.105 g kg^{-1}	Cyanidin-3-O-glucoside Pelargonidin-3-O-glucoside Peonidin-3-O-glucoside Cyanidin-3-O-(6-malonylglucoside) Pelargonidin-3-O-(6-malonylglucoside)	(Chen et al. 2017)
Black rice Varieties (*Oryza sativa* L.)	109.52 to 327.60	Cyanidin-3-glucoside Peonidin-3-glucoside	(Abdel-Aal et al. 2006; Pedro et al. 2016; Sompong et al. 2011)
Purple sweet potato	24.36	Cyanidin- ACN Peonidin- ACN	(Ano et al. 2005; He et al. 2015)

Red cabbage (*Brassica oleracea* L.)	109-322	Cyanidin-ACN	(Ahmadiani et al. 2014; Wu et al. 2006)
Saffron Petals	1712.19±60 mg delphinidin/L	Delphinidin-ACN	(Khazaei et al. 2016)
Blue Maize	1.6- 4.7	Cyanidin 3-glucoside Cyanidin 3,5-diglucoside pelargonidin 3-glucoside	(Escalante-Aburto et al. 2016)
Hibiscus sabdariffa L. (calyces) *Hibiscus sabdariffa* L. (flowers)	2.5 g 100 g−1 DW in calyces	Calyces Delphinidin 3-sambubioside Cyanidin 3-sambubioside Flowers Delphinidin-3-O-glucoside, Delphinidin-3-O-sambubioside, Cyanidin-3-O-glucoside Cyanidin-3-O-sambubioside	(Cisse et al. 2009; Cissé et al. 2011, 2012; Da-Costa-Rocha et al. 2014; Sang et al. 2018)
Edible flowers	0.3-14.4 cyn-3-glu eq.	Cyanidin 3-glucoside	(Benvenuti et al. 2016)
Nitraria tangutorun Bobr. Seed meal *Nitraria tangutorun* Bobr., a unique kind of fruit from Qinghai–Tibet Plateau.	65.04 mg/100 g of dry seed meal powder ~ 240 mg/ 100g FW of fruit (Malvidin-3,5-O-diglycoside equivalent)	Seed meal (Eight anthocyanins) Cyanidin-3-O-diglucoside Pelargonidin-3-O-diglucoside Cyanidin-3-O-(caffeoyl)-diglucoside Delphinidin-3-O-(p-coumaroyl)-diglucoside Cyanidin-3-O-(cis-p-coumaroyl)-diglucoside isomer Cyanidin-3-O-(trans-p-coumaroyl)-diglucoside isomer (18.17 mg/100 g) Pelargonidin-3-O-(p-coumaroyl)-diglucoside Delphinidin-3-O-(p-coumaroyl)-hexose Fruits (Nine anthocyanins) Main are Cyanidin-3-O-(trans-p-coumaroyl)-diglucoside (215.76 ± 22.91 mg/100 g FW) and pelargonidin-3-O-(p-coumaroyl)-diglucoside (5.13 ± 0.35 mg/100g FW).	(Sang et al. 2017; Zheng et al. 2011b)

Vireya Rhododendron species Rhododendron *javanicum*, R. jasminiflorum, R. *konori*, R. *malayanum*, R. *retusum* and R. *seranicum*	0.12–0.29 mg equivalent cyanidin-3-glycoside/g dried powder	--------	(Rafi et al. 2018)

2.2 HYDROXYCITRIC ACID AND THEIR SOURCES

Garcinia species like *Garcinia cambogia, Garcinia indica, Garcinia cowa* and *Garcinia atrovirdis* (Jayaprakasha and Sakariah 2002; Jena et al. 2002b) and tropical plant *Hibiscus sabdariffa* (Yamada et al. 2007) are known to produce and contain a higher concentration of acids in form of HCA. The HCA and HCA-lactone are mainly seen in leaves and rinds of Garcinia species generally called garcinia acid. However, there is very rare literature available for the exact concentration of these components present in the sources. The preparations of HCA extract (50 to 70 %, not is highly pure form) are commercially available in India as a nutraceutical product for enhanced weight loss applications as it is known for its antiobesity property (Nayak et al. 2010a). It significantly reduces the bodyweight lowering the synthesis of fatty acids and cholesterol. The lower in cholesterol synthesis and lower lipid accumulation ensures the cardio protection. HCA lowered body fat level with no loss of body protein or lean mass in test animals. The main active component in kokum is HCA which is now regarded as a fat reducer (Hegde 2019).

HCA is the major type of acid present in kokum fruits along with minor quantities of HCA lactone and citric acid. HCA is similar to citric acid with a hydroxyl group at the second carbon atom. As the carbon atom at position 2 is chiral, different isomers are possible for HCA. There are four isomers of HCA, (+) - and (-)-hydroxycitric acid, and (+)- and (-) - allo-hydroxycitric acid. The (-)-hydroxycitric acid isomer is the one found in Garcinia species. These stereoisomers can form a γ-lactone ring called lactones and both HCA non-lactone and lactone forms are present

in solution (Yamada et al. 2007). The HCA is present in the form of (-)-HCA which is an alpha-hydroxy tribasic acid. The molecular weight of HCA is 208 g/mol.

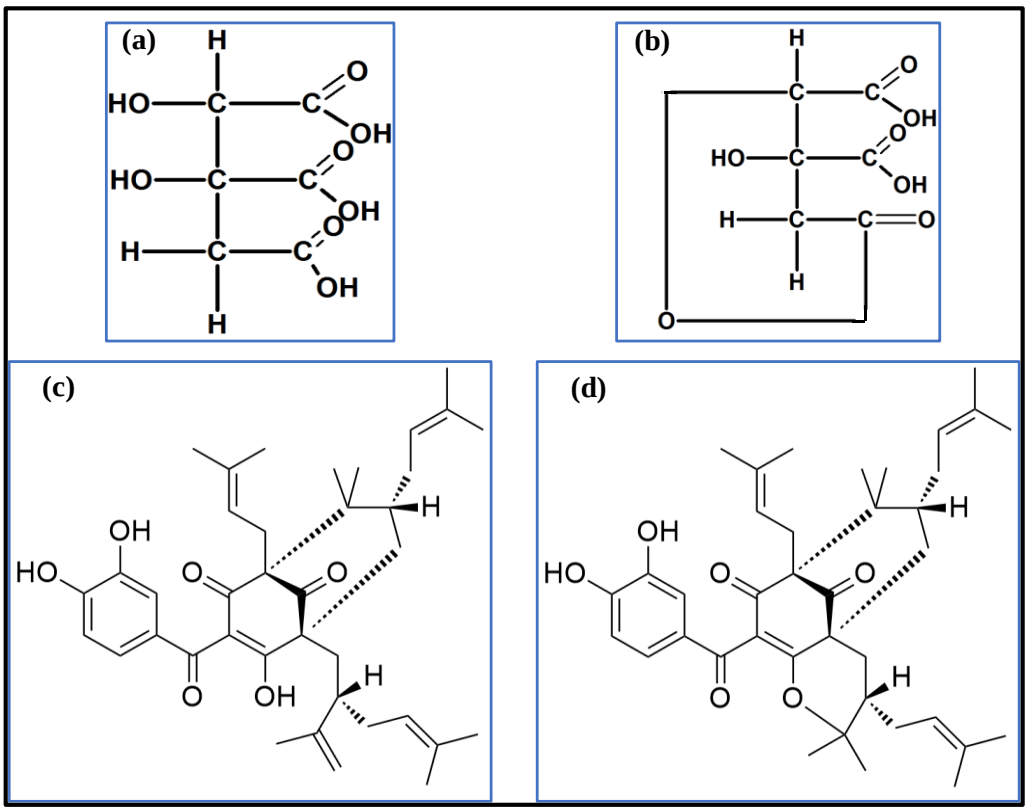


Figure 2.2 The chemical structures of (a) Hydroxycitric acid; (b) Hydroxycitric acid lactone form; and (c) Garcinol and (d) Isogarcinol present in kokum rinds (*Garcinia indica*) (Padhye et al. 2009; Schobert and Biersack 2019)

The structure of HCA in both free and lactone forms is displayed in Figure 2.2 (a, b). (-)- HCA can be converted to its lactone form by lactonization during purification, evaporation, and concentration. It may be converted to its sodium, potassium, or calcium salts, lactones, or esters for their applications. The dried rinds of the species are reported to contain (-)-HCA up to 20 to 30 percent on a dry basis (Swami et al. 2014). There is a higher scope to extract and purify these HCA bioactive compounds from kokum fruits for food and nutraceutical applications. HCA can be determined by HPLC or by titration method. The liquid chromatographic method has been developed to determine the amount of HCA and HCA-lactone in Garcinia leaves as well as rinds.

2.3 GARCINOL AND ISOGARCINOL AND THEIR SOURCES

Kokum fruit contains polyisoprenylated benzophenone derivatives such as garcinol, isogarcinol, contains phenolic hydroxyl groups. These phenolic hydroxyl groups make them active antioxidant molecules. Garcinol structure is similar to xanthochymol and can be called as camboginol. The molecular formula and the absorption spectral data indicate that the compound is related to isomeric isoxanthochymol and more appropriately, because of the sign of optical rotation, to Cambogin (isogarcinol) (Tang et al. 2013). The structures of garcinol and isogarcinol found in *Garcinia indica* are shown in Figure 2.2 (c, d). The garcinol resembles a well-known antioxidant i.e. curcumin because of the presence of β-diketone moiety (Sang et al. 2001). These compounds show their presence in the rinds of kokum varying between 2-3% of dry weight basis. The molecular weight of Garcinol is 602 and its melting point is 122°C (Nayak et al. 2010a). The Garcinol crystals can be dissolved in many organic solvents such as ethanol, methanol, acetone, dimethyl sulfoxide, acetonitrile, ethyl acetate, chloroform, and hexane, but has very poor aqueous solubility (Yamaguchi et al. 2000b).

Garcinol and isogarcinol were also detected in and isolated from plants of the *Garcinia* species such as G. *Cambogia*, G. *hanburyi*, G. *morella*, G. *yunnanensis*, G. *xanthochymus* and G. *travancorica*. Recently these compounds were known to present in the stem bark of the G. *buchananii*. Isogarcinol was also isolated from G. *punctata* and G. *ovalifolia* (Schobert and Biersack 2019). Maximum yields of garcinol can be obtained from dried *Garcinia indica* rinds compared to other species. While garcinol can be easily converted to isogarcinol under acidic conditions. Garcinol is a yellow colour fat-soluble pigment due to this they are used as a natural compound to impart a yellow colour to butter and ghee (Nayak et al. 2010a).

It was found to be the potent inhibitor of the enzyme histone acetyltransferase. It has shown promising antioxidative, antiglycation, and free radical scavenging activities. Garcinol also possesses anti-inflammatory, antibiotic, anti-cancer, anti-bacterial, and anti-ulcer activities. Isogarcinol also shows biological activities similar to that of garcinol and has been claimed to be an anti-inflammatory and antitumor

compound, a lipase inhibitor, an antiobesity agent as well as an antiulcer agent (Padhye et al. 2009). Garcinol was shown to have both chelating activity and free radical scavenging activity and is a lipid-soluble superoxide anion scavenger. The hexane extract, benzene extract, and garcinol from *Garcinia indica* have the equivalent antioxidant capacity to that of ascorbic acid determined as evaluated by the phosphomolybdenum method and by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method and among the three Garcinol is the highest. Garcinol partially inhibited the effect of peroxynitrite on platelet and plasma lipid peroxidation, measured as thiobarbituricacid reactive substances (TBARS) - a marker of this process, suggesting that garcinol can also be useful as protecting factors against diseases associated with oxidative stress (Kolodziejczyk et al. 2009).

2.4 STRATEGIES EMPLOYED FOR EXTRACTION AND PURIFICATION OF ANTHOCYANINS

The natural bioactive compounds including ACNs are expressed in different parts of plants like leaves, petals, fruits, bulbs, rhizomes, or stems. They are known to deposit inside the cells or vacuole of cells in tissues. The extent of their accumulation varies from tissue to tissue of plants. The intensity of expression and production depends on genotypic difference, growing conditions, stressed or senescent (Silva et al. 2017). To separate these bioactive compounds located inside the cells, an external force is required to extract them towards the external medium from a solid matrix. For example, as ACNs are hydrophilic, their polarity facilitates dissolution into solvents when they come in contact with cells or membranes. The availability of the apparatus, solvent, sample complexity, and concentration of bioactive compounds in the sample influence on the selection of these extraction methods. The flow chart given below in Figure 2.3, explains the methods employed for extraction and purification right from source till final product of bioactive compounds including ACNs along with characterization techniques used (Figure 2.3).

2.4.1 Extraction of Anthocyanins (ACNs)

Extraction is a basic step in the isolation and identification of phenolic compounds including ACNs also. These extraction methods vary and there is no single and standard extraction method available for a particular bioactive compound.

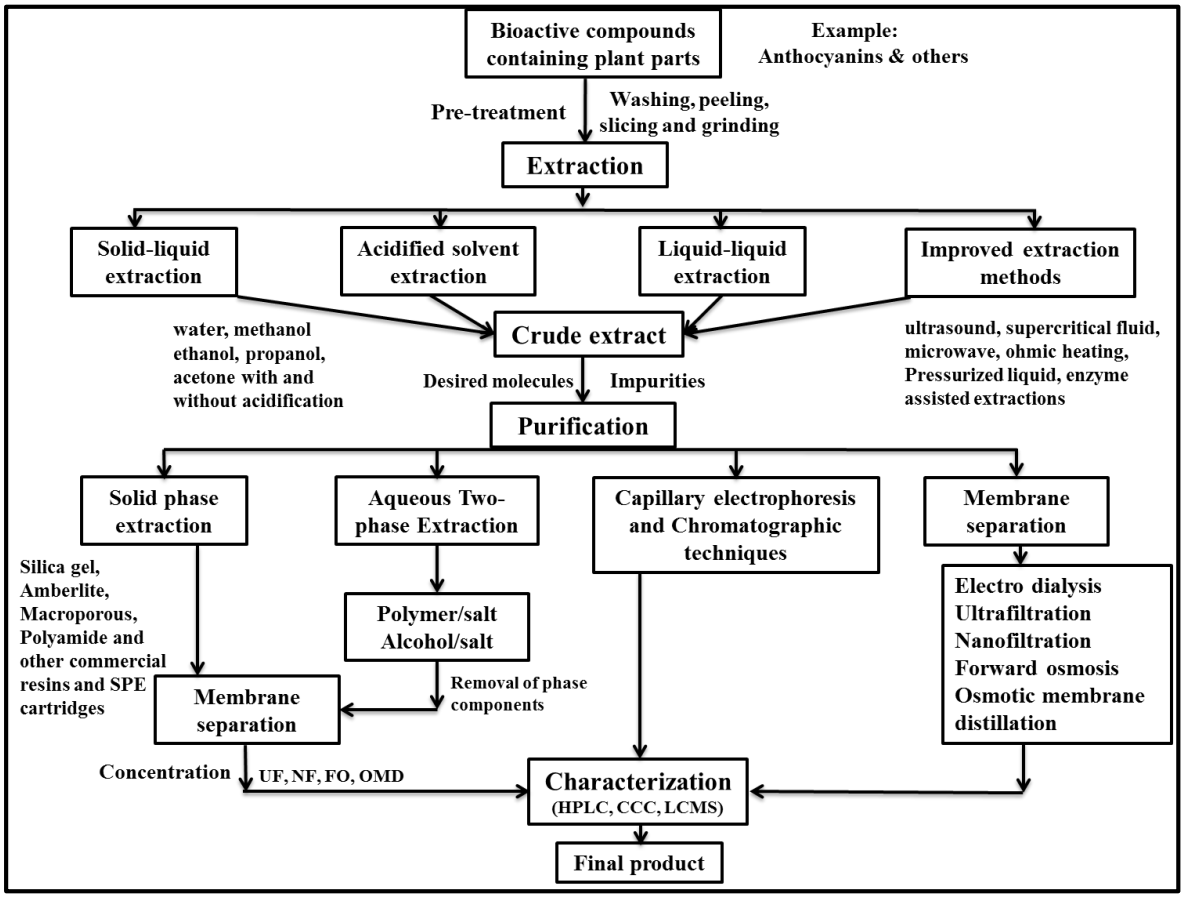


Figure 2.3 The flow chart representing the possible ways utilised and strategies applied for extraction and purification of bioactive compounds including ACNs from natural plant parts by solvent extraction, solid-phase extraction, aqueous two-phase processes, and membrane separations and their integrated processes

The phenolic compounds either hydrophilic or hydrophobic can be extracted by grinding, drying or lyophilizing fruits, vegetables, and leaves or only by soaking, stirring, homogenisation of plant parts with subsequent solvent extraction (Ignat et al. 2011). Solvent extraction as a classic approach used in recovery or to extract bioactive compounds from a solid matrix or biomass like leaves, fruits, flowers, etc. can be named as solid-liquid extraction. ACNs extraction and purifications strategies were carried out from natural sources like grapes (Corrales et al. 2009; Jeong et al. 2015;

Vidal et al. 2004; Wu et al. 2014), berries (haskapberries, black, blue and mulberries) (Celli et al. 2015; Liu et al. 2004; Wen et al. 2016; Wu et al. 2011; Xu et al. 2016), kokum fruits (*garcina indica*) (Nainegali et al. 2017; Nayak et al. 2010b; Nayak and Rastogi 2010a), jamun fruits (*Syzygium cumini L.*) (Chandrasekhar and Raghavarao 2015; Jampani et al. 2014), cranberry juice (Husson et al. 2013b; a), Sweet cherries (Grigoras et al. 2012), red cabbage (Coutinho et al. 2004; Jampani and Raghavarao 2015; Xu et al. 2010), purple sweet potatoes (Heinonen et al. 2016; Liu et al. 2007, 2013a; Yin et al. 2017), black rice (He et al. 2017; Kang et al. 2014; Pedro et al. 2016; Wen et al. 2016), purple corn (Muangrat et al. 2017), roselle extract (*Hibiscus sabdariffa)* (Cissé et al. 2011, 2012), saffron petals (Khazaei et al. 2016), black bean canning wastewater (Wang et al. 2014c), among others.

2.4.1.1 Solid-liquid extraction (SLE)

Solid-liquid extraction works on a mass transport phenomenon which facilitates the migration of solids present in a solid matrix into a solvent when it comes into contact with the matrix. It is a commonly used method to recover ACNs from plant tissues as their polarity and hydrophilicity nature facilitates dissolution into polar solvents like methanol, ethanol, propanol, acetone, acetonitrile, and water (Castañeda-Ovando et al. 2009; Silva et al. 2017). As ACNs are more stable at lower pH, acidification of solvents prior to extraction improved the extraction efficiency. Both organic and inorganic acids like HCl, citric acid, acetic acid, propionic acid, trifluoroacetic acid (TFA), tartaric, and formic acid with their combinations also used till now to acidify the solvents used for the extraction of ACNs. The methanol acidified with HCl led to higher extraction of ACNs from grape pulp compared to acetone, ethanol, and water alone (Kapasakalidis et al. 2006). But Barnes et al. showed that the non- acidified ethanol is a better extractant from blueberry samples and TFA is the better acid in case of acid-dependent extraction (Barnes et al. 2009). However, the acidification of solvents using stronger HCl and TFA may destroy the glycoside bonds and the acylated anthocyanins might be hydrolysed. Ethanol and organic acids are preferred over methanol as it is toxic to use in food applications (Castañeda-Ovando et al. 2009; Kapasakalidis et al. 2006).

The most influencing parameters for the ACN extraction are type and volume of solvent, temperature, particle size, solvent/mass ratio, and time. The combination of these factors at the optimal conditions is important to obtain a maximum yield of ACNs (Pedro et al. 2016). The best extraction conditions for strawberry fruit were found to be methanol as solvent, 30 min as extraction time and 1:1 (v: w) as liquid: solid ratio (Karaaslan and Yaman 2017) but for cherry and cranberry fruits the acidified acetonitrile as a solvent, 30 min as optimum extraction time and 2:1 (v: w) as a solvent: solid ratio were reported as optimum condition (Karaaslan and Yaman 2016). The acidified 70% ethanol with 1% citric acid could able to extract the ACNs from elderberry fruit within 30 min (Salamon et al. 2015). Recently in 2018, as a green approach β-cyclodextrin-based extraction is used for *Lycium ruthenicum* Murr ACNs. β-CD solutions produced higher extraction yields of petunidin-3-O-(trans-p-coumaroyl)-rutinoside-5-O-glucoside and total ACNs from L. r*uthenicum* fruit than pure water. aqueous hydroxypropyl-β-cyclodextrin (HPβ-CD), ethanol, and methanol solvents. The extraction at 50 °C for 30 min using 1.65% β-CD solution and a liquid/solid ratio of 15:1 was reported as an optimal condition for the extraction (Zhang et al. 2018). Overall, conventional extraction methods are a simple technique that needs a relatively simple and cheap apparatus and has the ability to extract phenolics and ACNs effectively. However, the longer extraction time, labour-intensive, large amount of solvent wastage, low product extractability, and less selectivity are some of the disadvantages associated with the process (Kang et al. 2014). The consumption of an enormous quantity of solvents also does not confirm the ecological and environmental protections of industries (He et al. 2016).

2.4.1.2 Advanced extraction methods

The greater demand for efficient extraction process with reduced solvent consumption, low environmental impact, high extraction yield, and shorter extraction time leads to the exploration of new improved extracting methods for the extraction of ACN since the last decade. The ultrasound, supercritical fluid, microwave, ohmic heating, pressurized liquid, enzyme assisted extractions were developed and studied (Celli et al. 2015; Chen et al. 2017; Corrales et al. 2009; Garcia-Mendoza et al. 2017; He et al. 2016; Loypimai et al. 2015; Ongkowijoyo et al. 2018; Silva et al. 2017; Xu

et al. 2010; Zhu et al. 2016). Some of these techniques are considered as ‘‘green techniques’’ (Bubalo et al. 2018; Heffels et al. 2015) and have advantages over conventional extraction process like effective mixing of solvent and solid matrix, faster energy transfer, reduced thermal gradients and extraction temperature, better selectivity, reduced equipment size, higher productivity and lesser number of process steps.

The 85% ACNs were extracted from grape skin using CO_2 as the supercritical fluid along with aqueous acidified ethanol as co-solvent (Bleve et al. 2008) and further optimization of supercritical fluid extraction was carried out by Ghafoor et al. in 2010, who could able to extract 1.176 mg/mL of ACNs from grape peel at lower temperature and 6% ethanol as a modifier with CO_2 (Ghafoor et al. 2010). The similar conditions are used for the extraction of ACNs from elderberry pomace, and blueberry residues. Though higher temperatures have been used in the extraction of other phenolic compounds, the temperatures above 40 °C was not employed for the extraction as the ACNs are not stable at relatively high temperatures (Azmir et al. 2013; Ghafoor et al. 2010).

Generally, ultrasound-assisted extraction (UAE) is the time-efficient method for ACN extraction with no or low degradation. The sonication showed better extraction of ACNs from grape skins using an ultrasonic bath at a frequency of 35 kHz for 30 min, followed by stirring at a temperature of 70 °C in a water bath for 2.5 h (Silva et al. 2017). The solvent consumption and extraction time were significantly decreased when ultrasound was applied. It was observed that the vibration and mechanical action during sonication had no impact on the structure of ACNs (Chen et al. 2017). Recently, Sang et al. achieved the extraction of 65.04 mg/100 g of ACNs and 947.39 mg/100 g of polyphenols from N. *tangutorun* seed meal using the ultrasound-assisted process with green solvent (51.15% ethanol) at 70 °C for 32.73 min. Later identified eight different ACNs in the crude extract and found that the cyanidin-3-O-(trans-p-coumaroyl)-di glucoside (18.17 mg/100 g) was the main ACN among them (Sang et al. 2017). In another UAE process case 92.45% of yield with 3.625 ± 0.105 g kg^{-1} total ACNs content was obtained at the optimal extraction conditions like, solid-liquid ratio 1:8, the effective extraction time of 35 min with the

ultrasonic time of 90 min and ultrasonic power of 400 W. The scaled-up process with 21 L 95% ethanol, 15.75 L distilled water and 5.25 L 0.1 mol L^{-1} citric acid mixed with the 5 kg purple corn bran (3.411 g kg^{-1} ACN concentration) showed the feasibility of this extraction process at a larger scale with the yield of 86.99%, which was 5.46% less compared to laboratory level (Chen et al. 2017). Besides, pulsed electrical field extraction as a non-thermal technique without any temperature impact is also recommended to increase the yield of ACN extraction (Chemat et al. 2011; Pingret et al. 2012).

Despite the limitation of high pressure and temperature, the pressurized liquid extraction has been carried out for the blueberries, red onion, and sweet potato. The solvent extractor operated at a pressure of 10 MPa could able to enhance the extraction yield of ACN at a temperature between 80-120 °C with aqueous acidified methanol or ethanol (150 mg/100 g powder of purple-fleshed sweet potato) (Bubalo et al. 2018; Cardoso et al. 2013; Truong et al. 2012). Microwave-assisted ACN extraction is tried for purple corn and 185.1 mg ACNs/100 g was achieved with 95% aqueous acidified ethanol as a solvent with a ratio of 4:1 (mL/g) in 19 min by maintaining the solid-liquid ratio of 1:20 and applying 555 W irradiation power. The 43.42 mg ACNs/100 g fresh red raspberries got extracted just within 12 min with the microwave power of 366 W (Yang and Zhai 2010). Meanwhile, few reports are also available on the ohmic heating assisted extraction of ACNs. The ACNs are extracted from black rice bran (*Oryza sativa* L.) by utilizing the ohmic heating assisted extraction in one of the research (Loypimai et al. 2015). However, the method may cause a similar degradation of bioactive compounds as reported for conventional heating. These special techniques require their special requirements and equipment, facilities and may add higher costs in industrial production (Wu et al. 2011; Yin et al. 2017).

2.4.2 Purification of anthocyanins (ACNs)

The crude extract obtained from the conventional extraction contains large amounts of other compounds such as organic acids, sugars, sugar alcohols, amino acids and proteins mother than ACNs, (He and Giusti 2011; Sariburun et al. 2010; Wen et al. 2016) that affects for functionality and stability of pigments (Jampani et al.

2014). The ACNs get easily oxidised and decomposed under the influence of sugars, light, oxygen, pH, temperature, enzymes, and solvents. The free sugars are one among them which degraded themselves and the degraded products may lead to Maillard reactions to form brown compounds (Liu et al. 2004). Therefore, after the extraction process, the removal of impurities stands as an important step, particularly in the study of ACN properties and their usage (Castañeda-Ovando et al. 2009; He and Giusti 2011). Hence, the purification of ACNs is very much desirable to maintain the stability and also to facilitate their application in food processing and nutraceutical applications, where pure ACN molecules are needed (Jampani et al. 2014; Wen et al. 2016). In accordance with these issues, pure ACN isolation and purification from the crude extract of plant sources is mandatory for their accurate quantification and biological application (Chorfa et al. 2016).

2.4.2.1 Solid-phase extraction (adsorption technique)

The adsorption principle is considered for the partial purification of bioactive compounds in a single step allowing the elimination of polar, non-phenolic impurities. The selection of stationary phase (for adsorption) and eluent (for desorption) is important to maintain selectivity, such that the interactive surface of the solid should remain constant. Changes in the selectivity of adsorbents can result in chemical degradation and chemical fouling (Kraemer-schafhalter and Pfannhauser 1998). The resins are polar or nonpolar polymers having characteristics of good selectivity, different surface properties, and high mechanical strength with fast adsorption characteristics (Sandhu and Gu 2013). Theoretically, adsorbent resins could absorb a wide variety of compounds from solutions employing van der Waals, hydrogen bond forces, different molecular sizes through their porous structures; on the other hand, these molecules adsorbed by the resins could be further eluted with ethanol, methanol, and saline solutions. As the binding forces between the adsorbed molecules with eluents are greater than those between the molecules and resins, the molecules are inevitably and selectively eluted (Gao et al. 2007; He et al. 2017; Sun et al. 2012). Various adsorbents like silica gels, macroporous adsorbent resin, cation exchange resins, and polyamide resins have been investigated quite much for purification of ACNs from different sources (Buran et al. 2014; Chandrasekhar et al. 2012; Heinonen

et al. 2016; Kang et al. 2014; Liu et al. 2004, 2007; Wen et al. 2016), where ACNs can strongly bond through their hydroxyl groups and separated subsequently from other compounds by increasing the polarity with different solvents (Da Costa, Horton, & Margolis, 2000; Tian et al. 2015).

The macroporous resins are more suitable for food industry applications with merits of low energy consumption, easy regeneration and long life, environmentally friendly and considered as safe (approved by the U.S. Food and Drug Administration for food contact) (Buran et al. 2014; Wang et al. 2014c). The macroporous adsorption resins can be suitable for natural product purification on a large scale (Gao et al. 2007; He et al. 2017; Wan et al. 2014). However, column chromatography has been demonstrated to achieve efficient large-scale separation of ACNs (Cao et al. 2010; Heinonen et al. 2016; Wang et al. 2014c). Among 16 materials screened for selectivity of ACNs, which includes three macroporous resins employed to purify the ACNs like D101 (nonpolar), AB-8 (weakly polar), DM301 (moderately polar) and silica gel (nonpolar), Amberlite IRC 80 (weak acidic anion exchanger), Amberlite IR 120 (weak acidic cation exchanger), DOWEX 50WX8 (strong acidic cation exchanger), Amberlite XAD4 ((He et al. 2017; Kraemer-schafhalter and Pfannhauser 1998). The Amberlite XAD7 (non-ionic acrylic ester resins with moderate polarity) was used for the purification of ACNs from red cabbage and Jamun fruits (Chandrasekhar et al. 2012; Jampani et al. 2014). The reversed-phase silica gels, non-ionic acrylic polymer adsorbents such as Serdolit PAD IV and Amberlite XAD-7, DM301 of moderate polarity turned out to be most suitable for purification of ACNs (He et al. 2017; Kraemer-schafhalter and Pfannhauser 1998). The moderate polarity resins proved superior to other resins that could strongly adsorb ACNs and showed the highest adsorptivity and desorptivity. The Amberlite XAD-7HP showed the highest adsorption capacity of about 0.84 mg/mL of resin, desorption ratio of 92.85% with 60% acidified ethanol as the best eluent. The highest adsorption capacity of ACNs was obtained from purple-fleshed potatoes with XAD-1600 and showed better purification to remove 90% of impurities (Liu et al. 2007). The FPX-66 and XAD-16N used for the extraction of ACNs from muscadine pomace showed the highest adsorption and desorption capacities among the five different Amberlite (macroporous adsorbent) resins studied (Sandhu and Gu 2013). Weak polar

macroporous adsorbent AB-8 resin was also found suitable for the ACN separation among the resins tested and showing a 19.1 fold increase in purity with optimized parameters (Yang et al. 2015). Scale-up experiments were performed with RP silica gel 100 and C-18 and Amberlite XAD-7 at 6.34 and 3.86 gL^{-1} concentration, respectively in a 36 litre column (Kraemer-schafhalter and Pfannhauser 1998). A potential industrial adsorbent was screened with 5 types of macroporous resins (Diaion Hp20, Sepabeads Sp70, Sepabeads Sp207, Sepabeads Sp700, and Sepabeads Sp710) where Sp700 found most potential for industrial purification of ACNs from black bean canning wastewater with adsorption capacity 39 ± 4 mg/g.

Recently, polyamide resins have attracted researchers because of its high adsorption and desorption capacity, low cost, easy regeneration properties, and works based on the affinity of hydrogen bonding interactions (Li et al. 2014). Polyamide resins were applied for the purification of various flavonoids (Gao et al. 2011; Xiao et al. 2013) and proved to have more specific selectivity for flavonoids. It could be an excellent adsorbent for the separation of ACN in the industrial scale extractions from plants (Wen et al. 2016). The recovery of ACNs using polyamide resin with different mesh sizes was investigated and 60–100 mesh polyamide resin with the adsorption and desorption capacities of 4.41 and 3.29 mg mL^{-1}, respectively recovered a higher quantity of ACNs compared to that of other polyamide resins. The adsorption capacity of polyamide resin observed to be more about 432 mg of anthocyanins per gram of resin compared to the cation exchange resin 160 mg g^{-1} and macroporous adsorbent resin adsorption 176 mg g^{-1} reported elsewhere.

Purification of ACNs from *Garcinia indica* was carried out using sorbent C-18 Sep-Pak cartridge which was activated with methanol and 0.01% aqueous HCl prior to adsorption. Adsorbed ACNs were collected using acidified methanol (0.01%, v/v HCl) after the separation of impurities with 0.01% aqueous hydrochloric acid (Nayak et al. 2010b). These partially purified ACNs were further subjected to sephadex LH20 column for characterization studies. The modified silica gel and functionalized strong cationic exchange resin (DSC-SCX) were also employed to purify ACNs from blueberries and identified by preparative-HPLC equipped with a photodiode array detector (Chorfa et al., 2016). *Schisandra chinensis* (S. *chinensis*) ACNs were purified with macroporous resins combing gel filtration chromatography to improve

purity. The purity of ACNs increased six-fold from 5.08% to 30.43% with the ACNs recovery of 96.5% using HPD-300 nonpolar resin and further isolated with Bio-Gel P2 gel filtration chromatography for identification.

2.4.2.2 Membrane separations

Membrane systems can be attractive options for separation and concentration of ACNs and other polyphenols from plant extracts due to their industrial favourable properties like ambient operating temperature, high selectivity, low energy consumption and no any chemical changes (He et al. 2017; Husson et al. 2013b; Nayak and Rastogi 2010b; Zambra et al. 2014). As the ACNs are used for functional foods, nutritional purposes and are a matter of health, the membrane technologies can be the ideal process with economic benefits to food and drug industries (Cissé et al. 2011). The application of ultrafiltration (UF) and nanofiltration (NF) operations, pressure-driven membrane processes, to concentrate the fruit juices and plant extracts are not explored well. The ultrafiltration and nanofiltration were employed to concentrate the ACNs from roselle extract (*Hibiscus sabdariffa* L.). Membranes with nominal MWCOs ranging from 0.2 to 150 kDa were tested with transmembrane pressures between 0.5 and 3.0 MPa, while keeping all other operating conditions constant. The retention values of total soluble solids, acidity, and ACNs found to increase with transmembrane pressure. At higher transmembrane pressure of 3 MPa, the UF membranes retained 80% of the ACNs, and 95% of ACNs were retained in the NF membranes at 2 MPa. Higher scale concentration trials using a 2.5-m^2 filtration surface could concentrate roselle extract from 4 to 25 g about six-fold enhancement with 100% retention of ACNs without any significant damages (Cissé et al. 2011).

The combination of electrodialysis with ultrafiltration membrane (EDUF) has been studied for the separation of biomolecules such as catechins or peptides and proteins (Doyen et al. 2011, 2014; Firdaous et al. 2010) and can be a promising method for the separation of polyphenols from plant sources. With an attempt, the feasibility of enrichment of natural polyphenols from cranberry juice by the EDUF method was studied. The migration of polyphenols towards the cathode side was observed throughout the ultrafiltration membrane because of the electric field applied.

This enables the selective migration of polyphenols or ACNs in the enriched juice compartment due to their positive charge at the cranberry juice pH (2.54) and the cut-off of the UF membrane used. The lesser efficiency was observed because of the depletion of polyphenols in raw juice after treatment and membrane fouling due to the interactions of polyphenols and polysaccharides with membranes (Bazinet et al. 2009, 2012). However, it was overcome by increasing the filtration area by stacking 3 polyethersulfone UF membranes in an EDUF configuration and resulted in 22 % enrichment of cranberry juice after 1 h 30 min treatment (Husson et al. 2013a). The polyvinylidene fluoride (PVDF) and polyethersulfone (PES) with a cut-off of 150 kDa and 500 kDa respectively were tested for enrichment of cranberry juice and about 24 % enrichment yields of ACNs was obtained (Husson et al. 2013b). The implementation of the above-discussed membrane processes on large scale is very limited for natural colour extracts because of some limitations like the requirement of high pressure, limit to maximum attainable concentration, concentration polarization, and membrane fouling.

The reverse osmosis technique can be used as an alternate but higher pressure was required to concentrate the product, which may damage the membrane (Nayak and Rastogi 2010b). Alternatively, new athermal non-pressure driven membrane processes such as forward osmosis (FO) and osmotic membrane distillation (OMD) which are capable of concentrating liquid foods at ambient conditions without product deterioration (Raghavarao et al. 2005) are employed to concentrate the liquid foods, vegetable and fruit juices (Babu et al. 2006; Kujawski et al. 2013; Zambra et al. 2014; Zhao et al. 2012) and also natural colours (Nayak and Rastogi 2010a; b). They use dense semi-permeable hydrophilic or hydrophobic membranes, which separates two solutions (extract and osmotic agent solution) having different osmotic and vapour pressures. The difference in osmotic pressure and vapour pressure between solutions is responsible for separation and enrichment in feed for FO and OMD, respectively. These methods offer several advantages upon conventional membrane processes such as low energy consumption, higher retention of thermally sensitive components without any product damage (Tang and Ng 2008; Zhao et al. 2012). The sodium chloride, calcium chloride, and mixed solution of sodium chloride and sucrose were

used for the concentration of vegetable and fruit juices (Babu et al. 2006; Zambra et al. 2014). Rastogi and his group worked on these athermal processes like osmotic membrane distillation and forward osmosis methods for ACN enrichment using kokum extract as feed material and sodium chloride as an osmotic agent (Nayak and Rastogi 2010b). OMD and FO were compared by studying the effect of feed and osmotic agent flow rate on transmembrane flux with an osmotic solution consisting of 26% sodium chloride. The transmembrane flux for forward osmosis was found higher and achieving 49.63 mg/l to 2.69 g/l in 18 hours as compared to OMD with the lower concentrating ability (Nayak and Rastogi 2010b). In the case of kokum (*Garcinia indica*) extract FO showed 54 times increase in concentration along with decreasing the non-enzymatic browning index and degradation constant. Considering the tolerance limit of the osmotic agent in the final product and disadvantages of the use of thermal concentration because of maintaining stability, FO and OMD techniques are potential for concentration of natural colourants.

An integrated process, aqueous two-phase extraction (ATPE) consisting of polyethylene glycol (PEG) 6000 and magnesium sulphate followed by membrane separation was employed to separate impurities and enrich Jamun ACNs from the extract (Chandrasekhar and Raghavarao 2015). Partial purification was achieved in ATPE removing sugars and further OMD and FO were applied to concentrate ACNs. OMD and FO processes enriched the concentration of ACNs from 430.1 mgL^{-1} to 790.3 mgL^{-1} and 2890.3 mgL^{-1}, respectively. The integrated processes like ATPE and FO, ATPE and OMD, UF and FO, the ATPE, and FO combination followed by ATPE and OMD found suitable for extraction and concentration of ACNs when compare to the thermal evaporation (Chandrasekhar and Raghavarao 2015).

2.4.2.3 Capillary electrophoresis and Chromatographic techniques

HPLC is the most popular and widely used technique for the analysis of polyphenols and flavonoids. But capillary electrophoresis (CE) can also be an alternative due to its high separation efficiency, small sample requirement, and rapid analysis in leaser time compared to HPLC. CE methods are left with some disadvantages of lower sensitivity and lesser reproducibility when compared to HPLC

(Valls et al. 2009). Most of the literature focuses on chromatographic and capillary electrophoresis (CE) techniques of ACN separation for identification and quantification, but preparative-scale separations of these pigments are scare.

2.4.2.3.1 Capillary electrophoresis (CE)

Capillary electrophoresis works on the common principle of differences in electromigration between analytes in a given electric field. Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) are commonly used modes for the separation and analysis of polyphenols including ACNs by changing electrolyte mixture. ACN analysis by CZE was introduced in the year 1996 by Bridle et al and used to separate mixtures of ACNs qualitatively with fused silica capillaries and alkaline borate as running buffer (pH-8). The anionic quinoidal base molecules also detected by absorption at 580 nm (Bridle, P. et al. 1996). This method had some limitations as the larger requirement of the sample due to the lesser amount of pH-sensitive ACN molecules to reach the UV-Vis absorption zone at pH-8. Further, separation of ACNs in a fruit-juice was studied in acidic media employing silica capillary column and different buffers like 0.25 mM cetyltrimethylammonium bromide (CTAB) in 160 mM NaH_2PO_4/H_3PO_4 (pH-2.1) and sodium phosphate running buffer containing 30% (v/v) acetonitrile at an apparent pH of 1.5 (Bicard et al. 1999; Costa et al. 1998). The acidic buffers stabilize the absorbent flavylium cation, which improves the sensitivity of the detection by UV–vis absorption at 520–560 nm. The addition of an organic modifier (methanol or acetonitrile) greatly improved the separation of blackcurrant anthocyanidins and strawberry ACNs. The analysis time can be shortened when cationic surfactant CTAB was used below its critical micelle concentration, which was used to reverse the direction of electroosmotic flow. The ACNs are analyzed in flavylium form (Bicard et al. 1999). However, the higher concentration of CTAB has no role in improving the selectivity.

Various capillary electrophoresis modifications were optimised for the quantitative determination of ACNs and their types from wine and other fruit sources (Bednar et al. 2003; Bednář et al. 2005; Calvo et al. 2004; Castañeda-Ovando et al.

2009). The MEKC procedure has been applied for the separation of non-charged components. The neutral solutions of ACNs are in noncharged quinoidal form and MEKC has been applied for their separation from elderberry fruits (Watanabe et al. 1998). As ACNs are known to be relatively stable at pH range 2–7, the acidity of running buffer should be maintained in that range or additives such as the solutions of anionic micelles, sodium dodecyl sulphate are added to stabilize and migrate the ACNs in the column. Elderberry pigments showed only one peak after CZE at pH 2.5 with phosphate buffer, while similar results were obtained when compared with HPLC and MEKC. MEKC with SDS solutions in a phosphate-borate buffer at pH 7.0 was successful for the separation of elderberry ACNs. Simultaneous separation of six ACNs from grape skin extracts (malvidin-3, 5-diglucoside, malvidin-3-glucoside, malvidin-3-galactoside, pelargonidin-3-glucoside, cyanidin-3, 5-diglucoside and cyanidin-3-galactoside) was achieved using MEKC. The pH 7.0 buffer was optimised to improve the selectivity in separation, which was 30 mM phosphate + 400 mM borate-TRIS, supported with 50 mM sodium dodecyl sulphate. The high content of borate was beneficial to separate diastereomeric pair malvidin-3-glucoside and malvidin-3-galactoside (Bednar et al. 2003).

As an alternative to HPLC, a method based on CZE was proposed for the quantitative determination of wine anthocyanin monoglucosides. The fused-silica capillary and a 50 mM sodium tetraborate buffer of pH 8.4 with 15% (v/v) methanol as a modifier was used for the separation (Saenz-Lopez et al. 2003). The basic medium allowed a faster separation than the acidic medium. The lower sensitivity for this basic medium could be improved by adding SO_2 to the samples. Thirteen wine ACNs of a Tannat wine, including acylated and non-acylated ACN monoglucosides, pyranoanthocyanins, and flavanol derivates, were separated using the CZE method (Calvo et al. 2004). Since the ACNs are anions at the pH of the running buffer, they migrate to the cathode (and to the detector) because of the mobility of positive electroosmotic flow. ACNs with a lower molecular mass have a higher charge/size and show longer migration times. Also, cyanidin-, delphinidin- and petunidin-3-O-glucoside have ortho-hydroxy groups form the complexes with tetraborate molecules, which increase their charge and then their charge/size ratio. In contrast, the non-

orthohydroxylated ACNs, malvidin- and peonidin-3-O-glucoside cannot form complexes. Hence, the malvidin-3-O-glucoside showed the shortest migration time, followed by peonidin-3-O-glucoside, which preceded petunidin-3-O-glucoside and delphinidin-3-O-glucoside, although they have higher molecular mass, and finally cyanidin-3-O-glucoside. The CZE method showed good sensitivity and reproducibility provided minimal set-up time, reduced costs and reagent consumption, as well as higher separation efficiencies in a shorter analysis time. Therefore, it was used as an alternative to LC for the analysis of ACNs.

2.4.2.3.2 Chromatographic techniques

Chromatographic techniques such as solid-liquid chromatography like HPLC, UPLC, or RPLC and liquid-liquid counter-current chromatography (CCC) were commonly used for identification and quantification of ACN molecules with numerous modifications. The HPLC analysis of ACNs was performed mostly with a reversed-phase C18 column and acidified organic solvent mobile phases. The formic acid-methanol, formic acid-methanol-acetonitrile, 0.01% to 0.1 % trifluro acetic acid-acetonitrile, phosphoric acid-acetic acid-acetonitrile, water-methanol both acidulated with 0.0037% HCl are some of the combinations reported as the mobile phases. Gradient mode elution (binary solvent system) is preferred with different gradient program/profiles. UV-Vis photodiode array detector (HPLC-DAD, HPLC-PDA) and mass spectrometry detection (HPLC-DAD-ESI-MS-MS) were commonly used for detection of ACNs in range of 510 to 530 nm (Canuto et al. 2016; Chandrasekhar et al. 2012; Heinonen et al. 2016; Ignat et al. 2011; Liu et al. 2004; Wang et al. 2014a; Welch et al. 2009; Yao et al. 2015).

Recently, the preparative chromatographic methods also employed for the separation and purification of ACNs. After the partial purification and concentration of kokum ACNs with sorbent C-18 Sep-Pak cartridge, the samples were loaded (0.8 ml) onto a Sephadex LH20 column and eluted with a mixture of methanol/water/trifluoroacetic acid at a ratio of 20:79.5:0.5. The separated fractions from the Sephadex LH20 column were pooled and were concentrated using a lyophiliser (Nayak et al. 2010b). The purified kokum ACNs were subjected to HPLC, mass, and NMR spectroscopy analyses and confirmed that the pigment essentially

contains two anthocyanins, which were identified as cyanidin 3-glucoside and cyanidin 3-sambubioside. Blood orange ACNs were extracted with selected NKA-9 macroporous resin and purified using the Toyopearl TSK HW-40S column using a mobile phase of 35% acidified methanol with 2% formic acid (Cao et al. 2010). A novel solid-phase extraction method using mixed-mode cation-exchange chromatography was developed and used for the purification of ACNs from fruits and vegetables. This novel cation-exchange/reversed-phase combination isolated 99 % of ACNs with high purity compared to normal SPE technique and also removed the non-ACN phenolics efficiently (He and Giusti 2011).

The purification strategies for ACNs from plant materials have been explored using techniques such as solid-phase extraction, high-speed counter-current chromatography (HSCCC), column chromatography (CC), and preparative high-performance liquid chromatography. Application of Amberlite XAD-7, NKA-9, C18, and Sephadex LH-20 resin columns as column chromatography has been widely used to isolate monomeric ACNs (Cao et al. 2010; Coutinho et al. 2004; He and Giusti 2011; Myjavcová et al. 2010; Takeoka et al. 1997). However, most of the extraction procedures are restricted to partial purification due to the lesser purity of products. Some of these methods involve toxic organic solvents, which makes them not likely to consider for the scaled-up commercial application.

Recently, integrated chromatographic methods received extensive attention of the researchers to achieve the efficient purification of bioactive components. Environmental friendly isolation of ACNs from sweet cherries was reported in 2012 by combining the solvent-free microwave-assisted extraction procedure with semi-preparative column chromatography (Grigoras et al. 2012). Sweet cherries were subjected to microwave irradiation at 1000 W for 4 cycles of 45 s each without adding solvent. The extracts collected were loaded to Hypersil H5 C18.25F column using a safe and biodegradable isocratic mobile phase mixture consisting of water/ethanol acidified with 1 % formic acid followed by UV detection at 280 nm. High purity cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside was recovered. Wang and et al. tried to isolate ACNs from wild blueberries using a combination of column chromatography and semi-preparative HPLC. Amberlite XAD-7HP column, the most

suitable adsorbent for ACNs is used initially to isolate fractions with acidified aqueous ethanol for elution (0.01% HCl and 35:65). First step purification showed about 32 % purification based on UV–Vis analysis. The purity of anthocyanin mixture is further increased to 59% to 68% in second step purification using the Sephadex LH-20 column and 25 % ethanol and 75 % water as the mobile phase. Three types of ACNs were isolated successfully namely malvidin-3-O-glucoside, petunidin-3-O-glucoside, and delphinidin-3-O-glucoside by semi-preparative HPLC and estimated purities of each monomer were 97.7%, 99.3%, and 95.4%, respectively (Wang et al. 2014a). The purification of S. *chinensis* ACNs was attempted with the combined method of macroporous resin plus gel filtration chromatography (Yue et al. 2016). Purification by dynamic adsorption and desorption was performed with HPD-300 nonpolar copolymer styrene type resin having a saturation capacity of 0.475 mg/g dry resin. At the 39 mLh^{-1} (3 BV/h) flow rate of extract, 0.517 mg/mL of S. chinensis ACNs was adsorbed in 4 hours and completely removed by 2.5 BV of 90% (v/v) ethanol solution at a flow rate of 13 mLh^{-1} (1 BV/h). The concentration of ACNs increased from 47.6 mg/g to 128.4 mg/g with six-fold purity. Subsequently, the sample was loaded to the glass column packed with Bio-Gel P2 and equilibrated with acetic acid and then eluted with acetic acid, pH 2.5 at a flow rate of 0.8 mL/min. The eluted samples were collected and subjected to a spectrophotometer at 520 nm for the identification of ACNs. Similarly, double solid-phase extraction followed by fractionation with preparative HPLC was employed for purification and isolation of ACN monomeric isomers from wild blueberries of Lake Saint-Jean region, Quebec, Canada (Chorfa et al. 2016). The ACNs were eluted from two successive solid phase extractions on hydrophobic silica gel (DSC-C18) and cationic exchange resin (DSC-SCX) with ethanol gradient (30% and 60% v/v) prepared in 5% aqueous formic acid pH 2–3. ACN molecules with purity up to 100% were isolated after fractionation by preparative HPLC. The HPLC methods used till now are beneficial to obtain pure ACNs, meanwhile implementation of them in large scale process may be difficult and economically not viable.

Counter-current chromatography (CCC) can be an alternative to conventional solid-liquid chromatographic methods. CCC is a versatile bi-phasic liquid-liquid

preparative chromatography separation method that separates the components of a mixture between two immiscible solvents. It consists of a long length open tube as a column which rotates in planetary motion along with rotor (Valls et al. 2009). The centrifugal force moves both the liquid phases in the opposite direction. The lighter mobile phase move towards one end of the column and the heavier stationary phase will move by displacement to the opposite end of the column. This principle allows the use of two liquids to flow in the counter-current direction in an open column and hence called as counter-current chromatography. The phase density difference and the centrifugal field are the only parameters allowing the equilibrium between the two liquid phases (Ignat et al. 2011). It can be operated in a dual-mode where the mobile phase and stationary phase's movement can be interchanged. Some of the advantages of CCC are the dual-mode capability of operation, no irreversible adsorption on active surfaces as no solid stationary phase is used, high injection capacity, less wastage of solvents and enables non-destructive isolation of natural compounds (Grigoras et al. 2012).

The high-speed counter-current chromatography (HSCCC) has been applied for the separations and isolation of ACNs with polar biphasic solvent systems like tert-butyl methyl ether/n-butanol/acetonitrile/water (2:2:1:5 or 1:4:1:5 or 1:3:1:5) acidified with trifluoroacetic acid (Degenhardt et al. 2000; Du et al. 2004; Montilla et al. 2010; Qiu et al. 2009; Schwarz et al. 2003; Vidal et al. 2004). This mixture was found to be a suitable solvent system for ACN separation and applied for various sources like red cabbage, black currant, black chokeberry and roselle extract, grapes, purple sweet potatoes, *Tradescantia pallida* leaves, purple corn, elderberry juice, red wine, and blackberries etc. The use of n-butanol maintains the high polarity in the biphasic system, which is required for ACNs separation. The TFA or other weak acids are used to lower the pH to maintain the ACN molecules in their equilibrium flavylium ion for displacement in the system. The concentration of TFA was used almost 0.1% for both solvent A and B, and in some cases 0.1 % and 0.01% for solvent A and B, respectively. The less-dense layer was always considered as a stationary phase and the elution mode is from head to tail direction. The monoglycosylated, acylated, and highly glycosylated derivatives of ACNs are successfully isolated using

this method. They showed the rapid fractionation of crude mixtures and help the enrichment of minor constituents in the separated CCC fractions (Winterhalter 2007).

Factors like the type of solvent system (Chen et al. 2014b; Qiu et al. 2009; Ying et al. 2011), the structure of ACNs (Salas et al. 2005), number of coils in the equipment (Degenhardt et al. 2000), and the flow rate (Schwarz et al. 2003) influences the separation in CCC. Various solvent systems and their ratio can improve the fractionation and purification of ACNs. The solvent system was optimized by changing the volume of n-butanol for the separation of ACNs from blue honeysuckle fruit crude extract. 100 mg crude extract loaded to the optimised biphasic system containing tert-butyl methyl ether/ n-butanol/ acetonitrile/ water/ trifluoroacetic acid (2:2:1:5:0.01, v/v) yielded 22.8 mg of cyanidin 3-glucoside (C3G) with 98.1% purity (Chen et al. 2014b). While in another study methyl tert-butyl ether–n-butanol–acetonitrile-water–trifluoroacetic acid with volume ratio (1:3:1:5:0.001) is successful to isolate five ACNs from mulberry fruit (Du et al. 2008). The polymeric ACNs and diglucosides are eluted faster with hydrophobic solvent systems; while less hydrophobic mobile phase enables the elution of monomeric ACNs. The separation also depends on B-ring substitution of anthocyanidin, where the acylated ACNs will move faster than non-acylated ACNs in head-to-tail separations (Du et al. 2008; Renault et al. 1997). A multi-step HSCCC method also experimented, where the co-eluting fractions are again subjected to CCC under different conditions to improve their separation. A two-step HSCCC method was successfully developed to separate and purify ACNs from purple sweet potatoes (Ying et al. 2011). In a first step, two compounds were collected with a two-phase solvent mixture containing n-butanol/ethyl acetate/0.5% acetic acid (3:1:4, V/V), at a flow rate of 2.0 mL min^{-1} and injection volume of 300 mg. Further, in second HSCCC refining step, the two compounds were separated and purified using 0.2% trifluoroacetic acid/ n-butanol/ methyl tertiary butyl ether/ acetonitrile (6:5:2:1, V/V) mixture with the upper phase as the stationary phase and the lower phase as the mobile phase at a flow rate of 1.5 mL min^{-1} and an injection volume of 100mg. By this two-step preparative HSCCC method, 63 mg of compound 1 was collected with a purity of 98.5%, and 48 mg of compound 2 was obtained with a purity of 96.7% respectively. Renault and others (Renault et al. 1997) obtained peonidin-3-glucoside and malvidin-3-glucoside from

one step purified fractions from grapes and the pure cyanidin-3-glucoside was isolated in the second step. Cyanidin-3-glucoside (C3G) and cyanidin-3-rutinoside (C3R) were isolated by high-performance CCC using a two-phase solvent system composed of tert-butyl methyl ether/ n-butanol/ acetonitrile/ water/ trifluoroacetic acid (1 : 3 : 1 : 5 : 0.01, v/v) to give pure C3G (34.1 mg) and C3R (14.3 mg) from 1.5 g crude mulberry fruit extract (Choi et al. 2015). Even though preparative scale HSCCC were successfully used to isolate ACNs in several hundred milligrams with higher purity, it is not utilised in large scale due to the toxicity of the solvents used and their harmful effect to human and environment even at the consumption of traces of these solvents (Grigoras et al. 2012).

2.5 EXTRACTION OF HYDROXYCITRIC ACID

Moffett and others have developed a process for the aqueous extraction and purification of (-)-HCA from Garcinia rinds (Moffett et al. 1997) which has been patented (U.S. Patent No. 5,536,516). The aqueous extract was loaded on to an anion exchange column for the adsorption of (-)-HCA, and subsequently, it was eluted with sodium/potassium hydroxide for release of (-)-HCA. The extract was further passed through a cation exchange column to yield a free acid. Jena and others extracted the acid of the rind fruits of G. *Cowa* by using the soxhlet method with acetone and methanol solution (Jena et al. 2002a). A process for large-scale isolation of HCA from the dried rinds of Garcinia *indica,* Garcinia *combogia,* and Garcinia *atorvirdis* (U.S. Patent No. 6,147,228) was demonstrated by Ibrahim and co-workers (Ibnusaud et al. 2000a). In which, the dry rinds of the fruit were sliced and soaked in boiling water for about 20 hrs and aqueous sodium hydroxide was added at around 80°C. Methanol was then added to the extract until two layers are formed. The lower layer contained the sodium salt of HCA, which was separated and neutralized by hydrochloric acid. Further, acetone was added to filter the pure crystals of HCA. The leaves of *Hibiscus* species was also demonstrated by Ibrahim and co-workers using a similar extraction method (Ibnusaud et al. 2000a; b).

The athermal process was employed by Ramakrishnan and co-workers to obtain concentrated HCA using osmotic membrane distillation (OMD). Garcinia

extract was prepared from Garcinia fruit rinds with deionized water. The extract was concentrated using the OMD process in a co-current flat membrane module using a micro porous hydrophobic polypropylene membrane. HCA was concentrated from 6.0–62°Brix, without any formation of HCA lactones. This process involves no phase change and can be operated at ambient temperature and pressure and hence no thermal damage of the desired product takes place (Ramakrishnan et al. 2008) (patent no US 20070154578 A1). The detailed research on the extraction of HCA at different conditions like the extraction time, ratio of solvent (R/L), or other extraction conditions are not much available. The water solvent steamed in a pressure cooker and soxhlet extraction with organic solvents as acetone and methanol are the two main methods adopted for the extraction of HCA in general. The limited reports available on the extraction of HCA from plant sources are summarized in Table 2.2.

2.6 EXTRACTION OF GARCINOL AND ISOGARCINOL

The procedure for the separation of GL from *Garcinia indica* was described in various literature (Sang et al. 2001; Yamaguchi et al. 2000a; b). The dried fruit rind was extracted with ethanol and then fractionated stepwise with 70-80% (v/v) ethanol using preparative octadecyl silica (ODS) column chromatography. The ethanol with the required fraction was concentrated and dried in a rotary evaporator under 50 °C. The dried material was dissolved again in hexane and the solution was cooled at 5 °C for 2 days. The yellow amorphous powder was collected from the solution and washed with cold hexane on a glass filter. After drying in a vacuum desiccator, the amorphous substances were solubilized in hot acetonitrile and recrystallized at room temperature; pale yellow crystal needles were obtained from the solvent. The crystals can be dissolved in many organic solvents such as ethanol, methanol, acetone, dimethyl sulfoxide, acetonitrile, ethyl acetate, chloroform, and hexane, but has very poor aqueous solubility.

In another studies, *Garcinia indica* rinds were first extracted with water to remove HCA. Further, dried powdered rinds were successively extracted with hexane and benzene. The extracts were purified onto a silica gel column using chloroform and methanol as eluent with increasing polarity and all fractions were concentrated

and analysed by TLC. Further, fractions were loaded onto the Diaion HP-20 column and the column was eluted with water, mixtures of water and methanol, methanol, and acetone. The fraction eluted with methanol gave a single spot on TLC, and it was crystallized to get GL compounds (Negi and Jayapraksha G. K. 2006). Further, the dried celite containing the methanol extract was successively extracted with hexane, chloroform, and ethyl acetate respectively, and subjected to column chromatographic separation and obtained the extra yield of GL and IGL from *Garcinia indica* (Kaur et al. 2012). The implemented procedures are a bit tedious and there are no well-established chromatographic methods yet to be used universally for GL extraction. Further, the lower yield, the complexity associated with the processes, and the use of rash chemicals are the disadvantages, which restrict the implementation of the processes on a large scale. The limited reports available on the extraction of GL, IGL, and HCA from plant sources are summarized in Table 2.2.

Table 2.2 The extraction of GL and HCA from plant parts

Sources of Garcinol	**Method**	**References**
Kokum Rinds (*Garcinia indica*)	Extracted in ethanol followed by column chromatography with ethanol gradient as eluent and redissolved in hexane after evaporation which is further recrystallised using acetonitrile to get the yellow crystal from the solvent	(Sang et al. 2001; Yamaguchi et al. 2000b).
	Hexane and benzene extraction and silica gel column	(Negi and Jayapraksha G. K. 2006)
	The dried celite containing the methanol extract was successively extracted with hexane, chloroform and ethyl acetate respectively and after column chromatographic separation	(Kaur et al. 2012).
Sources of HCA	**Method**	**References**
Garcinia indica	The extract was loaded on to an anion exchange column for adsorption of (-)-HCA, and it was eluted with sodium/potassium hydroxide for release of (-)-HCA. The extract was passed through a cation exchange column to yield a free acid	(Moffett 2008; Moffett et al. 1997, 2001) (U.S. Patent No. 5536516)
Garcinia. Cowa	Soxhlet extraction method with acetone and methanol solution	(Jena et al. 2002a)

Leaves of *Hibiscus* species	The dry rinds of the fruit were sliced and soaked in boiling water for about 20 hrs. The aqueous sodium hydroxide was added to the extract at around 80°C. Methanol was added to the extract until two layers are formed. The lower layer contained the sodium salt of HCA, which was separated and neutralized by hydrochloric acid. Further, acetone was added to the filtrate to obtain pure crystals of HCA	(Ibnusaud et al. 2000a; b) (U.S. Patent No. 6147228 and 6127553).
Garcinia fruit rinds	Garcinia extract was prepared from Garcinia fruit rinds with deionized water. The extract was subjected to concentrate using osmotic membrane distillation (OMD) in a co-current flat membrane module using a microporous hydrophobic polypropylene membrane. HCA was concentrated from 6.0–62°Brix, without any formation of HCA lactone in this process.	(Ramakrishnan et al. 2008) (US patent no. US7431951B2)

Few analytical methods are reported for the identification and quantification of GL and IGL in crude extracts of different Garcinia species. A reverse-phase HPLC method has been developed by Chattopadhyay and Kumar for qualitative and quantitative analysis of Xanthochymol and Isoxanthochymol in the fruit rinds and leaves of *Garcinia indica* using PDA detector, LC/electrospray ionization (ESI)-MS method (Chattopadhyay and Kumar 2006). The separation was achieved isocratically on a reverse-phase (RP) C18 column using a solvent system consisting of a mixture of acetonitrile-water (9: 1) and methanol - acetic acid (99.5:0.5) in the ratio of 30:70 as mobile phase at a flowrate of 0.4mL/min and comboginol was quantified using a multiple reaction monitoring (MRM) method. The authors' Song and others reported an HPLC method for polyprenylated xanthones in gamboge resin of G. *Hanburyi* (Song et al. 2007). The reversed-phase (RP-HPLC) method for the novel analysis of garcinol and isogarcinol in Garcinia indica with the internal standard has been developed in 2013. The analysis was carried out on a C18 reversed-phase column, using a mixture of acetonitrile and 0.1% orthophosphoric acid in water as the mobile phase and di-n-butyl phthalate was used as the internal standard (Praveen Kumar et al. 2013).

2.7 AQUEOUS TWO-PHASE EXTRACTION (ATPE)

The exploitation of conventional separation/ purification methods for valuable proteins, enzymes, and other natural compounds like pigments, antioxidants were often limited due to several problems such as a greater number of operational stages, losing biological activity, selectivity, handling, high purification cost and failure for scale-up etc. To overcome these issues, aqueous two-phase extraction, surfactant-based micellar and reverse micellar extraction, and membrane separations were considered for the purification of valuable products like proteins, peptides, natural pigments, enzymes, antioxidants and many others as an individual purification method or in a combination of these processes. These processes may be cost-effective, environment friendly, and possible to scale-up to industrial scale.

Aqueous two-phase extraction is an interesting cost-efficient separation technique with selective separation characteristic and used as the single purification method or used as an early processing step when it is combined and integrated with other operations (Peters 1987). This powerful and versatile downstream processing technique has been employed in the past three decades for the separation of many biomolecules. The primary point of interest in the ATPE strategy is that it generously decreases the number of primary steps of downstream processing. The high-water content (>70 % (w/w) water) in the system, low interfacial tension, non-flammable and safer environment, overcomes limited solubility and denaturation problems were the additional advantageous associated with the process. Furthermore, ATPE offers many distinct advantages over other purification processes such as high throughput, low process time, low energy requirements, the scope for continuous operation, good resolution, high separation yield and the ability to recycle the phase forming components (Patil and Raghavarao 2007; Raghavarao et al. 1998).

ATPS can be formed by combining aqueous solutions of two incompatible polymers or polymer/ salt or surfactant/ salt or ionic liquid/ salt or alcohol/salt or acetonitrile/polyols at a concentration above a critical level. Either of the phase forming component predominates in a particular phase (Top or bottom) with an enriched water content of 70- 80% in both the phases (Großmann et al. 1995;

Iyyaswami et al. 2012; Zafarani-MoattarTaghi and Salabat 1998; Zhao et al. 2011). PEG is majorly used as one of the phase forming components due to its non-toxic, non-immunogenic, non-antigenic, and highly soluble nature in water and other solvents. These PEG-based systems were widely reported in the literature which is extensively studied for partitioning and purification of various biomolecules ranging from proteins, nucleic acids, enzymes to antioxidants and antibodies, nanoparticles, pigments and also heavy metals recovery from microbial, plant and industrial wastes (Asenjo and Andrews 2011; de Barros et al. 2016; De Brito Cardoso et al. 2013; Hamta and Dehghani 2017; Jiang et al. 2009; Kalaivani and Regupathi 2013, 2015; Kula 2012; Liu et al. 2012; Luechau et al. 2009; Mehrnoush et al. 2012; Nagaraja and Iyyaswami 2015; Nagaraja and Regupathi 2013; Reis et al. 2012; Rosa et al. 2009; Santos et al. 2016; Saravanan et al. 2007) due to its mild environment, provides biostability and biocompatibility. For the scale-up operation, polymer/ salt systems are more preferable than polymer-polymer ATPS as they offer lower viscosity, lower interfacial tension, and higher density difference between the phases, low chemical cost, and faster phase separation. Besides, enriched biomolecules can be easily separated by simple dialysis techniques. A particular concentration of salt and polymer is required to effectively partition a specific biomolecule and faster phase separation, which depends on the molecular weight and type of polymer and type of salt and it's salting-out strength, which depends on its position in Hoffmeister series. The hydration of polymers in the system is highly responsible for the phase separation (Raghavarao et al. 1995). Despite their huge applications some issues like the high cost of the polymers, problems due to their higher viscosity and difficulties in the separation of the polymer by back extraction and recycling issues made them less economic to use in the large-scale application (Aydoğan et al. 2011).

The different combinations of phase forming compounds have been successfully identified and studied for the replacement of the traditional ATPS, such as the pairs of alcohol–salt, acetonitrile–salt, ionic liquid–salt, ionic liquid–polymer and ionic liquid–carbohydrate (Dilip et al. 2016; Freire et al. 2011; Neves et al. 2009; Pereira et al. 2013; Reis et al. 2012). Surfactant/ salt and ionic liquid/ salt systems were recently developed as a novel ATPS; however, these systems are highly suitable

for low volume and high-value compounds like recombinant technology products, pharmaceutical, aromatic compounds, and enzymes. Further, these ATPS may not suitable for large scale applications due to the higher price of the surfactant and ionic liquids when compared to the polymers.

ATPS based on acetonitrile-carbohydrates and acetonitrile-polyols has also been reported where sugars like mono- and disaccharides are used to prepare novel ATPS with acetonitrile (De Brito Cardoso et al. 2013, 2014). Different carbohydrates were investigated, including monosaccharides (glucose, mannose, galactose, xylose, arabinose, and fructose), disaccharides (sucrose and mannose) and also commercial food-grade sucrose, fructose, and glucose. The acetonitrile/carbohydrate system has been applied to partition the common antioxidant, vanillin. The vanillin extracted is migrated towards the acetonitrile rich phase with partition coefficients higher than 3.0, and vanillin has been recovered up to 91 % in a single-step ATPS. Carbohydrates are proven to be a potential substitute for inorganic salts and polymers in the formation of new ATPS. Novel aqueous two-phase systems based on tetrahydrofuran and carbohydrates have also been reported in 2017 (Sousa et al. 2017).

2.7.1 Partitioning of natural compounds in ATP systems

ATPS has been successfully used for separation and purification of various biomolecules such as enzymes/ proteins (Nadar et al. 2017; Perumalsamy and Murugesan 2012), and human antibodies (Rosa et al. 2007). The ATPS are also utilized to extract some of the value-added biomolecules from various sources such as invertase from tomato, papain from papaya latex, α and β amylases from Zea mays malt, lysozyme from chicken egg white (Goja et al. 2013), proteins from cheese whey (Kalaivani and Regupathi 2013), bromelain and polyphenol oxidase from pineapple, α-lactalbumin and β-lactoglobulin from whey, therapeutic proteins from tobacco extract (Aguilar and Rito-Palomares 2010), etc. It is also employed for the separation of proteins from various wastewaters importantly globular and soluble proteins from tannery wastewater (Saravanan et al. 2006, 2007), cheese whey proteins from dairy effluents (Perumalsamy and Murugesan 2012), value-added proteins from shrimp waste (Ramyadevi et al. 2012) and fish proteins from fish industry effluent. Recently,

ATPS is also successfully applied for the extraction and partial purification of natural pigments from different plant sources such as ACNs from red cabbage, jamun fruits, grape juice, Blueberry, Mulberry etc., (Jampani and Raghavarao 2015; Sang et al. 2018; Tang et al. 2017; Wang et al. 2014b), betacyanin from beetroot extract (Chandrasekhar et al. 2015; Chethana et al. 2007), C-phycocyanin from *Spirulina platensis* (Chethana et al. 2015; Patil et al. 2008; Zhao and Jia 2014). Extraction and recovery studies were performed using ionic liquid-based aqueous two-phase systems for natural red colourants from the fermented broth of *Penicillium purpurogenum* DPUA 1275 (Ventura et al. 2013). The aqueous two-phase extraction of biomolecules in the bench-scale prototype has been successful with potential commercial applications. However, the scale-up of the ATPS of biological products were not well reported in the literature (Goja et al. 2013).

2.7.2 Alcohol-salt based ATP systems

The alcohol/salt ATPS is formed by mixing alcohol and salts solutions (Wang et al. 2011). The systems composed of ethanol/ 1-propanol/ 2-propanol with salts like $(NH_4)_2SO_4$, K_2HPO_4, Na_2SO_3, NaH_2PO_4 were used in recent years for extracting and purifying phenolic compounds and polyphenols (Feng et al. 2015), lignans (Cheng et al. 2016), allicin from garlic (Li et al. 2017), anthocyanins (Liu et al. 2013a; Nainegali et al. 2017; Sang et al. 2018), anthraquinones derivatives (Tan et al. 2013), alkaloids like capsaicin (Cienfuegos et al. 2017). The alcohol/salt ATPS has been used to purify the intracellular human recombinant interferon-α2b (IFN-α2b) from E. coli with nine biphasic systems with a combination of alcohol-based top phase (ethanol, 1-propanol and 2-propanol) and salt-based bottom phase (ammonium sulphate, potassium hydrogen phosphate, and monosodium citrate). The 18 % (w/w) of 2-propanol and 22 % (w/w) of ammonium sulphate in 1.0 % (w/w) of sodium chloride found to be optimum with purification factor and yield of 16.2 and 74.6 % respectively (Lin et al. 2013). An alcohol-salt-based ATPS was also applied to purify serine proteases from mango peel and 2, 3-butanediol from fermentation broths using an ethanol/ammonium sulphate system with a highest partition coefficient (7.10) and recovery of 2,3-butanediol (91.7%) were obtained by a system composed of 32% (w/w) ethanol and 16% (w/w) ammonium sulphate (Yau et al. 2015). The new system called microwave-

assisted aqueous two-phase extraction (MAATPE) has been formed by zhang and others to utilize the di-electric constant effect along with the ATPE for better extraction and demixing effect. This method is applied for the extraction of alkaloids from *Sophora flavescens* using ethanol/ammonium sulphate system, which showed low viscosity, easy demixing and solvent recycling. The MAATPE process is claimed to be environmental-friendly process compared to other ATPS, which are mostly high viscos and will cause difficulty to form transparent solutions in the preparation of ATPS (Zhang et al. 2015). By removing the alcohol through evaporation, the target protein can be easily extracted and recovered after the ATPS partitioning. The alcohol/salt ATPS has less toxicity impact to the environment and cheaper compared to the conventional ATPSs (Goja et al. 2013; Yau et al. 2015).

2.7.3 Differential Partitioning of ACNs in the ATPE Systems

2.7.3.1 PEG-based systems

ATPE has been applied for the separation of ACNs because of its mild conditions and high capacity. The ATPSs used for the extraction of ACNs till now are summarized and reported in Table 2.3. ACNs showed higher stability and partitioning towards the PEG rich top-phase in the PEG-salt system formed with ammonium sulphate, magnesium sulphate, and sodium sulphate salts because the pH of these systems was around 5 (where ACNs are stable). Degradation was the drawback with other salts like potassium phosphate, sodium citrate studied because of higher pH (Jampani and Raghavarao 2015). Mostly hydrogen bonds may play an important role to drive the ACNs to PEG rich top-phase. ATPE based on PEG-4000/ $MgSO_4$ and PEG-6000/ $MgSO_4$ was found to be the most suitable system for effective differential partitioning of red cabbage and Jamun fruit ACNs towards top phase, contaminants, and sugars towards bottom phase (Chandrasekhar and Raghavarao 2015; Jampani and Raghavarao 2015). PEG 6000/ magnesium sulphate with the composition of 16.0/ 10.9 % w/w was found best with TLL of 34.2% and 0.76 phase volume ratio, partitioning almost 100 % of anthocyanins in PEG-rich (top) phase and removing 72.9 % sugars to the salt-rich (bottom) phase, thus purifying ACNs. Multistage ATPE enhanced the removal of sugars to 96.1 % (Chandrasekhar and Raghavarao 2015). Similarly, multistage ATP studies for red cabbage ACNs showed the yield of ACNs

and sugars in the PEG-rich (top) phase about 91.35% and 3.56%, respectively after the fourth stage with the composition of PEG 4000/magnesium sulphate (14.8%/10.3% w/w), tie line length of 32.61% and the volume ratio of 0.73 (Jampani and Raghavarao 2015). It is important to concentrate/ purify these pigments using evaporation or membranes etc. after the partitioning. The stability of ACNs is known to improve when it is concentrated further (Giusti and Wrolstad 2003). Hence, the membrane processes such as OMD and FO were employed after the ATPE step.

2.7.3.2 Alcohol based systems

Ethanol-$(NH4)_2SO_4$ /NaH_2PO_4 /K_2HPO_4 and propanol-$(NH4)_2SO_4$ /$MgSO_4$ /Na_2SO_3 /$MnSO_4$ /$Na_3C_6H_5O_7$ combinations were studied for the partitioning of ACNs where K_2HPO_4 and $Na_3C_6H_5O_7$ systems showed grey colour precipitation because of higher pH and are not preferred. Among various alcohol systems, ethanol–ammonium sulphate and propanol-magnesium sulphate systems are well reported for the separation of ACNs from mulberry (Wu et al. 2011), purple sweet potatoes (Liu et al. 2013a), blueberry fruits (Hua et al. 2013), grape juice (Wu et al. 2014) and kokum fruits (Nainegali et al. 2017). The list of ATPE systems used for ACN purification are given in Table 2.3. The ethanol concentration of about 25 to 30 % w/w with ammonium sulphate ranging from 19 to 22 % w/w found in three cases studied for different sources. Five different extraction methods for ACNs were compared in case of V. *uliginosum* residue like extraction in water at room temperature (pH=3.03), extraction in 19% (w/w) $(NH4)_2SO_4$ solution at room temperature (pH=3.03), extraction in 50% ethanol solution at room temperature (pH=3.47), extraction in acidified 50% (w/w) ethanol at 50°C (pH=3.47) for 2 h (Heat reflux extraction by ethanol solution), and aqueous two-phase extraction with 30% (w/w) ethanol/19% (w/w) $(NH4)_2SO_4$ system at room temperature, respectively. The yields of these methods were 7.56%, 42.78%, 51.86%, 100.00%, and 92.34%, respectively. ATPE, the yield was slightly lower compared to heat reflux extraction by ethanol solution because of small amounts of ACNs remained in the bottom phase of ATPS. It is showed that ATPE having advantages of no heating and higher removal efficiency of sugars and proteins with a yield of 4 times higher concentrated ACNs in the top phase. Multistep ATP extractions are used to enhance the recovery of ACNs but there

was no obvious increase seen as most of the molecules separated in a single step or two steps (Wu et al. 2011) and decrease in recovery was also observed in some cases (Wu et al. 2014). Therefore, multiple extractions in ATP were efficient to remove sugars from top-phase at each stage and concluded to be used till two or three steps depending upon the ACN's retention in top-phase. In the ATPE of grape ACNs (GA), the removal of the total sugar was 75% in the first step, but in successive after the third step, it reached nearly 98.81% and ACNs recovery was 99% which is decreased to 95.60% in the second step itself (Wu et al. 2014). Similarly, the top phase containing a small amount of free sugars about 6.8 mg from the first partition reached 3.4 mg and 1.9 mg after second and third partition, respectively during the ATPE of mulberry ACNs (Wu et al. 2011). Numbers of steps is chosen to retaining ACNs in a good amount in top-phase and remove the sugars by minimum level.

Ethanol-ammonium sulphate system is well-studied system under the alcohol-salt category for ACNs but the range of system combinations formed is very narrow (lesser two-phase area). The concentrations of salt ranging from 15 to 26% and ethanol from 20 to 30 % can work out in some minimum combinations (Qin et al. 2017) like when ethanol concentration is fixed at 20%, the concentration of $(NH4)_2SO_4$ should be lower than 26%, higher than this will salt out the system. An ultrasound-assisted ATPE method was used recently for the extraction of ACNs from *Lycium ruthenicum Murr* applying ethanol/ammonium sulphate system (Qin et al. 2017). The optimal conditions obtained as a salt concentration of 20%, an ethanol concentration of 25%, extraction time of 33.7 min, extraction temperature of 25°C, a liquid/solid ratio of 50:1 w/w, pH value of 3.98, and an ultrasound power of 600 W. When comparison made between solvent extraction, UAE, APTE and UA-ATPE the ATPE and UA-ATPE proved to be better for the purification and gave significant purity of 25.3 % with a yield of 4.73 mg/g of dry sample, 21.63 partition co-efficient and 98.95 % recovery (Qin et al. 2017). The partition behaviours of total ACNs and different polar ACNs in ATPS containing ethanol- NaH_2PO_4 /$(NH_4)_2SO_4$ from four plant species. The salt NaH_2PO_4 has higher selectivity then $(NH_4)_2SO_4$ giving higher yield in the top phase. The K values of total ACNs significantly differed and followed the series H. *sabdariffa* < M. *atropurpurea* < N. *tangutorun* < L. *ruthenicum*. The

yields are significant about 98.91% of N. *tangutorun* ACNs and 99.84% of L. *ruthenicum* ACNs with 28% NaH_2PO_4 and 26% ethanol (w/w) system (Sang et al. 2018). The phase separation abilities of ATPS among investigated alcohols with most salts fall in order of ethanol < 2-propanol < 1-propanol (Guo et al. 2012a; Wang et al. 2010c, 2011). Propanol-salt systems may also play an important role to separate out natural compounds. In this regard, only one basic attempt has been made to separate ACNs from kokum fruits source recently (Nainegali et al. 2017). 1-propanol - magnesium sulphate system was found to be more beneficial when comparing ethanol and PEG systems for kokum ACNs. Higher concentrations of 1-propanol and $MgSO_4$ of about 30 % (w/w) and 32 % (w/w) showed the yield of ACNs nearly 98.51 % with a partitioning coefficient of 70.36 and reduces 85.92 % sugars in bottom phase in a single step.

Table 2.3 ATPE systems used for separation of anthocyanins

Sources of Anthocyanins	**System and composition (%w/w)**	**Kp**	**Recovery/ Yield (%)**	**pH**	**Reference**
Purple sweet potatoes *(Ipomoea batatas Lam)*	Ethanol (25 %)/Ammonium sulphate (22%) system	19.62	90.02	3.3	(Liu et al. 2013a)
Grape juice	Ethanol (25 %)/sodium dihydrogen phosphate (28%) system	---	99.35	---	(Wu et al. 2014)
Red cabbage *(Brassica oleracea L.)*	Polyethylene glycol 4000/ magnesium sulphate (14.8/10.3%, w/w) system and concentration with forward osmosis	---	508.05 mg/L (10^0 Brix) to 3123.45 mg/L and (43^0 Brix)	---	(Jampani and Raghavarao 2015)
Mulberry *(Morus atropurpurea Roxb.)*	Ethanol (30 %)/Ammonium sulphate (20%) system	3	85.1	4.5	(Wu et al. 2011)
Blueberry *(Vaccinium uliginosum Linn)*	Ethanol (30 %)/Ammonium sulphate (19%) system	10.67	96.09	4.3	(Hua et al. 2013)
Jamun fruits *(Syzygium cumini)*	Polyethylene glycol 6000/ magnesium sulphate (16.0/10.9%)	---	~ 100	---	(Chandrasekhar and Raghavarao 2015)

Kokum Rinds (*Garcinia indica*)	1-propanol 30 % (w/w) and MgSO4 32 % (w/w)	70.36	98.51	3.9	(Nainegali et al. 2017; Nayak et al. 2010b)
Dulan (*Lycium ruthenicum Murr*)	**Ultrasound-assisted aqueous two-phase extraction** Ethanol 25% and ammonium sulphate 20%, extraction time of 33.7 min, extraction temperature of 25°C, a liquid/solid ratio of 50:1 w/w, pH value of 3.98, ultrasound power of 600 W	21.63	Yields of anthocyanins reached 4.71 mg/g dry sample	3.98	(Qin et al. 2017; Zheng et al. 2011a)
Grape Juice (Pinor Vermei type)	**L44-NaH2PO4** Optimal conditions: L44 - 26%, NaH2PO4 - 24%, Grape juice - 4.1 g in total quality of 15 g, no adjustment of pH, 10^0 C in thermostatic water bath for 12 hr incubation.	K_1 for anthocyanin-998 and K_2 for VLP 0.18.	Anthocyanin yield reached 99.8% in top phase and 90.3% vitis linn polysaccharide (VLP) in bottom phase.	---	(Tang et al. 2017)
1. *Hibiscus sabdariffa* L. 2. *Morus atropurpurea* Roxb. 3. *Nitraria tangutorun* Bobr. and 4. *Lycium ruthenicum* Murr. ATPE for both crude (total) and purified main anthocyanins was performed.	**NaH_2PO_4 /$(NH_4)_2SO_4$ - ethanol** aqueous two-phase systems (ATPS) optimum conditions: Extraction at 65 °C for 45 min and at 45.5 °C for 45 min using 28% NaH_2PO_4 and 26% ethanol (w/w) NaH_2PO_4-ethanol ATPS gave higher selectivity and total anthocyanin yield than the $(NH_4)_2SO_4$ - ethanol system.	K values of total Anthocyanins in 4 species **1 < 2 < 3 < 4**	Recovery of 98.91 ± 0.03% of N. *tangutorun* (3.62 ± 0.05 mg/g) and 99.84 ± 0.01% of L. *ruthenicum* anthocyanins (13.16 ± 0.29 mg/g) from raw material 70% of total sugars were removed in a single step.	---	(Sang et al. 2018)

2.7.4 Factors Influencing Partitioning of ACNs in the ATPS

The movement of the desired biomolecule in an ATPS depends on the biomolecule properties itself and various system properties like the type and properties of the phase forming components, ionic strength, hydrophobic characteristics, and other physical and chemical properties, system pH, TLL, phase volume ratio etc., (Brooks et al. 1985; Chandrasekhar and Raghavarao 2015; Peters 1987). According to the reports, the chemical structures of ACNs vary with the pH value of the solution. If the pH value of the solution is higher than seven (in an alkaline environment), the ACNs would degrade depending on their substituent groups. The selection of a suitable salt is necessary for the efficient separation and purification of ACNs. Mainly ATPSs composed of different salts namely potassium phosphate, sodium citrate, ammonium sulphate, magnesium sulphate, sodium dihydrogen phosphate and sodium sulphate along with PEG polymer, propanol and ethanol were reported and discussed elsewhere. The ATPS formed with salts like $(NH_4)_2SO_4$ solution (pH 4.10–5.30), NaH_2PO_4 and $MgSO_4$ solution (pH 3.50–4.10) and Na_2SO_4 (pH ~5.00) and 5.5 for PEG or 6.50 or more for alcohol, provide an acidic environment, which can protect the ACNs from degradation. But in the case of potassium phosphate (pH 7 to 8) and sodium citrate (pH 8 to 9) degradation of ACNs was observed. The pH influence studies from 1 to 5 revealed that the pH of ATPS has an important influence on partition behaviour (Wu et al. 2011). The partition coefficient of mulberry ACNs increased from 1.5 to 3.7 and the recovery varied in the range of 73–83% with the pH increasing from 1 to 5. At the same time, the partition coefficient of sugars was reduced from 0.7 to 0.1, and their recovery in the bottom phase was improved from 43% to 90%. The highest pH observed to be 4.5 at optimal other conditions, which showed the highest recovery of mulberry ACNs in the top phase. Liu and others discussed a similar increased pattern of K from pH 3 to 4 which was possibly due to the gradual weakening of the binding force of the top phase with decreasing pH. However, the increase in pH can increase the electrical potential, while the electric potential was proportional to the partition coefficient, but the effect was not that much dominant (Liu et al. 2013a). Similar findings were reported for *Lycium ruthenicum Murr* ACNs with increasing pH from 2.0 to 4.0, the yield also increased and further decreased at 5 and beyond (Qin et al. 2017). All three

investigations are supported each other and may work as mentioned above in case of pH (Guo et al. 2012b; Liu et al. 2013a; Qin et al. 2017; Wu et al. 2011) but recovery almost remained unchanged and got optimum values with no pH adjustment or the pH range of 2.0–4.2 for NaH_2PO_4 according to Yingchun Wu and team (native pH 3.31). On one hand, the addition of acid or alkaline solution can change the existing form of NaH_2PO_4 in the ATPS, which will further influence the phase equilibrium and the partition behaviours of ACNs and sugars. They claimed that the change of system pH will lead to adverse effects in the process (Wu et al. 2014). The temperature of the system has a minor influence on the partitioning of ACNs and sugars. Sugar partitioning in the bottom phase is higher at elevated temperature compared to ACNs in top-phase because at a higher temperature more salts are dissolved in the increased phase volume to accommodate more sugars. In case of mulberry ACNs purification, recovery of sugars reached 89.5% at 35 ºC and the partition coefficient of mulberry ACNs hardly varied with a value of 2.0–2.3 and the recovery fell in a narrow range of 73–76% when the temperature increased from 25 to 50 ºC (Wu et al. 2011) .

The increase in PEG molecular weight showed an increase in the transfer of ACNs towards the polymer-rich phase till 6000 and decreased with further increase. Sugars showed the opposite trend as the yield of sugars in the top phase as well as partition coefficient were found to decrease with an increase in the molecular weight of PEG (Chandrasekhar and Raghavarao 2015). Similar findings were obtained in the case of betacyanin pigments from *Beta vulgaris* with a PEG 6000/ammonium sulphate system (Chethana et al. 2007). When the molecular weight increases hydrophobicity in top-phase increases along with the decrease in relative free volume, which may be the reason for the limited transfer of ACNs to bottom-phase. Similarly, the phase components and TLL has a significant effect on the partitioning of ACNs (Chandrasekhar and Raghavarao 2015; Jampani and Raghavarao 2015).

In case of alcohol systems, an increase in alcohol and salt concentrations (TLL) made ACNs to transfer towards top-phase and sugars/ polysaccharides to the bottom phase (Hua et al. 2013; Nainegali et al. 2017; Sang et al. 2018). At higher ethanol and 1-propanol concentrations, an increase in salt concentrations led to the good recovery and partition co-efficient (Qin et al. 2017; Wu et al. 2011). It was

observed from the reports that the higher salt concentration makes the lower content of ACNs in the bottom phase because of its lower solubility in the bottom phase at higher salt concentration and maybe also the salting-out effect. The dissolving capacity of ACNs in the top phase is related directly to the alcohol concentration and seen that higher concentrations are preferred for better partitioning. The partition behaviour is similar in the case of ATPE performed with ethanol and ammonium sulphate for phenolic compounds from fig fruits (Feng et al. 2015) and ACNs with ethanol- NaH_2PO_4 (Sang et al. 2018). Much data is not available regarding studies on volume ratio except for one report. Increase in phase volume ratios (0.39, 0.76, 2.05, and 5.3) increases the yield of ACNs from 92.8% to 100% and remained practically constant with the further increase but sugars in the salt-rich phase decreased with an increase in the volume ratio which can be attributed due to the increase in top phase volume. The polarities and hydrophobicity of ACNs also play a role in partitioning, proved recently by sang et al. (Sang et al. 2018). Acylated ACNs are less polar and more hydrophobic. The ACNs with lower polarity and higher hydrophobicity exhibited a relatively high distribution ratio in ATPS. The low-polarity acylated ACNs were more suited for extraction using ATPE containing the ethanol-salt system (Sang et al. 2018). As a summary, the nature of ACNs in plant species, the concentration of phase components, size of polymer, pH, and TLL have major effects for ACNs which should be optimised for successful purification.

2.7.5 Simultaneous Aqueous Two-phase extraction of multiple compounds

ATPS offers the simultaneous selective partitioning of multiple compounds in a single step. Simultaneous extraction of oil and soy isoflavones from soy sauce residue, selective separation of flavones and sugars from honeysuckle, alkaloids from *Sophora flavescens* Ait, ACNs and Vitis linn polysaccharide from grape juice and extraction and enrichment of ACN, HCA, GL, IGL from *Garcinia indica* are few examples (Chen et al. 2014c; Liu et al. 2013b; Nainegali et al. 2019; Tang et al. 2017; Zhang et al. 2015) (Table 2.4). Grape juice is a rich source of vitis linn polysaccharides (VLP) compounds and ACNs as well. An attempt was made to separate these two components with L44 and NaH_2PO_4 two-phase system (Tang et al. 2017). The aromatic structures of ACN result in more hydrophobic than VLP.

Therefore, ACN transferred to the L44 rich phase because of the interaction between the aromatic structure of ACN and hydrophobic groups of L44 polymer. But the concentration of L44 showed less effect on the strong hydrophilic VLP hence retained in the bottom phase. The L44 concentration of 26% and the salt concentration of 24% were found to be optimum to yield 99.8% and 90.3% respectively. L44 was recycled in secondary ATP for the top phase to yield pure ACNs and dialysis was applied to the bottom phase to concentrate VLPs by removing impurities. The partition behaviours of different polar ACNs have been studied in ethanol- $(NH_4)_2SO_4$/ NaH_2PO_4 which paved the way for the selective separation of even different types of ACNs to either of the phases formed by the alcohol-based ATPS (Sang et al. 2018).

Table 2.4 Selective separation and simultaneous extraction of compounds in in ATP systems

Sources of compounds	Selective and Simultaneous extraction in ATP System	Partitioning and recovery in phases	Reference
***Sophora flavescens* Ait**	Microwave-assisted ATPE Ethanol 28% (w/w) and $(NH_4)_2SO_4$18% (w/w), solvent-to-material ratio 60:1, temperature 90 °C, extraction time 5 min, and microwave power 780 W	Mainly oxymatrine, matrine compounds and total alkaloids partitioned to top phase	(Zhang et al. 2015)
Honeysuckle powder	Single ATPE under the optimized condition of 4.66 g NaH_2PO_4, 36% ethanol (v/v, %) and pH 5.0 at 25 °C	Flavones in top phase and sugars in bottom phase	(Liu et al. 2013b)
Soy sauce residue	Ultrasonic-assisted two-phase solvent extraction Ethanol concentration -74.88%, ratio of ethanol/water phase to raw material 15:1, ratio of hexane phase to raw material 8.64:1, extraction time 20 min, and ultrasonic power 160 W.	Oil and Soy isoflavones in ethanol phase	(Chen et al. 2014c)
***Schisandra chinensis* Baill**	ATPS composed of 21% (w/w), ammonium sulphate and 22% (w/w) ethanol	Schisandrol A, Schisantherin A, Deoxyschisandrin, Schisandrin B and Schisandrin C Five types of lignins in ethanol rich top phase	(Cheng et al. 2016)
***Aloe vera* L.**	Ionic liquid based aqueous two-phase system	Aloe polysaccharides in bottom phase and proteins in top phase	(Tan et al. 2013)

Grape juice	L44 and NaH_2PO_4 two-phase system	ACNs transferred L44 rich polymer phase Hydrophilic vitis linn polysaccharides retained in bottom phase.	(Tang et al. 2017)

2.8 CONTINUOUS ATPE OF BIOACTIVE COMPOUNDS

Most of the ATP extractions both in lab-scale and higher scale were studied to date in batch mode, whereas continuous studies are limited. The knowledge developed in the batch process has to be utilised to develop the technology for continuous processes. As there is a huge demand that exists for the bioactive compounds in the market, the ATPE has to be implemented in a continuous or semi-continuous operation that has clear competitive advantages like reducing process time and costs and increasing process yields with higher efficiency (Espitia-Saloma et al. 2014). Meanwhile, different types of liquid-liquid extractors have been utilised for the continuous extraction of biomolecules in ATPE but are in limited numbers. The column extractors are the most studied and preferred extractors due to their reduced operational cost. Further, these column extractors are of agitated and non-agitated types. Spray and packed column extractors fall under non-agitated columns and used for the continuous extraction of BSA, other proteins, and enzymes (Jafarabad et al. 1992b; Rosa et al. 2012; Srinivas et al. 2002). The extractors like perforated rotating disk contactor, rotating disc contactor, sieve plate column, raining bucket contactor, york – Schiebel column etc. are the agitating column extractors and consist of a series of discs, turbines, caps or vanes attached to a central shaft which are responsible for the dispersion, coalescence, and breakage of drops. The drop breakage and their coalescence simultaneously happen in a single compartment made by the stator rings (Cavalcanti et al. 2008; Giraldo-Zuniga et al. 2006; Igarashi et al. 2004b; Jafarabad et al. 1992a; Kalaivani and Regupathi 2016; Porto et al. 2000, 2010; Sarubbo et al. 2003, 2005).

2.8.1 Continuous ATP extractors

To achieve a continuous extraction process, specific equipment is required that can efficiently mix the phase components to generate the phase dispersion and separate them into two phases (salt-rich continuous phase and PEG rich dispersed phase) continuously. The continuous phase separation was achieved under gravitational or centrifugal force. Many researches attempted to develop and study the conventional organic-aqueous phase extraction in the equipment such as spray column (Jafarabad et al. 1992b; Srinivas et al. 2002), packed bed column (Rosa et al. 2012), york-scheibel (Jafarabad et al. 1992a), graesser raining bucket contactor (Giraldo-Zuniga et al. 2006), sieve plate column (Igarashi et al. 2004b) and perforated rotating disc contactor (PRDC)/ rotating disc contactor (RDC) (Cavalcanti et al. 2008; Kalaichelvi and Murugesan 1998; Kalaivani and Regupathi 2016; Porto et al. 2010; Sarubbo et al. 2003, 2005) for the extraction of a verity of molecules. Later these equipment were modified and utilized for the extraction of biomolecules in the ATPS since both the extraction processes are similar. The selection of extraction column for ATPE depends on the biomolecule properties (hydrophobicity, molecular size, electrochemistry, and molecular confirmation) and type of system (polymer/ polymer or polymer/ salt) (Cavalcanti et al. 2008).

The spray columns, packed columns, York – scheibel contactors, and the static mixer configurations or mixer settlers were studied in the literature for the purification of biomolecules using ATPS. The spray columns were implemented to investigate the efficacy of the extraction of pure proteins evaluated in terms of hold up (H) and dispersed phase mass transfer coefficient ($K_D a$). Jafarabad et al. 1992 investigated the effect of column height, phase composition, and variation in the number of sparger holes, and dispersed phase velocity on H and $K_D a$ of BSA and amyloglucosidase using PEG/potassium phosphate and PEG/sodium sulphate. It was found that the increase in sparger holes and dispersed phase velocity increases the H and $K_D a$ whereas an increase in phase composition decreases H and $K_D a$. However, the extraction parameters were not influenced by the column height (Jafarabad et al. 1992b). The increase in dispersed phase velocity increases the H and $K_D a$ in presence of amyloglucosidase and alpha-galactosidase using PEG/sodium sulphate system. The

increase in the concentration of sulphate and PEG showed a decrease in extraction parameters studied. Srinivas et al (2002) studied the effect of NaCl concentration, orifice size, and dispersed phase superficial velocity on $K_D a$ and H for horseradish peroxidase using PEG/potassium phosphate and found that the increase in dispersed phase velocity, orifice size, and NaCl concentration increases the $K_D a$ and Hold up (Srinivas et al. 2002). A similar observation was found for xylanase mass transfer studies in aqueous two-phase systems using spray and sieve plate columns (Igarashi et al. 2004b).

Igarashi et al. (2004) investigated the performance of packed columns in xylanase extraction using PEG 4000 and dipotassium phosphate. The behaviour of xylanase extraction under the influence of dispersed and continuous phase velocities and in the presence of different packings such as raschig rings, glass spheres, and polystyrene rings was studied. Irrespective of packing material, $K_D a$ was increased with the increase in salt phase velocity. The mass transfer efficiency depends on the size of the packing material and the efficiency of extraction increases with the decrease in the size of the packing material. The extraction of xylanase increased with polystyrene rings of 3mm (75%) in comparison with 6 mm rasching rings and 5mm glass spheres (Igarashi et al. 2004a). The continuous extraction of human immunoglobulin from the supernatant of Chinese hamster ovary (CHO) was studied using PEG 3350/phosphate aqueous two-phase system. The wetting studies were conducted based on which stainless steel was selected as a material for the column packing bed. The mass transfer process was explained in terms of Whitman's two-film theory. Hydrodynamic studies were conducted and the experimental data was related to the holdup values using the Richardson–Zaki, and Mísek equations, generally used for the organic-solvent systems. Pump mixer-settler combination with packed column and the high flow rates favours the mass transfer of IgG (Rosa et al. 2012).

The continuous extraction of Bovine serum albumin (BSA) and amyloglucosidase in York – scheibel contactor was investigated with PEG 4000/potassium phosphate and sodium sulphate systems. The effect of column height, phase composition, impeller speed, and dispersed phase velocity on H and $K_D a$ was studied. It was observed that the values of H and $k_D a$ were independent of column

height and increase with the impeller speed but decrease with the phase composition (Jafarabad et al. 1992a). Some static mixer configurations or mixer settlers as novel separators are used for continuous extraction recovery of whey proteins (α-La and β-Lg) from whey protein extract (Vázquez-Villegas et al. 2011). The ability of the novel separator (consists of static mixer before tubular separator) for the partitioning of biomolecules was evaluated using dyes by optimize the tubular length and static mixer configurations. The model protein's (BSA) partition behaviour was also studied and then the system was implemented for the whey protein extraction. Both the proteins have different affinities for phase systems where α -Lg has more affinity towards the PEG phase and β -La towards the salt-rich phase. The maximum recovery of 90% of the proteins was observed in top and bottom phases with minimal precipitation at the interface. It was reported that the length of the tubular separator increases the interfacial contact between the phases and thereby increases the efficiency of separation (Vázquez-Villegas et al. 2011). Rosa et al. (2013) attempted to purify human immunoglobulin from the recombinant cells in the pilot plant mixer settler device. The set up consists of some mixer settler units as multi-stage, back extraction and washing. Cell culture supernatant was added to the phosphate phase and immunoglobulins were extracted to the PEG phase. The extracted immunoglobulins from different cell types resulted in more than 80% recovery and 99% purity (Rosa et al. 2013). Muendges and co-workers studied the continuous extraction of immunoglobulin GI in PEG/phosphate salt using a multistage mixer settler unit. Experiments were conducted with and without the addition of NaCl where, without NaCl, the extraction was performed as a washing step (washing of bottom phase repeatedly transfers contaminants towards top phase) whereas with NaCl extraction was performed as a stripping step. The immunoglobulin G1 has an affinity to the salt phase without NaCl and yields nearly about 90% recovery and 32% purity. Whereas with the addition of NaCl, immunoglobulin G1 showed affinity to the top phase. The performance of the extractor was analysed by varying factors such as throughput, phase ratio, and stage number. The purity of the immunoglobulin G1 increases with the increase in the number of stages from one to five and with the increase of the product phase volume and yields nearly 90% recovery and purity of 32% (Muendges et al. 2015).

2.8.2 Rotating disc contactor (RDC)

RDC is the major extraction column, which has been widely used in the process industries for the liquid-liquid extraction process. RDC consists of a cylindrical column, having several compartments created by a series of stator rings and the center rotor discs placed in each compartment and attached to a central rotating shaft. The central shaft is driven by an electrical motor and the speed of the motor is controlled by DC power supply. The continuous phase outlet is fitted with an adjustable limb, to maintain the interface level in the column. The lighter phase is introduced at the bottom of the column, and the heavier phase introduced at the top of the column, thus the counter-current flow is established due to the density difference between the phases. As the disc rotates, it generates toroidal vortices in each compartment causing turbulence in the liquid, in proportion to its speed. The performance of RDC is mainly affected by column diameter, rotor disc diameter, stator ring-opening, compartment height, number of compartments, and disc rotor speed. Hence, the performance of an RDC greatly depends on the hydrodynamic and mass transfer parameters, which play a vital role in the design of successful and efficient extractors (Kalaichelvi and Murugesan 1998; Murugesan and Regupathi 2006).

RDC was extensively used in chemical industries like hydrometallurgy, pharmaceutical, edible oils extractions, aromatics, and allied processes such as sulphur dioxide extraction, propane deasphalting, processing of nuclear fuel and removal of dinitrochlorobenzene from industrial residuals (Ferreira et al. 2007; Murugesan and Regupathi 2004). This influenced the application of RDC in biological processes like downstream operations to separate value-added molecules from fermentation broths (Kalaichelvi and Murugesan 1998). The RDC is recognized as basic extraction equipment in many process industries like biochemical, environmental, oil extraction from oilseed, and many foods and allied industries for desired product refining and recovery of raw materials.

2.8.2.1 Hydrodynamic studies in RDC

Holdup represents the fraction of the column volume occupied by the dispersed phase, which indirectly indicates the volume of the total drop population in the extraction column at the steady-state operation. Holdup mainly depends on the operating parameters (rotor speed, continuous and dispersed phase flow rates), physical properties (density, viscosity, and interfacial tension) and column internals (column height, disc and stator diameter, number compartments and compartment height) (Kalaichelvi and Murugesan 1998; Kalaivani and Regupathi 2016; Moris et al. 1997). The holdup is a complex function of various factors such as drop size, drop shape, drop velocity, rate of drop coalescence, drop break down, shear, and drag forces in the column. The study of mass transfer inside the LLE columns is of fundamental importance which provides the knowledge on the rate of mass transfer or extraction in LLE equipment's, which is dependent on the mass transfer coefficient, interfacial area, and concentration gradient (Sarubbo et al. 2003). The mass transfer coefficient depends on the rate of diffusion inside, across the interface, and outside the droplet. The mass transfer in the droplet life span in contactor may happen during three different stages which are drop formation at the distributor, drop travel through a continuous phase and column internals, and coalescence of drop at the bulk interface in the separation zone. In most of the agitating columns, these factors play a dependent role in the drop formation, coalescence, and re-dispersion.

2.8.2.2 Holdup studies in aqueous-organic system

Generally, RDC is operated in counter-current mode, in which lighter (dispersed) phase is introduced in the bottom of the column and heavier phase (continuous) introduced into the top of the column. The counter-current phase flow in the column is being developed by virtue of the density difference between the phases. The resulting flow and the rotor speed causes the recirculation and back-mixing of both phases in each compartment. Laddha et al. (1978) reported that the holdup was distinctly different in two regions (region 1: rotor speed less than critical rotor speed and region 2: rotor speed greater than critical rotor speed) and hence the effect of column geometry may be considered in the estimation of critical rotor speed. Critical rotor speed is defined as the rotor speed below the energy spent by the agitator not

being sufficient to overcome the interfacial tension and the breakup of dispersed phase droplets (Laddha et al. 1978). Authors also reported that holdup increases with an increase in rotor speed and dispersed phase flow velocity (Jeffreys et al. 1981).

Moris et al. (1997) studied the hydrodynamic behavior of water – kerosene system in RDC (72 mm in diameter, operating height of 1.1 m, 22 mixing compartments) using the two-phase system water-kerosene. The total holdup was found to increase with rotor speed, increased total throughput, and organic-aqueous phase flow ratio (Moris et al. 1997). Kalaichelvi and Murugesan (1998) carried out an elaborated study on dispersed phase holdup in RDC to understand the effect of various operating parameters such as rotor speed, phase flow rates, column geometry, and system physical properties. Moreira et al. (2005) studied the hydrodynamics of an RDC under low agitation conditions in the water- n-heptane system. Statistical analysis has revealed that the total holdup did not significantly vary with the rotor speed, however, it changed mainly due to dispersed phase flow rate (Moreira et al. 2005).

Kadam et al. (2009) studied the hydrodynamic and mass transfer characteristics of asymmetric rotating disc contactors (ARDC) with various aqueous - organic systems namely Water- Toluene, Aqueous PEG 4000 (13 % w/w) – Toluene, Water - Butyl acetate and Water – Butanol. Overall, dispersed phase holdup was found to increases drastically with increasing impeller speed, linearly with increasing dispersed phase velocity and nominally increasing with continuous phase velocity. A new correlation was proposed by considering the ratio of power input per unit mass to volume of the column (Kadam et al. 2009).

2.8.2.3 Holdup and mass transfer studies for ATPS

The holdup behaviour of ATPS in RDC also follows a similar trend of aqueous – organic systems. Table 2.5 provides the information about the mass transfer and holdup studies reported for RDC/ PRDC in the literature for different continuous extraction process with ATPS. Holdup increases with increasing PEG phase flow rate (dispersed phase), rotor speed as similar to aqueous – organic systems and further the holdup is highly influenced in ATPS due to distinctly different variation in physical properties (interfacial tension, viscosity and density difference between the phases)

with tie-line length (TLL). The increase in physical properties along with the increase of TLLs leads to an increase in the drop size and their velocity which decreases the dispersed phase holdup (Porto et al. 2000). Further, the dispersed phase holdup was found to increase with increasing rotor speed.

Table 2.5 Continuous ATPE of biomolecules in PRDC/RDC extraction systems

System/ Target	Target loaded phase	Operating variables	Outcome	Reference
PEG 550 and 10000/ Potassium phosphate BSA	Dis	Dis (1 – 3 mL/min) Con (1 mL/min) Rotor speed 140 rpm	H: 0.01–0.45 K_Da: 0.03–0.125 1/min	(Porto et al. 2000)
PEG 4000/ cashew-nut tree gum BSA	Dis	Dis (2 – 5 mL/min) Con (1 mL/min) Rotor speed: 60, 140 and 220 rpm	H: 0.28-0.56 K_Da: 0.12–0.28 1/min Opti: 220	(Sarubbo et al. 2003)
PEG 4000/ cashew-nut tree gum BSA	Dis	Dis (2 – 5 mL/min) Con (1 mL/min) Rotor speed: 60, 140 and 220 rpm	SE of 96% at 220 rpm	(Sarubbo et al. 2005)
PEG 550/ Potassium phosphate Aascorbic oxidoreductase	Dis	Dis (1 – 3 mL/min) Con (2 mL/min) Rotor speed: 220 rpm	K_Da: 0.043–0.073 1/min SE: 75–95% PF: 2–35	(Figueiredo Porto et al. 2004)
PEG 8000/ Potassium phosphate α-toxin	Conti	Dis (1 – 3 mL/min) Con (1 – 3 mL/min) Rotor speed: 35, 90 140 rpm	K_Da: 0.003 1/min H: 0.80 PF: 2.4	(Cavalcanti et al. 2008)
PEG (3350, 8000 & 20000)/ sodium citrate Ascorbate oxidase	Conti	Dis (2 mL/min) Con (1 mL/min) Rotor speed: 80 rpm	K: 3.35 PF: 1.46 SE: 54.98%	(Porto et al. 2010)
PEG 1000/sodium citrate α-lactalbumin and β-lactoglobulin	Conti	Dis (1.64×10^{-5} to 9.87×10^{-5}) Con (1.64×10^{-5} to 9.87×10^{-5}) Rotor speed: 1-6 rpm	Rec: 86%, Purity: 85.5% of purity of α-lactalbumin in PEG phase.	(Kalaivani and Regupathi 2016)
Dis: Dispersed phase flow rate, conti: continuous phase flow rate, H: holdup, KDa: mass transfer coefficient, PF: purification factor, SE: Separation efficiency				

The increase in the rotor speed delivers higher shear stress and leads to intense drop breakage near the disc's edge and an increase in the number of smaller drops. Consequently, the drops settle velocity was also found to decrease. The drops should also flow through an additional number of obstacles which further diminish the drops settle velocity (Sarubbo et al. 2003).

Cavalcanti et al. (2008) have reported the extraction of α-toxin using PRDC with maximum hold up value of 0.8. The result of this study demonstrated that the dispersed phase flow rate and rotor speed had a strong influence on the holdup and extraction performance of PRDC. The holdup decreased with increasing PEG molecular weight and concentration, due to the resulting increase in viscosity and the formation of large drops, which favoured the reduction of the holdup (Porto et al. 2010). Coimbra et al. (1998) studied dispersed phase holdup and characteristic velocity in a perforated rotating disc contactor using PEG 6000/ di-basic potassium phosphate system. The dispersed phase holdup experiments were carried on varying operational parameters such as flow free area of discs, rotor speed, and phase flow ratio. It was observed that the dispersed phase holdup was slightly influenced by the flow free area of discs and highly influenced by the rotor speed and phase flow ratio. Porto et al. (2000 and 2010) and Cavalcanti et al. (2008) observed that the dispersed phase flow velocity has a negligible effect on mass transfer coefficient in PRDC. Mass transfer coefficient decrease with increasing TLL due to an increase in physical properties which, in turn, decreases the diffusivity of the protein compromising the mass transfer. Sarubbo et al. (2003) and Porto et al. (2004) reported that the mass transfer coefficient increased with increasing dispersed phase flow rate and rotor speed due to the formation of smaller size drops and lead to a larger mass transfer area (Porto et al. 2010). The increased difference between the density, viscosity, and interfacial tension and total effect of these factors had a significant effect on the mass transfer coefficient in ATPS. The higher physical properties of the phases and the increased properties difference between the phases also restrict the diffusion of proteins (Espitia-Saloma et al. 2013).

Cavalcanti et al. (2008) studied the performance of PRDC to extract α-toxin from the fermented broth of Clostridium perfringens Type A using PEG/ phosphate

salts. The influence of three independent variables, specifically the dispersed phase flow rate (1 – 3 mL/min), the continuous phase flow rate (1 – 3 mL/min) and the disc rotational speed (35 – 140 rpm), were investigated on the holdup and mass transfer coefficient and the variables were further optimized through the statistical method. The separation efficiency and the purification factor were considered as the response variables. The optimized operating conditions α -toxin extraction in PRDC with PEG/ potassium phosphate ATPS were obtained at a dispersed phase flow rate of 3.0 mL/min and a disc rotational speed of 140 rpm. The maximum dispersed phase holdup and mass transfer coefficient values were 0.59 and 0.165 (1/h), respectively. Porto et al. (2010) have reported the extraction of ascorbate oxidase from cucurbita maxima by a continuous process in PRDC using PEG/ sodium citrate system. The influence of three independent variables PEG molecular weight (3,350, 8,000 and 20,000), PEG concentration (10, 15 and 20%) and sodium citrate concentration (15, 20 and 25%), were considered to establish a maximum partition coefficient, hold up, mass transfer coefficient, purification factor and separation efficiency by statistical method. The most favourable conditions for extraction of ascorbate oxidase from cucurbita maxima by PRDC were obtained at a high PEG molar mass of 20,000 and a concentration of 20% and citrate concentration of 15%. The maximum mass transfer and holdup values are 0.0586 1/min and 0.4, respectively.

Recently, conventional RDC was implemented by Kalaivani et al. (2016) for the differential partitioning of α lactalbumin (α-La) and β lactoglobulin (β-Lg) to PEG-rich and salt-rich phase respectively. The hydrodynamic and mass transfer studies were carried out in an ATPS of PEG 1000/sodium citrate. The effect of operating variables such as dispersed and continuous phase velocities and rotor speed were evaluated on dispersed phase hold up, volumetric mass transfer coefficients, and recovery and separation efficiency. The maximum dispersed phase velocity (9.86×10^{-5} m/s) and minimum continuous phase velocity (1.34×10^{-5} m/s) with high rotor speed (above 5 1/s) yields 86% of recovery and 85.5% of purity of α-lactalbumin in PEG phase (Kalaivani and Regupathi 2016).

The conventional perforated rotating disk contactor and rotating disc contactor for ATPE has been tried to utilize for the extraction of few biomolecules as reported,

but not successfully implemented yet in a large scale ATPE processes. Limited work was reported in the literature for RDC/ PRDC hydrodynamics along with the other studies like extraction performance analysis and mass transfer studies for the extraction of a specific biomolecule. Further, there are no reports available for the application of continuous extractors like RDC for alcohol-salt based ATP systems. Hence, there is a scope for the modification of these extractors and their operation to extract the biomolecules on the industrial scale.

Summary of literature review:

- Several research and review articles had been reported on the need and application of natural compounds as additives that can replace artificial/synthetic compounds available in the market for the food, pharma, cosmetic and nutraceutical applications.

- Huge numbers of bioactive compounds are widely spread in nature and more specifically in plants parts like leaves, fruits, vegetables, stem, roots etc. These value-added compounds had tremendous demand in the market due to their biological and functional properties, which are useful in food and pharmaceutical applications. The utilization of natural resources will overcome environmental issues and safer for mankind.

- The literature review suggested that most of the conventional separation methods were used to recover these bioactive compounds from plant sources and the main disadvantages were less purity, more labor, and cost-intensive. Achieving biologically active separation and continuous mode operation method is highly essential to meet the current demand.

- The kokum fruits are a blend of efficient bioactive compounds which include garcinol, isogarcinol, anthocyanins, and hydroxycitric acids which have numerous biological applications. The extraction of these compounds and their purification are well studied using both conventional and nonconventional methods specifically for anthocyanins but very few reports were available for other compounds like garcinol, isogarcinol, and hydroxycitric acids

- The literature survey indicates that the ATPS as a nonconventional and selective technique can be successfully applied for the simultaneous partitioning of two or more compounds from a single natural source. Even though ACNs are partitioned successfully from various sources, the ATPE was not studied for the simultaneous partitioning of the other bioactive components like HCA, GL, and IGL along with ACNs.

- The studies on hydrodynamics and mass transfer studies in the continuous extractors are carried out for non-conventional liquid-liquid extraction. Only a few literatures discuss the continuous mode of ATPE (PEG-based systems) of biomolecules that too mainly for enzymes and proteins. Specifically, no report is available on the implementation of alcohol-salt ATPE in the RDC. The detailed hydrodynamics and mass transfer characteristics are unexplored for RDC with ATPS.

2.9 RESEARCH GAP

- Four important bioactive compounds present in kokum *Garcinia Indica* (GI) fruit rinds namely GL, IGL, HCA, and ACN, which are very less exploited for industrial-scale production and only ACN pigments are under research from GI.

- The individual bioactive compounds GL, IGL, and HCA were tried to extract using complex methods and not used in the industrial scale yet because of the requirement of the associated complexity and the demand of highly pure and stable compounds for the application in food product and drug formulations.

- The extraction of anthocyanin compounds and their purification are well studied using both conventional and nonconventional methods but very few reports were available for the other compounds like garcinol, isogarcinol, and hydroxycitric acids

- The simultaneous extraction and purification of multiple compounds can ease the process cost. However, the conventional techniques separate these compounds individually by losing the other important compounds. The simultaneous extraction followed by separation and fractionation of multicomponent may be difficult due to the complications involved in the simultaneous extraction from the complex crude.

- The well-established methods like solid-phase solvent extraction and adsorption are not feasible for the simultaneous extraction and purification of ACNs, GL, IGL, and HCA in a large scale operation.

- There is a need for the development of economically feasible, scalable, mild downstream processes with the least number of steps, which can further integrate with other advanced purification processes to obtain pure natural compounds.

2.10 SCOPE AND OBJECTIVES OF PRESENT WORK

Kokum (*Garcinia indica*), is an underexploited fruit species distributed throughout the tropical Asian and African countries and has tremendous potential as a colourant and spice with medicinal value. It is found in India in tropical humid evergreen rain forests of the Western Ghats of south India as well as in northeast states of India. These kokum fruits are a blend of efficient bioactive compounds which include garcinol, isogarcinol, anthocyanins, and hydroxycitric acids which have numerous biological applications for humans that can be extracted and incorporated in food and nutraceuticals.

The conventional techniques were used to extract the ACN, HCA, GL, and IGL individually by losing other important compounds. However, the simultaneous extraction of all the four components together and followed by fractionating or purifying the individual components will be beneficial. The literature survey indicates that the ATPS as a nonconventional and selective technique can be successfully applied for the simultaneous partitioning of two compounds from a single natural source.

The application or capability of ATP process for four compounds is not yet analysed and reported in the literature. The detailed study on the ATPS partitioning of four compounds and its feasibility may be explored to understand the mechanism of ATPS by altering the operating parameters using PEG/salt and alcohol-salt ATPS. Only a few documents available on the continuous mode of operation of ATPE. The hydrodynamics and mass transfer characteristics are unexplored for RDC with ATPS which may be studied in the present work.

In this perspective, the following objectives were formulated to study the selective extraction and purification of bioactive compounds from kokum (*Garcinia indica*) fruits using aqueous two-phase systems.

2.10.1 OBJECTIVES

1. To simultaneously extract the four bioactive compounds namely, Anthocyanins (ACNs), Hydroxycitric acid (HCA), Garcinol (GL), and Isogarcinol (IGL) from kokum fruit rinds by identifying a suitable solvent system and to characterize the bioactive compounds present in the crude extract.
2. To select the suitable Aqueous Two-Phase System for the simultaneous partitioning and separation of bioactive compounds and to study the influence of process variables on the differential partitioning of ACN, HCA, GL, and IGL.
3. To optimize the significant process variables using Response Surface Methodology (RSM) to accomplish maximum possible recovery and purity of the desired bioactive compounds.
4. To enhance the recovery and purity of the desired bioactive compounds by employing the second stage ATPE and enrichment steps.
5. To implement the optimized ATPE system in a continuous extractor, Rotating Disc Contactor (RDC), and to study the efficacy of the RDC at different operating conditions by considering the hydrodynamic and mass transfer characteristics.

2.11 ORGANIZATION OF THE THESIS

The thesis is organised into five chapters. 'Chapter 1' presents the generic introduction of the thesis and 'Chapter 2' summarizes the technical information and research updates available to date and presented as Literature Review. 'Chapter 3' explains the 'Materials and Methods' adopted in the present work. The results obtained in different experiments were consolidated, analysed, and discussed in detail and presented in 'Chapter 4' as 'Results and discussion'. The important results are summarized, and the significant conclusions drawn in the research work is consolidated in the 'Chapter 5' as 'summary and conclusion'.

Chapter 1: Introduction

This chapter presents the generic introduction of the thesis which discusses the need of natural bioactive compounds in the present scenario for food, cosmetic, and nutraceutical industries and briefs about plant biological compound exploited from plant sources specifically the natural colourants and antioxidants. The importance of *Garcinia indica* fruits (kokum) and the presence of value-added bioactive compounds in it are discussed. The importance of these compounds and their potential uses and health benefits are highlighted. This section also introduces the merits and necessity of nonconventional technique aqueous two-phase extraction for the extraction of biomolecules.

Chapter 2: Literature review

This chapter summarizes the technical information and research updates available till date about the four bioactive compounds (ACN, HCA, GL, and IGL), their structure, availability, and the efforts made to extract and purify the bioactive molecules. The brief history of the aqueous two-phase system and current trends, various physicochemical parameters affecting two phase formation and partition of biomolecules, and utilisation of ATPE for the simultaneous extraction of two and more compounds in a single system are reviewed. A review of various types of extractors used for the continuous separation of biomolecules using the ATPE and the effect of operational parameters on the hydrodynamic behaviour of the columns along with mass transfer characteristics was also performed.

Chapter 3: Materials and Methods

This chapter lists the chemicals and materials used for the present study. The complete description of the experimental methodologies and the analytical procedures adopted for the present work is presented. This chapter also presents the constructional details and dimensions of the continuous extractor, and rotating disc contactor (RDC). The procedure adopted for the estimation of dispersed phase holdup and mass transfer coefficients at different operating conditions is also presented.

Chapter 4: Results and Discussion

The experimental results are discussed in four parts namely,

(i) Simultaneous Extraction of bioactive compounds from *Garcinia indica*, and Screening of suitable ATPS for the simultaneous partitioning of four bioactive compounds

The kokum fruit containing major bioactive compounds like ACN, HCA, GL, and IGL was attempted to simultaneously extract using water, solvent and water solvent mixtures and solid-liquid ratio studies are reported in this part. Further, screening for PEG-salt and Alcohol-salt ATP systems for partitioning and separation of bioactive compounds were reported and suitable systems that are successfully able to separate these compounds are presented in this part.

(ii) The selective separation and differential partitioning studies of bioactive compounds in alcohol-based ATPS

The suitable phase forming salts with alcohols are identified and the phase diagram with binodal curve for these ATPS also presented. The one variable experiment performed and detailed results on partitioning characteristics and extraction efficiency of bioactive compounds in ethanol-ammonium sulphate, 1-propanol-magnesium/ ammonium sulphate are covered in this chapter. Further, the RSM employed for 1-propanol-ammonium sulphate to optimise phase component concentration, crude loading as process variables, and the results of optimised system found are explained with possible discussion.

(iii) The Enrichment of the bio-active compounds employing second stage ATPE with optimised systems

The phase volume ratio, the purification step (second stage) ATP to enhance recovery and purity, and further fractionation of ACN, HCA are discussed.

(iv) Studies of continuous ATPE of bioactive compounds in rotating disc contactor (RDC).

Effect of various operating parameters such as continuous phase flow velocity, dispersed phase flow velocity, and rotor speed on dispersed phase holdup and mass transfer coefficient, separation efficiency, and recovery in the rotating disc contactor (RDC) were studied and reported for 1-propanol-ammonium sulphate system.

Chapters 5: Summary and Conclusions

This chapter summarizes the silent results with conclusions of the present study and lists out the future scope of the work.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 CHEMICALS AND PLANT MATERIAL

The kokum (*Garcinia indica*) dried fruits about 5 kg were procured from the local market in Mangalore, Karnataka, India. Standards like anthocyanin-Kuromanin chloride (cyanidin-3-O-glucoside), Hydroxycitric acid Garcinol, Isogarcinol are purchased from Cayman Chemical Company, USA and Sigma Aldrich now Merck, India. Analytical grade polyethylene glycols of molecular weight PEG 6000, 1-propanol, ethanol was also purchased from Merck, India. HPLC-grade 1-Propanol, Methanol, Acetonitrile, Trifluoroacetic acid (TFA) were procured from Merck India Ltd. Glacial acetic acid, Hydrochloric acid, Sodium hydroxide, and different salts ammonium sulphate, magnesium sulphate, sodium sulphate, zinc sulphate, trisodium citrate, sodium dihydrogen phosphate, dipotassium hydrogen phosphate and diammonium hydrogen phosphate salts (> 99%) were purchased from Merck, Mumbai, India. The double-distilled water was used throughout the experiments.

Refrigerating bath circulator (JEIO Tech, RW – 0525G, with the accuracy of ± 0.05°C), Digital viscometer (Rolling –ball viscometer Lovis 2000 M/ME, Anton paar) with an accuracy of ± 0.005 mPa.s, Digital densitometer (DDM 2911, Rudolph, USA, with the accuracy of ± 0.00005 $g.cm^{-3}$ and 0.03°C, precision ± 0.00001 $g.cm^{-3}$ and 0.01°C), Electronic analytical balance (Shimadzu, Japan) with a precision of ± 0.01mg, High performance liquid chromatography (HPLC) (Shimadzu, prominence) equipped with Reverse Phase C18 column, Shiseido Co., Ltd, Japan, UV/Vis Spectrophotometer (Double beam UVD3500, Labomed, USA), Refrigerated cooling centrifuge (Kubota 6930, Japan) and magnetic stirrer are some of the equipment's used for the study. Software like MS Excel, Origin pro 2016, Minitab and Matlab, were used for mathematical calculations, analysis of results.

3.2 EXPERIMENTAL METHODS AND CALCULATIONS

3.2.1 Preparation of crude extract

Dried kokum (*Garcinia indica*) fruits were properly cleaned after removing the seeds and the rind parts (pulp) were considered for the studies. Four different solvents like distilled water, acidified water (0.1% hydrochloric acid), 1-propanol, and ethanol were considered to simultaneously extract all the bioactive components in the crude form. For each experiment, 5g fruit rinds (solid) were mixed with these solvents (liquid) by maintaining a different solid-liquid ratio of 1:4, 1:6, 1:10, 1:20 w/v. Ascorbic acid (0.1%, w/v) was added to the extraction medium to inhibit the activity of polyphenol oxidase. The mixer was vortexed for 10 min and incubated for about 24 hours. Then the mixture was ground using Morton piston and filtered with a muslin cloth to remove coarse particles. The filtrate was centrifuged at 10000 rpm for about 20 min (Kubota 6930, Japan) to remove fine suspended impurities and the crude supernatant (mixture of bioactive compounds) was stored in a cold room at 4–5 °C. The volumes were recorded and subjected to the quantification of bioactive compounds. The extraction experiments were repeated with different aqueous solvent mixtures at varied concentrations of ethanol and 1-propanol with water (20%, 40%, 50%, 60%, 80%, 100% v/v). The concentration of bioactive compounds in the extract (yield, mg/g) was measured using the HPLC chromatograms and the extracts were stored at 4 °C for further experimentations. All the experiments were conducted in triplicate and average values were reported. The error in the analysis was within ± 2% for solid liquid ratio experiments and 1% for aqueous-solvent mixture experiments..

3.2.2 Analytical Methods

3.2.2.1 Estimation of bioactive compounds by HPLC analysis

Detection of bioactive compounds such as ACNs, GL, IGL and HCA and their quantification was performed with Shimadzu LC-20AD model high-performance liquid chromatography system (HPLC) (Shimadzu, Japan) using Reverse Phase C18 Shiseido Co., Ltd, Japan CAPCELL PAK C18 MGII S5 column (4.6 mm ID x 250 mm) with photodiode array UV-Vis detector (SPD-M20A). The ACNs were identified in kokum extract and samples by optimizing and modifying the method of Liu and co-workers (Liu et al. 2013a). The Acetonitrile (solvent A) and 0.1%

trifluoroacetic acid (TFA) in water (v/v) (solvent B) were used as mobile phases under binary gradient mode. The binary gradient is maintained as follows: 0 to 2 min - 95% B; 2 to 21 min - gradual decrease in B till 20 % and remains constant till 23 min; 23 to 27 min - B increased from 20 to 95 % B and finally, constant B of 95 % is maintained for equilibration till 35 min. The column temperature was 35 °C and absorbance was measured at 520 nm. The flow rate was maintained at 0.5 mL/min with a sample injection volume of 10 µL. The standard graph was developed using the different concentrations (12.5 to 125 µg/ml) of standard cyanidin-3-glucoside and cyanidin-3-sambubioside (Sigma Aldrich, USA, and Cayman Chemical Company, USA).

The chromatographic separation and quantitative analysis of GL (camboginol) and IGL (Isoxanthochymol) were performed using the reverse-phase (RP) C18 column with a modification in the method proposed by (Chattopadhyay and Kumar 2006)). The mixture of acetonitrile-water (90:10 v/v, solvent A) and methanol-acetic acid (99.5:0.5 v/v, solvent B) in the ratio of 30:70 was used as mobile phase at a flow rate of 0.5 mL/min. The column temperature was maintained at 25 °C and the GL and IGL were detected at 250 and 276 nm, respectively. The calibration is performed using the standard GL and IGL (Cayman Chemical Company, USA) between the concentrations of 50 µg/ml and 500 µg/ml.

The concentration of HCA was determined by HPLC analysis with initial calibration using standard HCA (Sigma Aldrich, USA) in a linear range from 50 µg/ml to 1000 µg/ml. The modified method of Jayaprakasha and Sakariah (Jayaprakasha and Sakariah 1998, 2002) was used. The HCA was eluted by 0.1 % HCl in water with a flow rate of 0.3 ml/min under the isocratic condition for about 30 min at 40 °C and detected at 210 nm. The standard curves of bioactive compounds were displayed in Figure A1.1 (a-d) (Appendix).

3.2.2.2 Determination of Protein and Total Sugars

Protein estimations were performed using Bradford assay using Coomassie Brilliant Blue G-250 (Bradford 1976) using BSA as standard. The absorbance of the mixture was measured at 595 nm (Labomed, UVD3500) after incubation at 37 °C for

5 min. The calibration curve was prepared using the BSA concentration ranging from 10 µg/ml and 100 µg/ml which is shown in Figure A1.2 (Appendix).

The total sugars present in the samples were determined through Dubois method (Albalasmeh et al. 2013; Dubois et al. 1956), Glucose was used as a standard for the determination of sugars. The absorbance of the sample was recorded in the double beam UV spectrophotometer (Labomed, UVD3500) at 490 nm. The calibration curve was prepared using the glucose concentration ranging from 10 µg/ml and 100 µg/ml (Figure A1.3 in the Appendix). The pH of various samples was measured using a digital pH meter (Eutech Instruments, Singapore).

3.2.3 Screening of ATP systems for the partitioning of bioactive compounds

Different salts like sulphates, citrates, and phosphates with the polymer (PEG-6000) and alcohols like ethanol and 1-propanol (Table 3.1) were considered for the simultaneous partitioning of bioactive molecules in ATPS. The partitioning studies were conducted by preparing each system with a total weight of 10g basis including 1g of crude extract in a 15 ml Falcon centrifuge tubes by considering the identified phase diagrams of the systems in the literature (Table 3.1).

Table 3.1 List of ATPS considered for the selection of suitable ATPS

ATP Systems	References
PEG6000-Magnesium sulphate ($MgSO_4$) PEG6000-Ammonium sulphate $(NH_4)_2SO_4$ PEG6000-Sodium sulphate (Na_2SO_4)	(Salabat 2001; Zhang et al. 2013)
PEG6000-Sodium citrate ($Na_3C_6H_5O_7$) PEG6000-di-potassium hydrogen phosphate (K_2HPO_4)	(Glyk et al. 2014; Mirsiaghi et al. 2010)
PEG6000-di-ammonium hydrogen phosphate ($(NH_4)_2$ HPO_4)	(Zhang et al. 2013)
1-Propanol-Magnesium sulphate ($MgSO_4$) 1-Propanol-Zinc sulphate ($ZnSO_4$)	(Guo et al. 2012a; Nainegali et al. 2017)
1-Propanol-dipotassium hydrogen phosphate (K_2HPO_4) Ethanol-dipotassium hydrogen phosphate (K_2HPO_4)	(Wang et al. 2011)
1-Propanol-Sodium citrate ($Na_3C_6H_5O_7$)	(Zafarani-Moattar et al. 2005)
Ethanol-Ammonium sulphate $(NH_4)_2SO_4$ Ethanol-Sodium dihydrogen phosphate (NaH_2PO_4) 1-Propanol-Ammonium sulphate $(NH_4)_2SO_4$ 1-Propanol-Sodium sulphate (Na_2SO_4)	(Khayati and Shahriari 2016; Wang et al. 2010c; Wu et al. 2014)

The pH of the system was adjusted to 5 before the addition of extract since ACNs are one among the bioactive compounds present in the GI extract, which is not stable in alkaline pH. The temperature maintained was 30 °C with the help of the thermostat. The contents were mixed in a vortex mixer for 10 min and allowed for phase separation for overnight to attain equilibrium. The phase equilibrium was confirmed when there is no change in concentrations of compounds in both the phases. After incubation, the top and bottom phases were separated carefully and volumes, and their weights were calculated. The concentrations of the bioactive compounds such as ACN, GL, IGL, and HCA in both the phases of ATPS were quantified from the chromatogram obtained from the HPLC analysis and partition coefficient (K_{ACN}, K_{GL}, K_{IGL}, K_{HCA}) and extraction efficiency (EE_{ACN}, EE_{HCA}, EE_{GL}, EE_{IGL}) in the top and bottom phases were calculated using equations 3.1, 3.2 and 3.3.

$$K = \frac{C_T}{C_B} \tag{3.1}$$

$$EE_t = \frac{C_T \ V_T}{(C_T \ V_T + C_B \ V_B)} * 100 \tag{3.2}$$

$$EE_b = \frac{C_B \ V_B}{(C_T \ V_T + C_B \ V_B)} * 100 \tag{3.3}$$

where C_T and C_B represent the equilibrium concentrations of compounds in the top and bottom phase, V_T and V_B represent the volume of the top and bottom phase, respectively. All the experiments were conducted in triplicate and average values were reported. The error in the analysis was within ± 1%.

3.2.4 Binodal curve construction and correlation

The alcohol - salt systems were identified as a suitable ATPS for partitioning the ACN and HCA in the salt-rich bottom phase and GL and IGL into the top phase from the crude extract of GI. The binodal curves of the ethanol - ammonium sulphate, 1-propanol - ammonium sulphate, 1-propanol – magnesium sulphate ATPS were developed at 30 °C (303.15 K) using cloud point titration method (Kaul 2000). Stock solutions of phase components were prepared and kept in a thermostatic bath to maintain the temperature at 30 °C. To construct the binodal curve, a jacketed glass vessel of working volume 200 cm^3 was used and the temperature was maintained by circulating water through a temperature-controlled water bath (JEIO Tech, model RW-0525G). The salt solution of known concentration was titrated against the 1-

propanol solution or vice versa until the clear solution turned turbid. The constant stirring was applied by using a magnetic stirrer to ensure the uniform concentration of the constituents of ATPS in the jacketed vessel. The weight of the mixture at each point of turbidity and the clear solution obtained with the addition of water were noted using an analytical balance with a precision of 0.01 mg (Shimadzu AUW 120D). Volume to mass conversion was done using the density of the solutions, which were measured at 30 °C in automatic digital density meter. These data points were used to calculate the concentrations of phase forming components for establishing binodal curves.

The binodal data resulted for ethanol-ammonium sulphate, 1-propanol-ammonium sulphate / magnesium sulphate ATPS are presented in Table A1.1 (Appendix). The binodal data were correlated with non-linear empirical equations reported in the literature (Eqs 3.4 – 3.6). The equation (3.4) was proposed by Merchuk et al., (Merchuk et al. 1998) and has often been used to correlate binodal data of polymer-based ATPSs (Zhao et al. 2011) and ionic liquid-based ATPSs (Han et al. 2012; Zafarani-Moattar and Hamzehzadeh 2011). However, the equation (3.5) and the (3.6) was proposed by Mistry et al., (Mistry et al. 1996)) has been applied mainly for alcohol-based systems (Guo et al. 2012a; Wang et al. 2010c; b) and found a relatively limited application for non-alcoholic systems.

$$w_1 = a \exp(b{w_2}^{0.5} - c{w_2}^3) \quad (3.4)$$

$$w_1 = a \exp(-w_2/b) + c \exp(-w_2/d) + e \quad (3.5)$$

$$w_1 = \exp(a + b\, {w_2}^{0.5} + cw_2 + d{w_2}^2) \quad (3.6)$$

where w_1 is the total composition of alcohol (ethanol/1-propanol) and w_2 is the total composition of salts ($(NH_4)_2SO_4$/ $MgSO_4$) in mass fraction and the coefficients a, b, c, d, and e are fitting parameters. The constants and coefficients of the binodal equations (Eqs. (3.4) – (3.6)) were obtained through the regression analysis of the binodal data and the fitting parameter values, correlation coefficient (R^2), the sum of mean square (SSE) and standard deviation (sd) of the three equations are given in Table 3.2. It was observed that the equation (3.6) proposed by Mistry et al., (Mistry et al. 1996) and equation (3.5) were able to represent the experimental binodal data with higher

accuracy based on the values of the R^2 (>99) and the sum of square error. Thus, these two equations can be successfully applied for correlating binodal data of alcohol-salt systems. Hence the Eq. (3.5 and 3.6) and their fitting parameters were further used for the calculation of equilibration concentrations of the phases.

Table 3.2. Values of the fitting parameters of the empirical equations (3.4), (3.5), (3.6) for the alcohol + salts + H_2O systems at 30 °C

Ethanol and $(NH_4)_2SO_4$								
a	**b**	**c**	**d**	**e**	**SSE[b]**	**sd[a]**	**R^2**	**Emperical Eqn. Utilised**
0.7591	0.3275	0.0838	0.0441	-0.2104	0.00218	0.0055	0.9994	Eqn. No. 3.5
1-propanol and $(NH_4)_2SO_4$								
a	**b**	**c**	**d**	**e**	**SSE[b]**	**sd[a]**	**R^2**	**Emperical Eqn. Utilised**
0.8440	0.0156	0.4341	0.0704	0.0370	0.000286	0.0019	0.9913	Eqn. No. 3.5
1-propanol and $MgSO_4$								
a	**b**	**c**	**d**	**e**	**SSE[b]**	**sd[a]**	**R^2**	**Emperical Eqn. Utilised**
0.7124	-3.9061	-1.5193	--	--	0.1081	0.036	0.9802	Eqn. No. 3.4
0.3416	0.1053	0.2017	0.0739	0.0528	0.0070	0.0092	0.9976	Eqn. No. 3.5
-0.4453	-0.9094	-8.1446	8.9638	--	0.00196	0.0048	0.9991	Eqn. No. 3.6

[a] sd = $(\sum_{i=1}^{n} (w_1^{cal} - w_1^{exp})^2/N)^{0.5}$, where w_1 and N represent the mass fraction of alcohol and the number of binodal data, respectively. w_1^{exp} is the experimental mass fraction of alcohol and w_1^{cal} is corresponding data calculated using equations. SSE[b] = standard mean square error.

3.2.5 Partitioning behaviour of bioactive compounds in three different ATPSs

To study the effect of phase component concentration and TLL on the partition coefficient and extraction efficiency of bioactive compounds, different ATPs systems were prepared with varying concentrations of 1-propanol and $(NH_4)_2SO_4$/ $MgSO_4$. The 10g systems including 1g of the extract on 100% on w/w basis were prepared to keep one of the phase components constant at a time and to vary another component according to two phase regions. In case of ethanol-$(NH_4)_2SO_4$ system, six different mixture points of ethanol - $(NH_4)_2SO_4$ (w/w) like (30%-16%, 28%-18%, 25%-19%, 23%-20%, 22%-22%, and 20%-24%) on 10g basis were considered for the partitioning studies due to the narrow biphasic area of the system (Qin et al. 2017). The contents were mixed thoroughly using a vortex mixer for 10 min and kept for incubation for phase separation for about 2 hours. The extractions were performed till the equilibrium is reached. As the ATPE is not a mass transfer controlled process (Instantaneous equilibrium process), the equilibrium attained is faster specifically in alcohol-salt systems. However, to ensure the equilibrium of the extraction, the mixture was incubated at least 2 hours as mentioned, where we found no any changes in the phase composition, as well as the solute composition for both the top and bottom phases beyond 2 hrs.

The top and bottom phase volumes were separated carefully and their weights, as well as volumes, are calculated. The concentrations of the bioactive compounds such as ACN, GL, IGL, and HCA in both the phases of ATPS were quantified from the chromatogram obtained from the HPLC analysis and partition coefficient (K_{ACN}, K_{GL}, K_{IGL}, K_{HCA}) and extraction efficiency (EE_{ACN}, EE_{HCA}, EE_{GL}, EE_{IGL}) in the top and bottom phases were calculated using Eqs. (3.1), (3.2) and (3.3) respectively. All the experiments were conducted in triplicate and average values were reported. The error in the analysis were reported as standard deviation which were within ± 1%.

3.2.6 Determination of TLL and Liquid-liquid equilibrium data

The ATPS formed of different compositions during the partitioning studies of natural bioactive compounds were used to determine the TLL (%) and construct the tie lines to know the effect of TLL on partitioning. The data of total composition and

mass of each phase after separating the equilibrated phases and fitting parameters of the binodal correlations are used to calculate the equilibrium concentrations (w_1^t, w_1^b, w_2^t, and w_2^b) by simultaneously solving the derived Eqs. (3.7) – (3.10) from Eq. 3.5 and derived Eqs. (3.11) – (3.14) from Eq. 3.6 using MATLAB. The MATLAB code/program and function are given in Appendix Va and Vb. A similar method of calculation of LLE was successfully performed for the alcohol–salt ATPSs (Caldeira et al. 2019; Guo et al. 2012a; Wang et al. 2010a) and ionic liquid–salt (Han et al. 2012). These equations are helpful to calculate LLE data quickly.

$$w_1^t = a_1 \exp(-w_2^t/b_1) + a_2 \exp(-w_2^t/b_2) + c \quad (3.7)$$

$$w_1^b = a_1 \exp(-w_2^b/b_1) + a_2 \exp(-w_2^b/b_2) + c \quad (3.8)$$

$$m^t.\sqrt{(w_2^t - w_2)^2 + (w_1^t - w_1)^2} = m^b.\sqrt{(w_2^b - w_2)^2 + (w_1^b - w_1)^2} \quad (3.9)$$

$$\frac{w_1 - w_1^t}{w_2 - w_2^t} = \frac{w_1 - w_1^b}{w_2 - w_2^b} \quad (3.10)$$

$$w_1^t = \exp\left[a + b\,(w_2{}^t)^{0.5} + c\,w_2{}^t + d\,(w_2{}^t)^2\right] \quad (3.11)$$

$$w_1^b = \exp\left[a + b\,(w_2{}^b)^{0.5} + c\,w_2{}^b + d\,(w_2{}^b)^2\right] \quad (3.12)$$

$$\frac{w_1{}^t - w_1}{w_1 - w_1{}^b} = \frac{m_b}{m_t} \quad (3.13)$$

$$\frac{w_2 - w_2{}^t}{w_2{}^b - w_2} = \frac{m_b}{m_t} \quad (3.14)$$

where w_1^t, w_1^b, w_2^t, and w_2^b represent the equilibrium compositions (in mass fraction) of alcohol (1) and salt (2), in the top (t), and bottom (b) phases, respectively. m_t/m^t and m_b/m^b are mass of top and bottom phases, respectively. Further, the tie-line length (TLL, %) and Slope of tie line (STL) at different compositions alcohol - salts were calculated using the Eq. (3.15) and (3.16).

$$\text{TLL}\,(\%) = \left[(w_1{}^t - w_1{}^b)^2 + (w_2{}^t - w_2{}^b)^2\right]^{0.5} \quad (3.15)$$

$$STL = \frac{(w_1{}^t - w_1{}^b)}{(w_2{}^t - w_2{}^b} \quad (3.16)$$

3.2.7 ATPE with 1-Propanol-Ammonium sulphate ATPS

3.2.7.1 Optimisation of process variables using Response Surface Methodology (RSM)

Response Surface Methodology (RSM) was performed to study the interactive effect of the three independent variables, namely concentration of 1-propanol, $(NH_4)_2SO_4$, and crude load on the partitioning of bioactive compounds. These phase components have wider and diverse effect on separation of these diversified compounds, so there is need to arrive at an optimised conditions to get maximum yields for all the compounds. pH, time of extraction and temperature are not considered in the optimisation. This is because, the presence of multiple compounds and phase components within the system where further addition of acid or base will increase the complexity of the system. Anthocyanins being pH sensitive needs acidic medium which is supported by both 1-propanol and HCA compounds. As alcohol-salts ATPs showed instantaneous phase separation and attained equilibrium faster, the time of incubation and temperature are not considered as major variables in the present study.

Partitioning conditions for the extraction were optimized to achieve maximum partition coefficient and extraction efficiency (K_{GL}, $K_{IGL,}$ EE_{GL}, EE_{IGL}) of GL and IGL in the top phase and that of ACNs and HCA (K_{ACN}, $K_{HCA,}$ EE_{ACN}, EE_{HCA}) in the bottom phase. The Central Composite Design (CCD), rotatable and has circular, spherical, or hyperspherical symmetry, was utilized for the design of experiments. Each factor is varied over five levels. Rotatability depends on the value of α chosen. The central point was repeated six times to allow estimation of pure experimental error. Factor and levels of variables were chosen based on the knowledge gained during one factor at a time analysis. The value of the factors was coded and varied over 5 levels: ± α (α = ± 2, fixed) (axial points) ±1 (factorial points) and the center point. The uncoded values of independent variables at five different levels are given in Table 3.3. Experimental design, statistical and graphical analysis were performed with a statistical software ‘‘Minitab, version 17.0’’. For the RSM studies the experiments were conducted in triplicate and average values were used for analysis. The error in the analysis were within ± 2.5%.

Table 3.3 Experimental design for 5 levels of central composite method (CCD) for 3 factors with 4 responses.

Variables	- α	- 1	0	1	+ α
A: Concentration of 1-propanol (% w/w)	15	20	25	30	35
B: Concentration of Ammonium sulphate (% w/w)	10	12	14	16	18
C: Crude Load (g)	0.5	1	1.5	2	2.5

Statistical analysis of the responses was carried out using ANOVA (Analysis of variance). The responses were fitted into the quadratic model with the highest coefficient of determination (R^2). The general quadratic model for the responses is given by Eq. (3.17).

$$Y_i = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (3.17)$$

where Y_i is the predicted responses (i = 1–8), β represents the coefficients, A, B and C denotes the independent variables, the concentration of 1-propanol, $(NH_4)_2SO_4$ and crude load, respectively. The significance of each term in the model was analysed by computing F-value and P-value. The generated models were used for numerical optimization to examine the feasible optimum conditions of the factors to satisfy the desired goal.

3.2.7.2 Effect of Phase Volume Ratio

The ATP system containing 15.202 % w/w 1-propanol, 10.242 % w/w $(NH_4)_2SO_4$ with 2.5 g of crude load was used to study phase volume ratio. The top phase and bottom phases were prepared separately using equilibrium data obtained (w_1^t = 40.81, w_1^b= 13.17, w_2^t= 3.82 and w_2^b= 10.79 % w/w). The phase volume maintained were from 0.101, 0.145, 0.256, 0.290, 0.414, 0.587, 0.744 and the partition characteristics and extraction efficiency were analysed. All the experiments were conducted in triplicate and average values were used for analysis. The error in the analysis were within ± 1%.

3.2.8 Enrichment of bioactive compounds in ATPS

3.2.8.1 Second-stage ATPE

Second-stage ATPE ($ATPE^2$) was performed to improve the purity of a specific bioactive molecule in a particular phase by removing the traces of other components. It was noticed that the ACN and HCA were partitioned in the salt-rich bottom phase and GL and IGL into the 1-propanol rich top phase during the first stage ATPE ($ATPE^1$).

The first step ATPE was performed with the optimised system obtained through desirability based multi-response optimization i.e., 15.202 % w/w 1-propanol, 10.242 % w/w $(NH_4)_2SO_4$ with 2.5 g of the crude load. The equilibrium compositions in top phase were w_1^t = 40.81, w_1^b= 13.17, w_2^t= 3.82 and w_2^b= 10.79 % w/w respectively corresponds to TLL 28.505. Similarly, the TLL, which shown maximum partitioning of the components consisting of TLL 27.56 and equilibrium concentrations (w_1^t = 43.11, w_2^t = 3.31, w_1^b = 17.62 and w_2^b = 13.8), was considered for the $ATPE^2$ of 1-propanol- $MgSO_4$. The new bottom phase and top phases were prepared at this TLLs, and the individual new phases were added to their opposite phases of $ATPE^1$, i.e., the new bottom phase was added with the top phase of $ATPE^1$, and the new top phase was added with the bottom phase of $ATPE^1$. The concentration of all the bioactive molecules in both the phases of $ATPE^2$ was analysed through HPLC and the Extraction Efficiency (EE), the total recovery (R_{Total}) of each bioactive compound were calculated (Eq. 3.18 and 3.19). The purity of these compounds was estimated using the Eq. 3.20 and 3.21. All the experiments were conducted in triplicate and average values were taken for calculations. The error in the analysis were within ± 2%.

$$\boldsymbol{R_{Total\ (ACN\ or\ HCA)}} = \left(\frac{Total\ amount\ of\ (ACN\ or\ HCA)\ after\ second\ ATPE\ in\ bottom\ phase}{Total\ amount\ of\ (ACN\ or\ HCA)\ in\ the\ system}\right) x\ 100 \qquad (3.18)$$

$$\boldsymbol{R_{Total\ (GL\ or IGL)}} = \left(\frac{Total\ amount\ of\ (GL\ \ or\ IGL)\ after\ second\ ATPE\ in\ top\ phase}{Total\ amount\ of\ (GL\ or\ IGL)\ in\ the\ system}\right) x\ 100 \qquad (3.19)$$

$$\boldsymbol{Purity\ (P_{ACN\&HCA})} = \frac{(ACN + HCA) in\ bottom\ phase}{Total\ of\ all\ bioactive\ compounds\ in\ bottom\ phase}\ x\ 100 \qquad (3.20)$$

$$\boldsymbol{Purity\ (P_{ACN\&HCA})} = \frac{(GL + IGL) in\ top\ phase}{Total\ of\ all\ bioactive\ compounds\ in\ top\ phase}\ x\ 100 \qquad (3.21)$$

3.2.8.2 Fractionation of ACN and HCA in 1-propanol-Ammonium sulphate ATPS

The ACN and HCA recovered in the bottom phase were tried to separate out by fractionating ACN back to top phase and retain HCA in the bottom phase. The separation ability and extraction efficiency of ACN in top and HCA in bottom phases were analysed. The ATP systems of different concentrations were formed using enriched bottom phase with the addition of 1-propanol (15 to 40 % w/w) at constant 15 w/w $(NH_4)_2SO_4$. The mass of each system was kept constant to maintain the desired concentration within the system. Similarly, further 1- propanol (35 % w/w) was constant at varied concentrations of $(NH_4)_2SO_4$ (10 to 20 % w/w). The mass and volume of phases and ACN and HCA concentrations were calculated in the top and bottom phases, respectively. All the experiments were conducted in triplicate and average values were used for calculations. The error in the analysis were reported as standard deviation which are below ± 1%.

The overall ATPE process including the first and second stage/ enrichment step is shown as a flowchart in Figure 3.1.

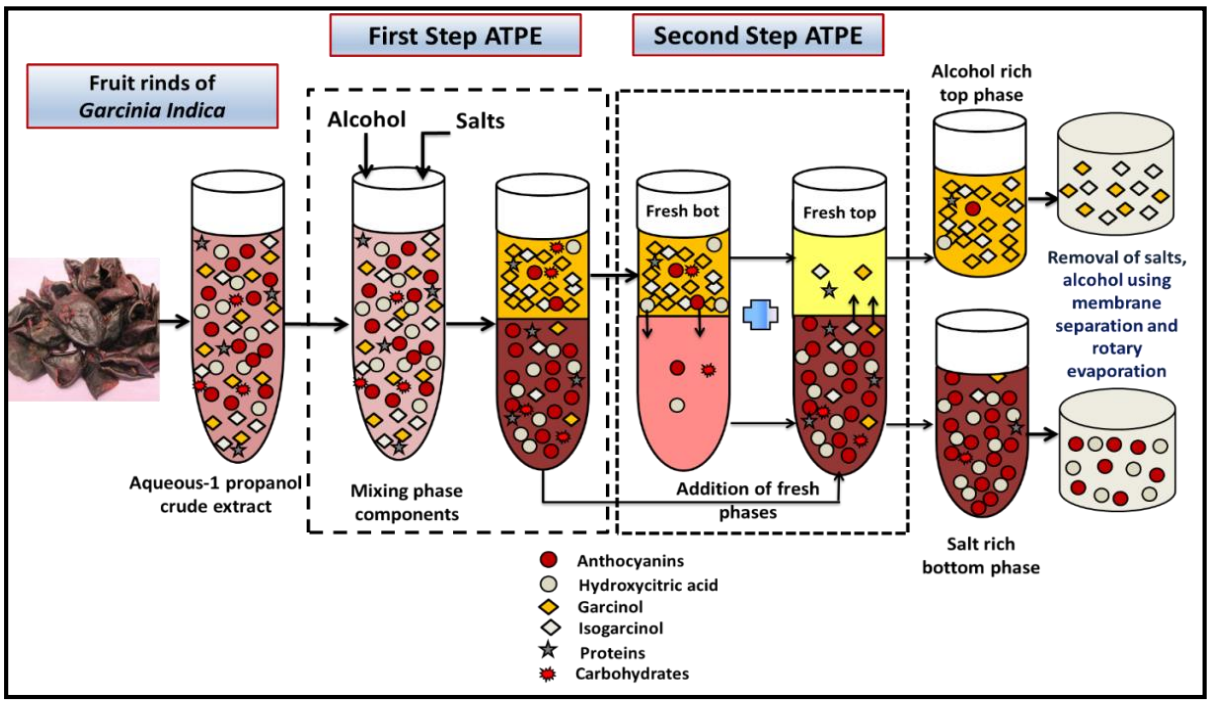


Figure 3.1 The overall flow chart of initial extraction and ATPE of bioactive compounds including the first and second stage ATPS

3.2.9 Removal and Recycling of phase components in 1-propanol-Ammonium sulphate ATPS

After the second step extraction or enrichment step, the enriched 1-propanol and $(NH_4)_2SO_4$ rich top and bottom phases were collected and stored at 4 °C. The small amounts of salt could be removed upon storage at a lower temperature. The enriched phases were mixed in different proportions with methanol to study the crystallization of the salts using the dilution crystallization method. Similar procedures have been followed in the literature (Li et al. 2017; Tan et al. 2013). The ratios maintained were 1:0.5, 1:0.75, 1:1, 1:1.25, 1:1.5, 1:1.75, 1:2, 1:2.5, 1:3 in pre-weighed vials. The mixtures were vortexed and incubated for 2 hours in a cold temperature. After incubation, the vials were filtered and kept for drying at 60 °C in hot air oven overnight. The vials were collected after removal of water and weighed again to calculate the mass of salts retained/recovered. The 1-propanol enriched top phase was subjected to rotary evaporation to remove the 1-propanol efficiently by leaving the partitioned GL and IGL to recycle.

3.2.10 Continuous ATPE studies in Rotating disc contactor

The rotating disc contactor column (RDC) is one of the widely used extractors under the mechanically agitated liquid-liquid extractor. The continuous ATPE was implemented in the RDC. The RDC column and their internals were fabricated by considering the dimensions proposed by Misek (1963) and Perry (7th edition). The experimental RDC column is schematically shown in Figure 3.2. The RDC column was made up of borosilicate glass to resist corrosive and reactive chemicals. All the column internals (shaft, stator ring, rotating discs, and connecting nozzles) were made up of stainless steel. The column consists of a cylindrical vessel with flanges at the top and bottom ends. The compartments were formed by placing the stator rings (horizontal doughnut-shaped) at equal distance and within each compartment, a rotating disc is provided which is mounted on a central shaft. The central shaft was driven by an electric DC motor with gearbox and the rotation of the central shaft was controlled by a voltage regulator. The speed of rotation was measured and digitally displayed by an electronic tachometer with higher precision.

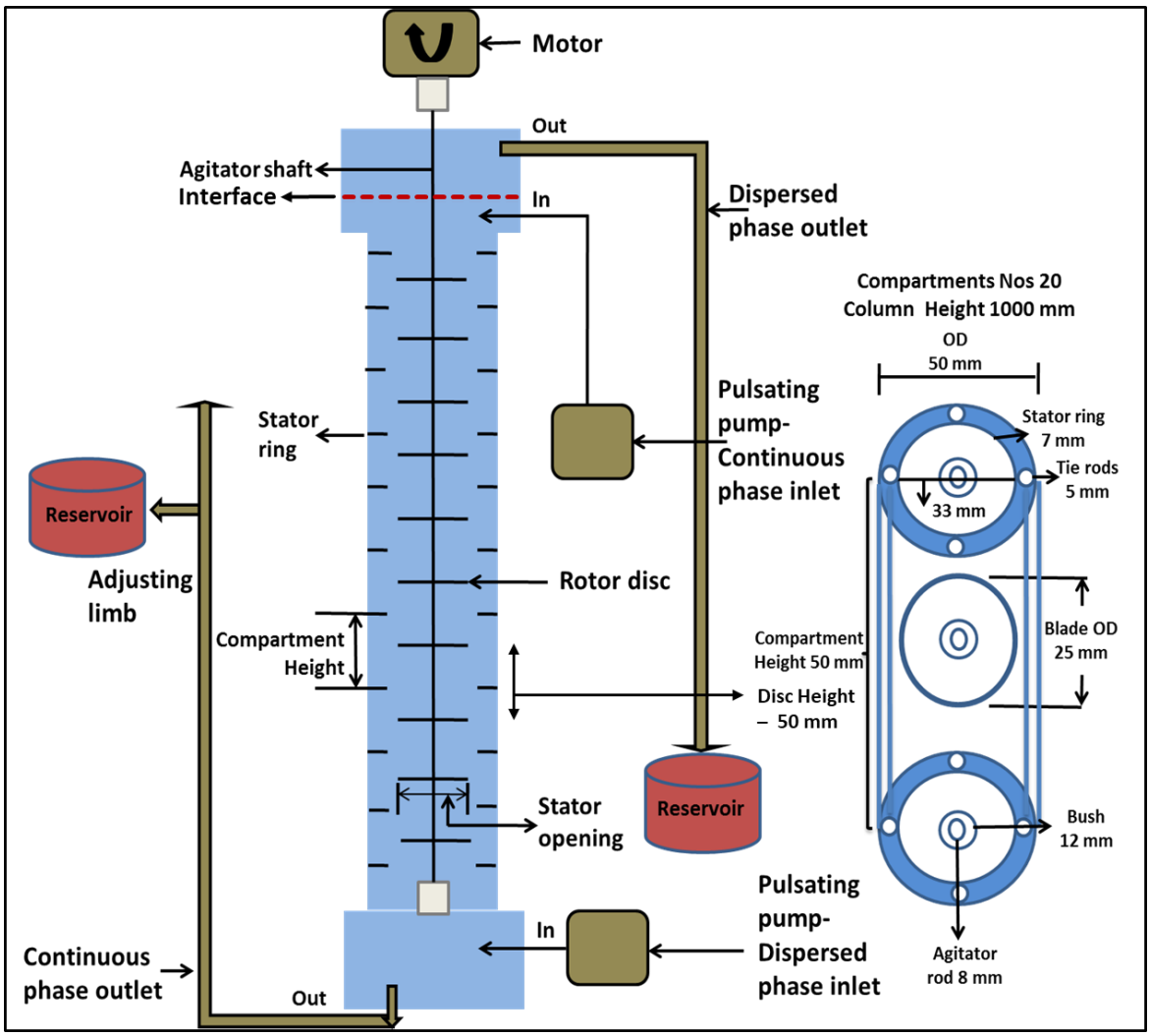


Figure 3.2 Schematic drawing of the rotating disc contactor (RDC) with stator ring and agitator blade and their dimensions.

Table 3.4 Experimental rotating disc contractor (RDC) dimensions

Column internals	Column internals Dimensions (mm)
Diameter of column	50
Height	1000
Rotor diameter	25
Stator ring diameter	33
Compartment height	50
Distance between discs	50
Number of compartments	20
Volume of column (mL)	3500

The column top and bottom flange were installed with the necessary pipe connections for the inlet and outlet of the dispersed and continuous phase solutions. The phase flow rates of dispersed and continuous phase were maintained by two peristaltic pumps. The continuous phase outlet was fitted with an adjustable limb to control the main interface at the top of the column. The dimensions of the RDC were mentioned in Table 3.4.

3.2.10.1 Dispersed phase hold up

Initially, the RDC column was filled with a continuous phase, which enters through the inlet provided at the top of the column. Then the dispersed phase was slowly admitted through the inlet provided at the bottom. The interface level was maintained just above the top stator ring by adjusting the continuous phase outlet limp.

The rotor was started and the desired rotor speed was fixed by adjusting the DC voltage regulator. The rotational speed of the rotating disc assembly was measured by an electronic tachometer. The desired flow rates of continuous and dispersed phases were maintained by controlling the pump head rotations. The interface was maintained at a particular point above the top stator ring. After attaining steady-state (stationary interface), inlet and outlet of the phases and rotating motor were simultaneously closed. The dispersed phase holdup was measured by the volume displacement method. All the dispersed phase droplets present in the internals of the column were brought to the interface by varying rotor speed. The interface level was reduced due to the accumulation of dispersed phase above the continuous phase. Then the accumulated dispersed phase was collected by admitting the continuous phase slowly till the interface reaches the initial position (steady-state position). Thus, collected dispersed phase volume was measured and the dispersed phase holdup was expressed as the ratio of the volume of dispersed phase to effective volume of the column (from the bottom of the column to interface) (Kalaichelvi and Murugesan 1998; Kalaivani and Regupathi 2016; Murugesan and Regupathi 2004). The dispersed phase holdup was measured for various combinations of phase flow velocities and rotor speed (Table 3.5) and it is calculated by Eq. 3.22. All the experiments in RDC

conducted in triplicate and average values were used for calculations. The error in the analysis are below ± 2%.

$$\text{Dispersed phase hold up} = \frac{\text{Volume of dispersed phase}}{\text{Effective volume of the column}} \quad (3.22)$$

Table 3.5 Operating parameters of 1-Propanol/ ammonium sulphate system for holdup studies

Parameters Range	Parameters Range
Dispersed phase flow velocity (m/s)	1 x 10^{-3}, 1.58 x 10^{-3}, 2.16 x 10^{-3}, 2.74 x 10^{-3}, 3.24 x 10^{-3}
Continuous phase flow velocity (m/s)	1 x 10^{-3}, 1.58 x 10^{-3}, 2.16 x 10^{-3}, 2.74 x 10^{-3}, 3.24 x 10^{-3}
Rotor speed (RPM)	0, 60, 90, 120, 150
TLL%	65.514

3.2.10.2 Separation of bioactive compounds in RDC and Mass transfer coefficient (K_Da)

Initially, the column was filled with a continuous phase, which contains 25% of the kokum crude extract through inlet provided at the top of the column. Then both continuous (salt solution with crude extract) and dispersed phase (an alcohol solution) were continuously fed into the column in the upward and downward direction through the inlets present at the top and bottom of the column, respectively at the desired flow rates. The interface level was maintained just above the top stator ring by adjusting the continuous phase outlet limb. The extract phase enriched with GL, IGL, and the raffinate phase with exhaust salt solution with ACN, HCA was collected from top and bottom outlets respectively. The column was operated in counter-current mode at varied dispersed and continuous phase velocities in the range of 1 × 10^{-3} to 3.24 × 10^{-3} m/s and at the rotor speed of 120 rpm and continuous phase velocity of 2.74 × 10^{-3} m/s. The column was operated until the steady-state was reached which was indicated by the stationary interphase. The continuous phase was prepared with a predetermined quantity with kokum crude extract according to the tie line. The transfer of GL as well as IGL takes place from continuous to dispersed

phase. The mass transfer can be studied for these compounds. The phase flow velocities and the rotor speeds were varied to understand their effect on the volumetric mass transfer coefficient ($K_D a$). The column was operated in counter-current mode to determine the mass transfer coefficient. After allowing a continuous and dispersed phase at desired velocities and rotor speed, the samples from the extract and raffinate phases were collected for every 5 minutes. The concentration of all the bioactive compounds was measured using HPLC analysis. The compounds concentration obtained in samples collected was found to increase with time and reaches a constant value after some time, which was considered for calculation. In the study, at the studied range of flow velocities and rotor speed, the time taken to achieve steady-state was nearly 15 minutes where no changes in equilibrium concentrations were observed. The following equation was used to calculate the K_D^a (Cavalcanti et al. 2008; Porto et al. 2010).

$$K_D{}^a = \frac{F_D}{V} \ln\left(\frac{C_{D_i} - KC_{C_i}}{C_{D_o} - KC_{C_i}}\right) \quad (3.23)$$

where K is the partition coefficient of bioactive compounds (GL and IGL) at equilibrium, V is the dispersion volume (effective volume of the column) (mL), K_D^a is the dispersed phase volumetric mass transfer coefficient (min^{-1}), F_D and F_C are the flow rates of dispersed and continuous phases (ml/min). C_{Di} and C_{Do} are initial (zero) and final concentrations (in outlet collected) of bioactive compounds in the dispersed phase (mg/L). C_{Ci} is the initial concentration of bioactive compounds in the continuous phase (an initial mixture of crude extract and salt phase) (mg/L).

The separation efficiency (SE) and Recovery (%) of bioactive compounds in the top and bottom phases were determined under steady-state conditions using the Eqs. 3.24, 3.25, and 3.26 (Cavalcanti et al. 2008; Porto et al. 2010).

$$SE = \left(\frac{C_{C_i} - C_{C_o}}{C_{C_o}}\right) x\ 100 \quad (3.24)$$

$$Recovery\ (\%)_{\ in\ top\ phase} = \left(\frac{F_D\ x\ C_{D_O}}{F_C\ x\ C_{C_i}}\right) x\ 100 \quad (3.25)$$

$$Recovery\ (\%)_{\ in\ bottom\ phase} = 1 - \left(\frac{F_D\ x\ C_{D_O}}{F_C\ x\ C_{C_i}}\right) x\ 100 \quad (3.26)$$

CHAPTER 4

4. RESULTS AND DISCUSSION

The development of a suitable extraction strategy for the simultaneous extraction of four bioactive compounds namely ACN, HCA, GL, and IGL in maximum concentrations was performed from *Garcinia indica* (GI). Further, the ATPE process for selective extraction/separation of these compounds from the crude extract was attempted in the eco-friendly ATPS through a methodical approach. It is well established that the separation of desired biomolecules from the complex sources can be achieved in Aqueous two-phase systems (ATPS) by the thoughtful selection of phase components wherein the contaminants and the desired biomolecules get partitioned based on their affinity towards different phases. Further, the recovery and purity can be enhanced through the selectivity by appropriate manipulation of the other key factors such as phase components concentration, addition of additive, system pH and volume ratio of the system. Initially, the suitability of different types of ATPS for the partitioning of desired compounds was analysed and thereafter, the selected ATPSs were studied in detail for the effect of process variables on the partitioning ability. Commercial implementation of the process necessitates scale-up and continuous operation which has been endeavoured in Rotating Disc Contactor (RDC). The experimental results are discussed in four parts namely,

(i) Simultaneous Extraction of bioactive compounds from *Garcinia indica*, and Screening of suitable ATPS for the simultaneous partitioning of four bioactive compounds

(ii) The selective separation and differential partitioning studies of bioactive compounds in alcohol-based ATPS

(iii) The Enrichment of the bio-active compounds employing second stage ATPE with optimised systems and

(iv) Studies of continuous ATPE of bioactive compounds in rotating disc contactor (RDC).

The simultaneous extraction of bioactive compounds ACN, HCA, GL, and IGL from *Garcinia indica*, and screen the suitable ATPS for the simultaneous

partitioning of four bioactive compounds were discussed in the first part. The simultaneous extraction at different operating conditions using the solvents like distilled water, acidified water (0.1% hydrochloric acid), 1-propanol and ethanol and their aqueous mixtures were explained in the first part of this section along with the screening and selection of suitable ATPS including polymer-salts and alcohol-salts systems. The partitioning studies and extraction yield are considered to select suitable ATPS. The second part of this section emphases the selective partitioning of bioactive compounds from the kokum (*Garcinia indica*) extract by analysing the efficacy of three selected alcohol-salt systems, such as ethanol-ammonium sulphate, 1-propanol-ammonium sulphate, 1-propanol-magnesium sulphate. The performance of the various process variables like phase components and TLL in the selective extraction of ACN, HCA, GL, IGL was assessed by partition coefficient (K), extraction efficiency (EE). This part also explains the multi-variate multi-objective optimization of the crucial factors to achieve the desired goals through response surface methodology (RSM).

The third section of the chapter demonstrates the enrichment process of bioactive components in the phases of ATPE by introducing the second stage ATPE. The second step ATP was employed to enhance the recovery and purity of bioactive compounds for 1-propanol-magnesium sulphate, and 1-propanol-ammonium sulphate ATPSs. Then the fractionation step applied to enriched bottom phase to fractionate (separate out) ACN to top and HCA towards bottom phase also discussed in this part. The possibility of removal/recycling of phase forming components present in the phases of the ATPS was evaluated and represented in this section. The stability of compounds after the ATPE was confirmed by HPLC and LCMS results.

The continuous ATPE in the RDC column was studied and the results were described in the fourth section of this Chapter. The effect of varied velocities of light phase and heavy phase at different rotor speeds in a system of 1-propanol-ammonium sulphate on the dispersed phase holdup and volumetric mass transfer coefficient were discussed in this section. The efficacy of RDC for separation of bioactive compounds in their continuous and dispersed phase was assessed with separation efficiency (SE), recovery (R) analysis and are reported.

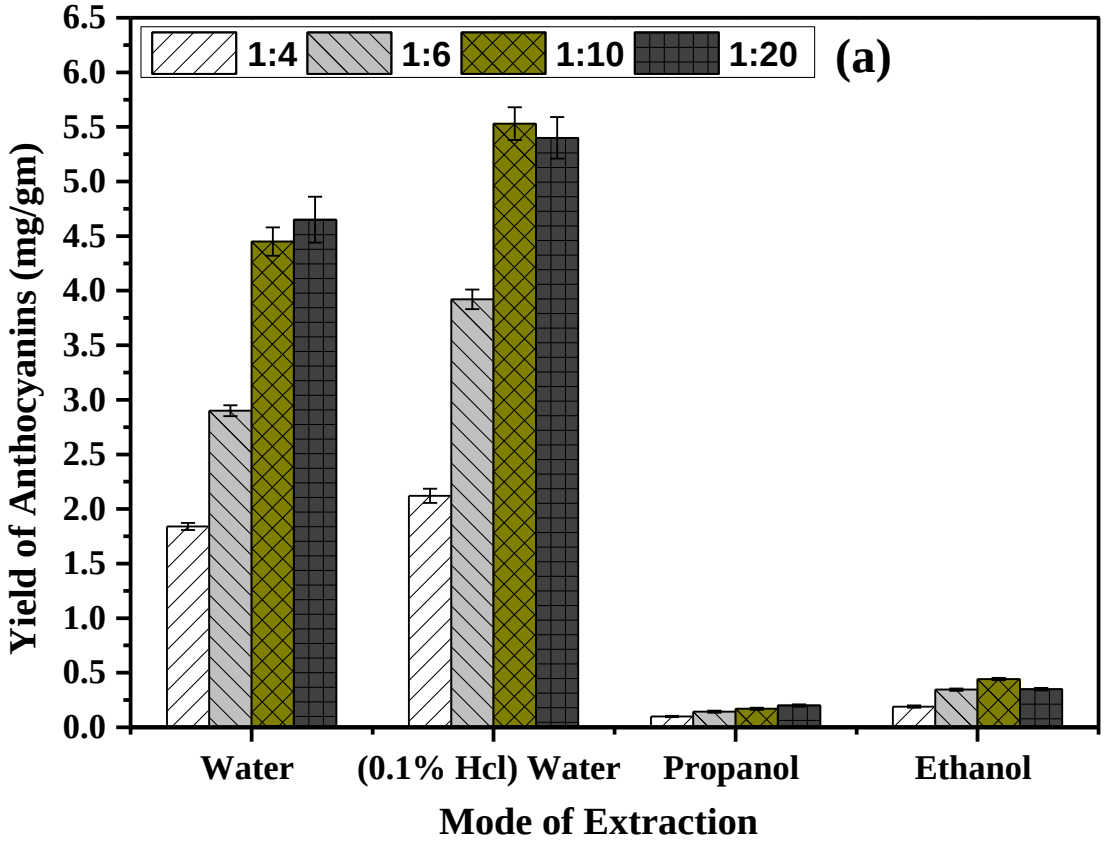
4.1 SIMULTANEOUS EXTRACTION OF BIOACTIVE COMPOUNDS FROM *GARCINIA INDICA*, AND SCREENING OF SUITABLE ATPS FOR THE SIMULTANEOUS PARTITIONING OF FOUR BIOACTIVE COMPOUNDS

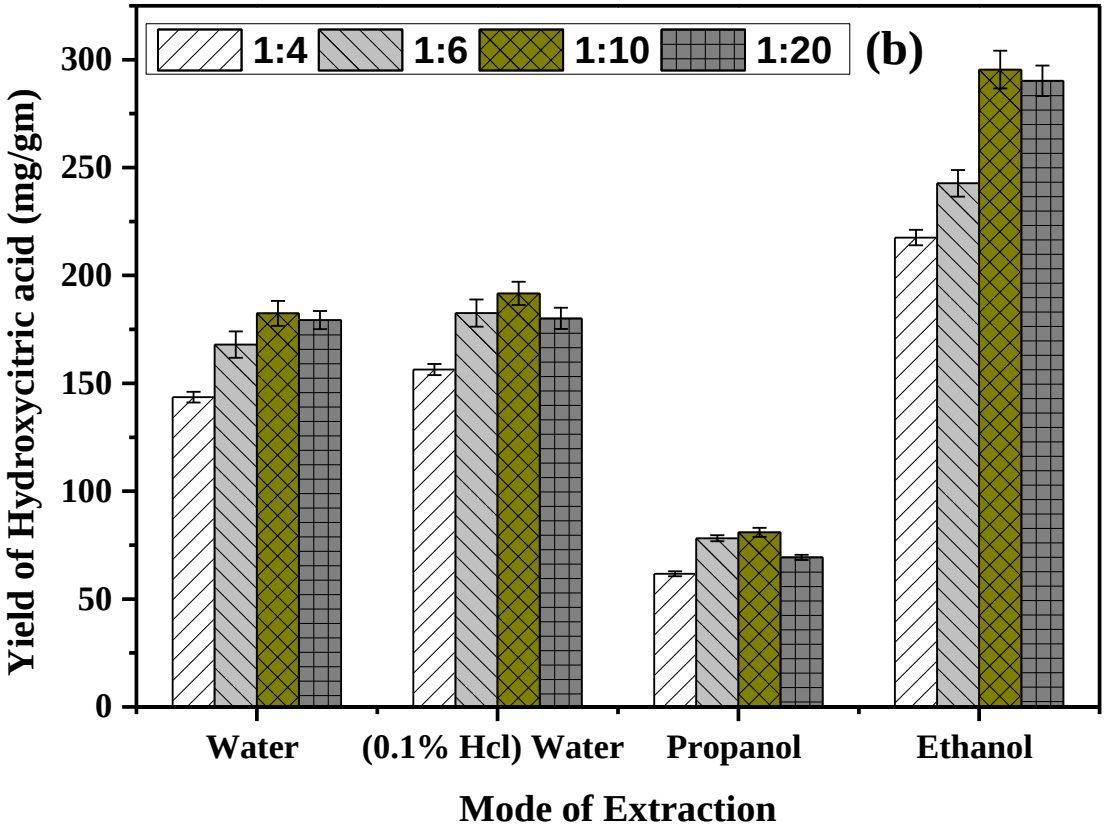
The kokum fruit contains efficient bioactive compounds like garcinol, isogarcinol, anthocyanins and hydroxycitric acids which have numerous biological applications. The well-known conventional separation and purification processes like solvent extraction and their improved techniques, adsorption processes, membrane processes, and chromatographic techniques, may not able to either selectively extract and purify a specific molecule or simultaneously extract all the four molecules or retain the bioactivity of the molecules from the *G. indica* due to the complexity associated with the processes and the use of rash chemicals. Even though extensive studies are reported for the extraction of ACN from GI including solvent extraction, limited studies are available for the extraction of other valuable bioactive components like HCA, GL, and IGL. The conventional techniques used to extract the ACN, HCA, GL, and IGL individually by losing other important compounds and purifying the individual components may increase the processing cost. The simultaneous extraction of all the four compounds together and followed by fractionating with an economically cheaper downstream technique will be the current requirement to make use of the natural resources. In this regard, the simultaneous extraction of ACN, GL, IGL, and HCA from the dried rinds of GI was tried initially with the pure solvents like water, acidified water, ethanol, 1-propanol, and their aqueous solution. The screening of possible ATPS suitable for the simultaneous partitioning of all four bioactive compounds was performed in this present study.

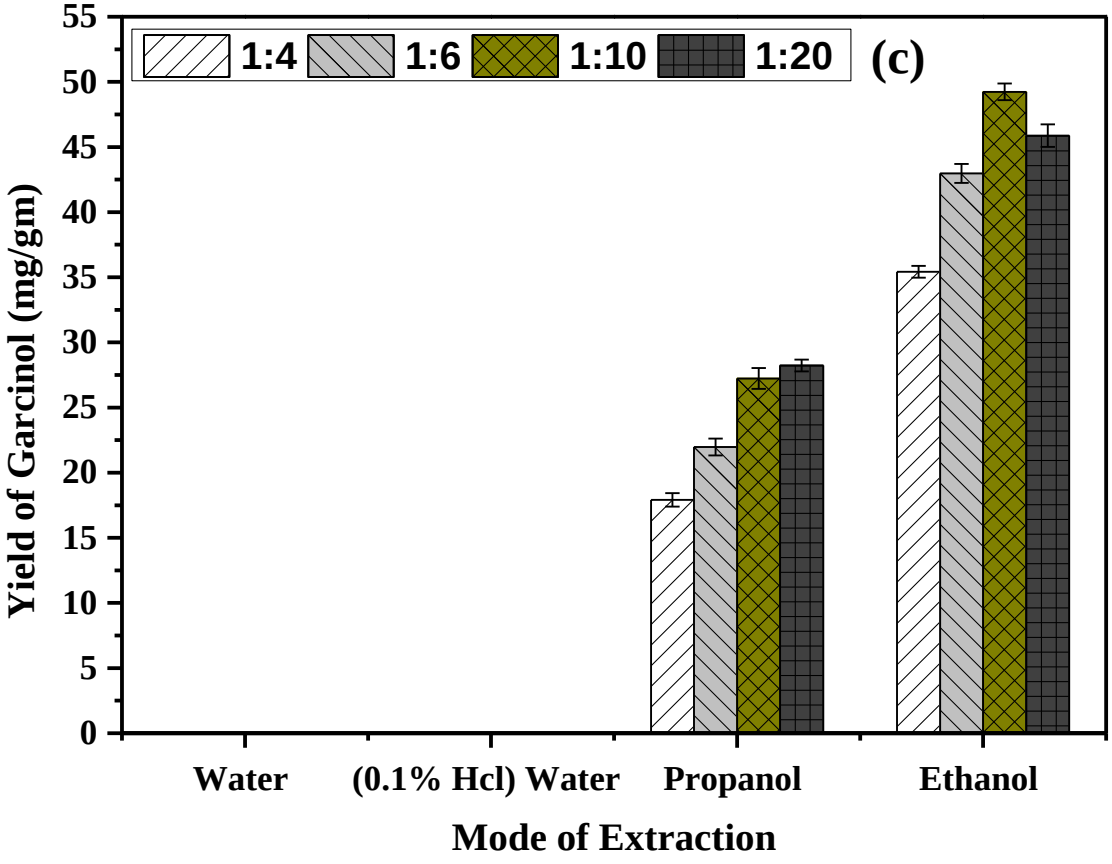
4.1.1 Extraction of bioactive compounds from GI: Solid-liquid (S/L) ratio

In any extraction studies from plant materials, a solid-liquid ratio can be the foremost important step to know the better extraction and higher productivity of required compounds. The simultaneous extraction of ACN, GL, IGL, and HCA from the dried rinds of GI was tried initially with pure solvents like water, acidified water, ethanol, and 1-propanol. The solid-liquid ratio was maintained by varying the ratio of dried rinds (solid) to extraction medium as 1:4, 1:6, 1:10, and 1:20, and the results are presented in Figure 4.1 (a-d).

All the experiments were conducted in triplicate and average values were reported. The error in the analysis was within ± 2%. It was observed from the figure that the significant amount of ACN (5.53 mg/gm) and HCA (191.66 mg/gm) was get extracted in water and acidified water due to their hydrophilic, moderately hydrophobic nature whereas comparatively no or lesser quantity of hydrophobic molecules, GL and IGL, was get extracted to water (Figure 4.1 a and b). The pure 1-propanol and ethanol showed limited extractions for ACN but HCA got released in sufficient quantities to ethanol compared to 1-propanol and water. Meanwhile, the ethanol and 1-propanol could able to extract the GL and IGL with the minor concentration of ACN due to their reduced polarity and the hydrophobicity of the GL and IGL (Figure 4.1 c and d). However, all the three compounds together (GL- 49.23 mg/gm, IGL- 5.10 mg/gm and HCA- 295.43 mg/gm) except ACN (0.44 mg/gm) were get extracted in the pure ethanol system. The 1-propanol system could able to extract all the four compounds together at lower concentrations when compared to the extraction of hydrophilic molecules (ACN and HCA) with water and acidified water and hydrophobic molecules (GL and IGL) with ethanol.




1:4
1:6
1:10
1:20
(b)
Yield of Hydroxycitric acid (mg/gm)
300
250
200
150
100
50
0
Water
(0.1% Hcl) Water
Propanol
Ethanol
Mode of Extraction


1:4
1:6
1:10
1:20
(c)
Yield of Garcinol (mg/gm)
55
50
45
40
35
30
25
20
15
10
5
0
Water
(0.1% Hcl) Water
Propanol
Ethanol
Mode of Extraction

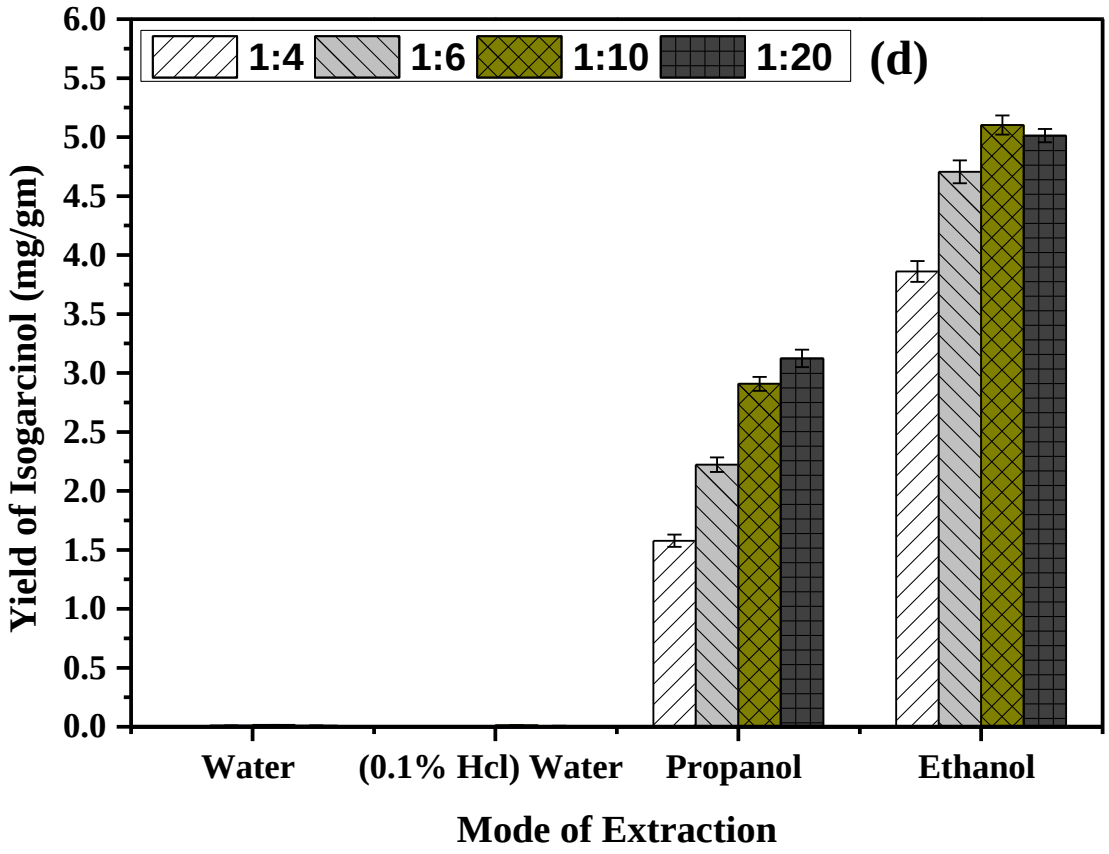


Figure 4.1 Simultaneous extraction of ACN (a), HCA (b), GL (c), IGL (d) from GI (kokum) with different extraction mediums like water, acidified water, 1-propanol and ethanol at different solid-liquid ratios

An increase in the concentration of all the four components was observed with increasing S/L ratio till 1:10, but a significant increase in concentration was not observed beyond the S/L ratio of 1:10. Extraction of HCA with water, HCl water, and 1-propanol showed a slight increase in extraction efficiency with solid-liquid ratio, and ethanol extraction has higher efficiency compared to others. The higher ratio of 1:20 showed not much difference, also it will increase the dilution of extracted bioactive compounds and can lead to higher water or solvent content which can become tedious to remove during concentration processes. The maximum concentration of ACN (5.53 mg/gm) was observed at S/L ratio of 1:10 with acidified water. However, the ethanol extract at a 1:10 S/L ratio provided the maximum concentration of GL (49.23 mg/gm), IGL (5.10 mg/gm), and HCA (295.43 mg/gm). The S/L ratio studies revealed that the maximum concentration of bioactive

components in the extract was possible with the S/L ratio of 1:10 irrespective of the solvents used for the extraction. Hence the S/L ratio of 1:10 was kept as constant for further studies.

4.1.2 Extraction of bioactive compounds from GI: Aqueous-Solvent mixture

Even though all the four bioactive compounds can be simultaneously extracted with 1-propanol and ethanol, the extraction of diversified components from GI may be improved by modifying the hydrophobicity and polarity of the solvents by considering the aqueous mixture of 1-propanol and ethanol at various concentrations. The concentrations of solvents varied from 0 to 100% (v/v) to study the extraction efficiency of four bioactive compounds and the results were reported in Figures 4.2 and 4.3. All the experiments were conducted in triplicate and average values were reported. The error in the analysis was calculated as standard deviation and reported which is found below ± 1%.

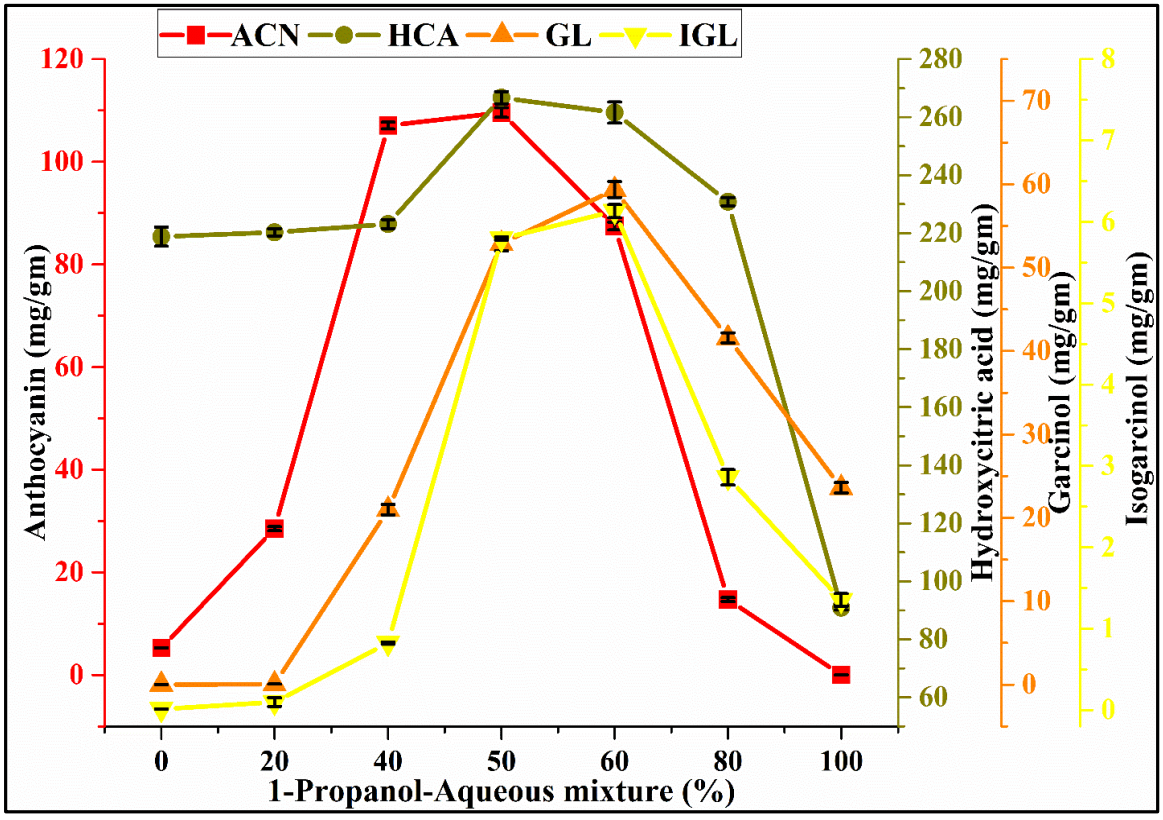


Figure 4.2 Extraction of bioactive compounds from fruits of GI (kokum) with various concentrations of 1-propanol in water. The extraction efficiency of 1-propanol for ACN (■), HCL (●), GL (▲), and IGL (▼)

The extraction of ACN was found to increase with increasing concentration of 1-propanol and reached the highest concentration of 109.57 mg/gm at 50% 1-propanol (Figure 4.2), when compared to the water and acidified water extraction which showed about 6.6 mg/gm. However, the ACN concentration was found to reduce gradually with increasing 1-propanol concentration beyond 50%. The aqueous ethanol also showed a similar trend for the extraction of ACN as seen in Figure 4.3. The optimal concentration of ACN reached about 43.44 mg/gm with 60% ethanol in water mixture but further increase in ethanol failed to extract this compound. As we saw in the last section water and acidified water has the ability to extract HCA molecules in good quantities yielded up to 247.17 mg/gm of HCA, however a slightly higher concentration of 266.71 mg/gm was achieved in presence of 50% 1-propanol-water (Figure 4.2). As a result, water as a pure solvent and 20 to 80% 1-propanol extraction showed not much variation in the extraction efficiency of HCA but the best productivity was found at 50% 1-propanol.

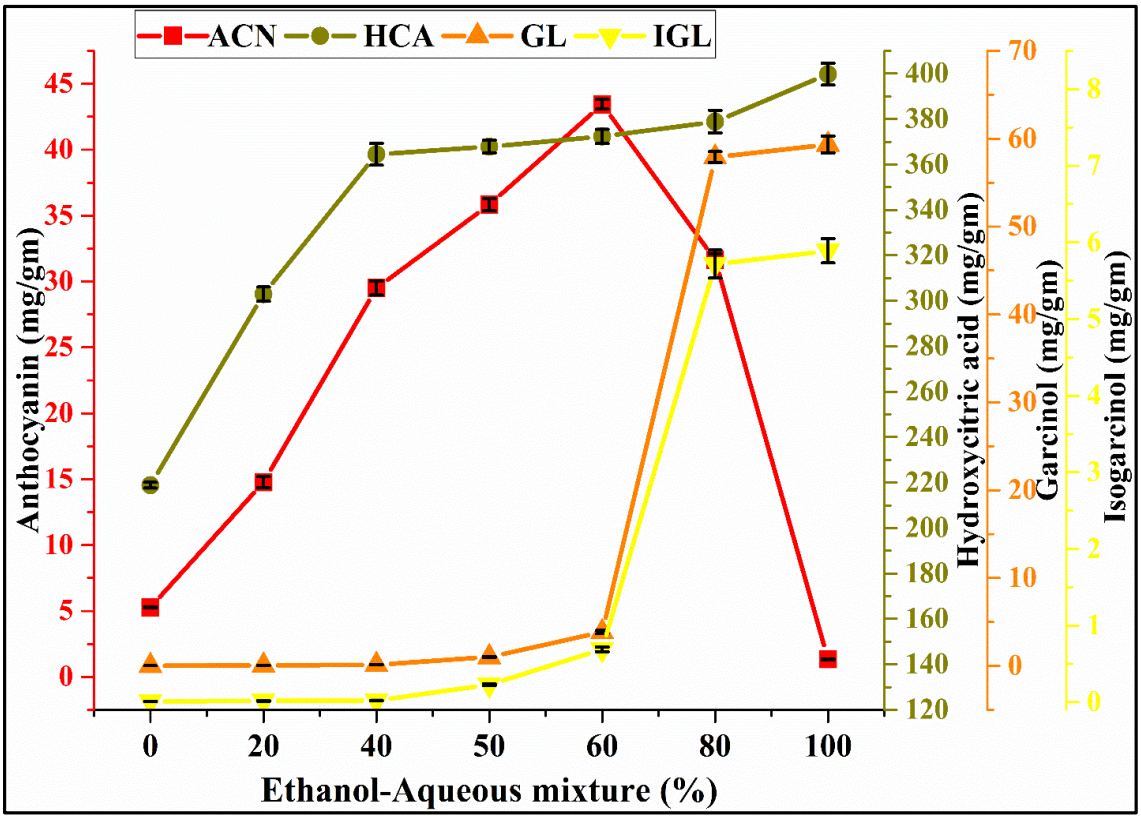


Figure 4.3 Extraction of bioactive compounds from fruits of GI (kokum) with various concentrations of ethanol in water. The extraction efficiency of ethanol for ACN (■), HCL (●), GL (▲), and IGL (▼)

As shown in Figure 4.3, the HCA concentration increases with an increasing concentration of ethanol, and a maximum concentration of 399.74 mg/gm of HCA was achieved with pure ethanol (100%). However, a significant increase in HCA was not observed (364.47 mg/gm HCA) beyond 40% of ethanol in water (Figure 4.3). The presence of GL and IGL was not noticed in the aqueous extract of GI, however, the extraction efficiency was found to increase with increasing concentration of the 1-propanol solvent medium. The effective extraction was observed from 40% to 80% of 1-propanol with the increasing GL concentration from 20.98 to 59.36 mg/gm and IGL of 0.82 and 6.13 mg/gm at 60 % 1-propanol as an optimal point. Further decrease in extraction was found beyond 60% 1-propanol as seen in Figure 4.2. On the other hand, a lower concentration of GL (3.85 mg/gm) and IGL (0.69 mg/gm) were extracted till 60% ethanol in water mixture and which increased further with higher ethanol content. The maximum extraction reached 57.90 to 59.34 mg/gm of GL and 5.72 to 5.89 mg/gm of IGL concentrations at 80 and 100% ethanol (Figure 4.3). Almost, similar extraction efficiency was observed in the case of GL and IGL but the solvent-water mixture is 60% with 1-propanol and pure ethanol respectively (Figure 4.2 and 4.3).

The hydrophilic and moderately hydrophobic molecules HCA and ACN were get extracted with a polar solvent, water, or acidified water. But the hydrophobic molecules GL and IGL were get extracted by decreasing the polarity of the solvent through the addition of ethanol and propanol. It was observed that the higher ethanol concentration (80 to 100%) was required to extract maximum concentration of all the bioactive molecules from the GI when compare to 1-propanol (50 to 60 %) due to the lower polarity of 1-propanol than the ethanol. The equal quantity of water and 1-propanol present in the mixture (approx. 50%) promote the simultaneous extraction of all the bioactive compounds since the diversified molecules interact with the aqueous and non-aqueous part of the solvent mixture at different intensity.

4.1.3 HPLC Analysis of extracts

Each extract was analyzed for four different bioactive components separately as per the HPLC methods explained in methods section 3.2.2. The concentration of each bioactive molecule was quantified based on the area of the chromatogram

resulted in the analysis (Figure 4.4) by referring the calibration curves generated (concentration v/s area) using the pure standards of biomolecules. The chromatograms obtained with each type of solvent extract for specific bioactive compounds are combined along with the chromatogram of standards and presented in a single graph for the comparison purpose (Figure 4.4). Two peaks were observed for ACNs namely cyanidin-3-glucoside (retention time 19.098 min) and cyanidin-3-sambubioside (retention time 18.752 min) which was in accordance with other reports (Nayak et al. 2010b) (Figure 4.4a).

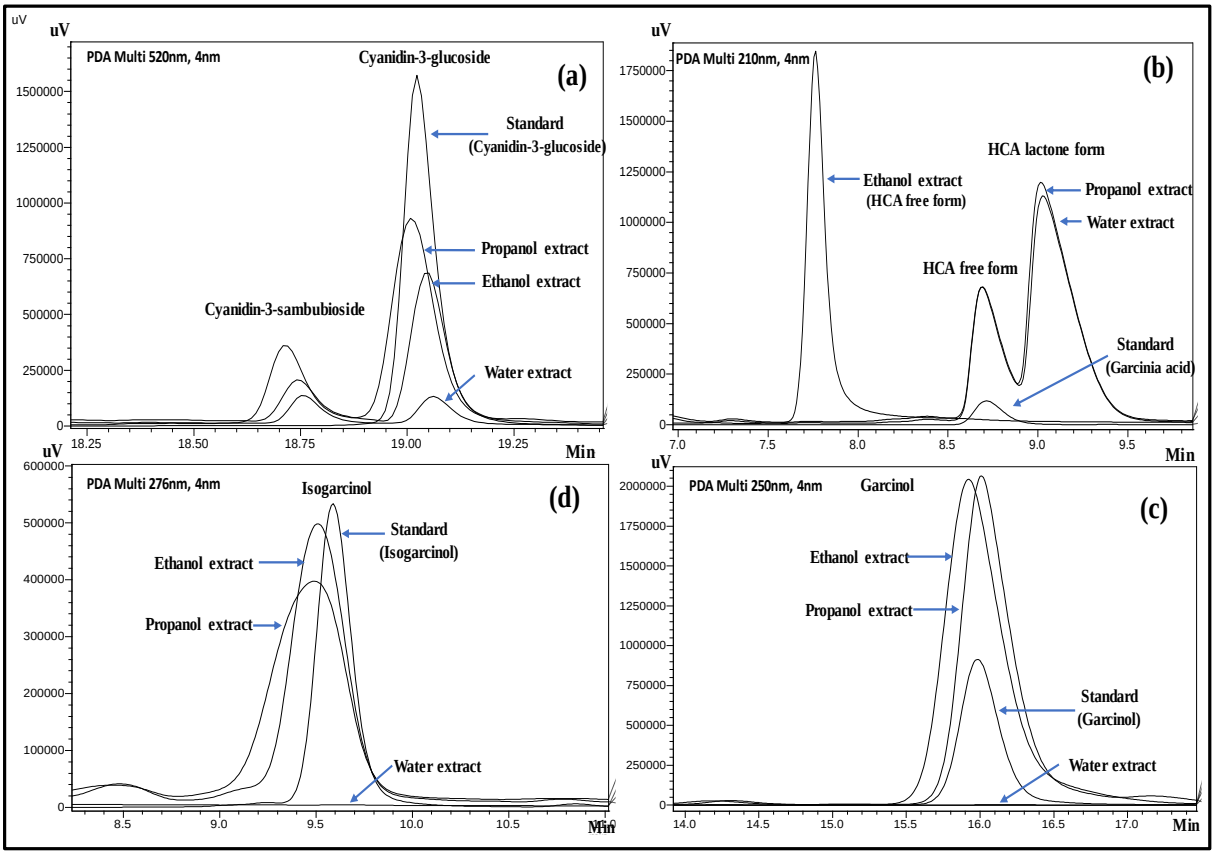


Figure 4.4 The chromatograms of ACNs (a), HCA (b), GL (c) and IGL (d), for the standards and crudes obtained using different solvents

The peaks for both free and lactone forms of HCA were obtained at 8.689 and 9.017 minutes with water and propanol extract, as reported in the literature. Yet, the peak for the free form of HCA alone was detected in the 100 % ethanol extract with the retention time of 7.763 min (Figure 4.4b). Garcinia acid as a standard displayed the retention time of 8.437 min. The free form of HCA in the ethanol extract was

eluted much before (7.763 min) the elution time of the standard. The standard molecules of IGL and GL eluted at 9.535 and 15.983 min, respectively. The water extract of GI failed to display any peak for GL and IGL (Figure 4.4 c and d). The GL and IGL were eluted at 9.489 and 15.922 minutes for GL and IGL, respectively for the GI extracts obtained using ethanol and 1-propanol as solvents.

4.1.4 Screening of Aqueous two-phase systems

The ATPS formed with PEG, ethanol, and 1-propanol – salts were individually studied for the simultaneous partitioning of bioactive molecules in either of the phases of ATPS. The ACN is found to be stable in the acidic pH and hence a grey colour precipitation was observed in the ATPS whose natural pH was greater than 5 with the addition of crude. The systems formed with the salts like K_2HPO_4, $(NH_4)_2HPO_4$, $Na_3C_6H_5O_7$ showed a pH greater than 5 and lead to the formation of precipitation. The pH of such systems was adjusted to 5 before the addition of crude extract to the system. The stable systems formed without any precipitation were identified and the partition coefficients of ACN, HCA, GL, and IGL in the respective system along with the extraction efficiency were reported in Table 4.1 and 4.2. The crude extract contains all the valuable bioactive compounds (ACN, HCA, GL, and IGL), which was obtained during the extraction with 55% 1-propanol as a solvent, was considered for the partitioning experiments. All the experiments were conducted in triplicate and average values were reported. The error in the analysis was calculated which were within ± 1.

4.1.4.1 Partition Characteristics of PEG-based ATPS

The partitioning characteristic of the bioactive compounds was initially analyzed in the ATPS formed with PEG6000 with different salts (Table 4.1) and found that the ACN was partitioned in the PEG-rich upper phase by leaving the HCA in the salt-rich bottom phase and failed to partition the GL and IGL into any of the phases. The HPLC analysis of GL and IGL in both phases confirms the non-presence of GL and IGL. The PEG- $MgSO_4$/$(NH_4)_2SO_4$ systems partitioned the ACN in the PEG-rich phase with partition coefficients of 24.56, 9.057, and yield of 96.98%, 92.31% respectively at the native pH of the system. However, the ATPS formed by Na_2SO_3, $Na_3C_6H_5O_7$ with PEG 6000 provided higher partition coefficients of 48.39

and 36.32 with nearly 97% yield in the top phase at the adjusted pH of 5. The ATPS formed with the salts like K_2HPO_4 and $(NH_4)_2HPO_4$ were partitioned lesser ACN in the top phase (Table 4.1). It is preferred to partition the HCA in the bottom phase to obtain the HCA free ACN as a by-product. However, the HCA was not significantly partitioned in any of the phases of the systems considered except the system formed with Na_2SO_3 and $Na_3C_6H_5O_7$. The salts like Na_2SO_3, $Na_3C_6H_5O_7$ can be a better option to partition ACN in top and HCA in bottom phases because the ATPS retains 74.96 % and 81.16% HCA molecules in bottom phase with a lower partition coefficient of 0.40 and 0.20, respectively. Even though both ACN and HCA are considered hydrophilic components, ACN is less hydrophilic than HCA because of the presence of an aromatic ring. Hence, HCA prefers the bottom phase compared to ACN and was strongly interacted with the salt-rich phase than the ACN. The higher concentration of HCA and the limited availability of solvent water for ACN in the bottom phase promote the partitioning of ACN in the PEG-rich top phase. The obtained results are following the observation made by Chandrasekhar and others for the ACN partitioning from red cabbage and Jamun fruit in PEG-4000/ $MgSO_4$ and PEG-6000/ $MgSO_4$ ATPS (Chandrasekhar and Raghavarao 2015; Jampani and Raghavarao 2015). As the PEG-salt systems failed to partition GL and IGL because of its hydrophobic and insoluble nature, these systems may be suitable for the simultaneous partitioning of HCA and ACN into the bottom and top phases, respectively. Specifically, PEG 6000-Na_2SO_3 and PEG 6000-$Na_3C_6H_5O_7$ ATPS may be extended for the better yield of HCA and ACN after the systematic analysis and optimization of different variables including, tie line length and phase volume ratio.

4.1.4.2 Partition Characteristics of Alcohol-based ATPS

The alcohol-based ATPS are considered to improve the simultaneous recovery and purification of all the bioactive components. Three salts namely, $(NH_4)_2SO_4$, K_2HPO_4, and NaH_2PO_4 only able to form the ATPS with ethanol (Table 4.2). In general, the hydrophobic molecules (GL and IGL) preferred to be in the alcohol-rich top phase and the hydrophilic molecules preferred to be in the salt-rich bottom phase during the partitioning.

Table 4.1 Screening of PEG-Salt systems for the partitioning of bioactive compounds from *Garcinia indica*

ATP Systems (% w/w)	Initial pH	Adjusted pH	Top phase		Bottom phase	
			Anthocyanin		HCA	
			K_{ACN}	EE_{ACN}	K_{HCA}	EE_{HCA}
PEG-Magnesium sulphate (12.5% - 8%)	4.8	--	24.563	96.98	0.764	52.46
PEG-Ammonium sulphate (12.5% - 10%)	4.9	--	9.057	92.31	0.938	47.35
PEG-Sodium sulphate (12.5% - 9%)	5.5	5	48.388	97.53	0.402	74.96
PEG-trisodium citrate (12.5% - 10%)	7.8	5	36.322	97.67	0.201	81.16
PEG-di-potassium hydrogen phosphate (12.5% - 10%)	8.1	5	4.564	85.09	0.844	48.67
PEG-di-ammonium hydrogen phosphate (12.5% - 9%)	7.9	5	1.564	65.08	0.981	46.68

Table 4.2 Screening of alcohol-salt systems for the partitioning of bioactive compounds from *Garcinia indica*

ATP Systems (% w/w)	Initial pH	Adjusted pH	Bottom phase				Top phase			
			Anthocyanin		HCA		Garcinol		Isogarcinol	
			K_{ACN}	EE_{ACN}	K_{HCA}	EE_{HCA}	K_{GL}	EE_{GL}	K_{IGL}	EE_{IGL}
Propanol-Magnesium sulphate (25% - 15%)	4.3	--	0.74	64.50	0.170	90.24	18.72	88.87	9.67	79.51
Propanol-Ammonium sulphate (25% - 15%)	4.9	--	0.39	83.54	0.072	96.78	18.61	90.28	10.57	80.89
Propanol-Sodium sulphate (25% - 15%)	5.6	5	0.74	67.22	0.079	95.86	36.89	96.14	8.75	85.51
Propanol-trisodium citrate (25% - 15%)	7.9	5	0.58	82.73	0.042	97.92	5.57	75.01	1.97	51.51
Propanol-Zinc sulphate (25% - 15%)	5.4	5	0.26	85.30	0.064	95.98	39.01	96.23	16.01	91.30
Propanol-dipotassium hydrogen phosphate (25% - 12%)	8.1	5	1.15	57.10	0.45	82.12	1.67	25.05	1.00	16.66
Ethanol-Ammonium sulphate (25% - 22%)	4.6	--	0.72	67.54	0.51	71.36	13.12	89.75	12.38	89.19
Ethanol-Sodium dihydrogen phosphate (25% - 20%)	4.7	--	1.16	57.53	1.40	23.59	6.80	90.29	7.41	91.03
Ethanol-dipotassium hydrogen phosphate (22.5% - 20%)	7.6	5	1.83	40.52	1.63	13.66	69.13	98.95	50.12	98.56

Hence the extraction efficiency of GL and IGL was considered in the top phase and ACN and HCA in the bottom phase of the ATPS. The ATPS consisting of $(NH_4)_2SO_4$ and ZnSO4 showed higher yield for ACN about 83.54 and 85.30% with 0.39 and 0.26 partition coefficient, respectively. At the adjusted pH (5≤) condition, the 1-proponal -$Na_3C_6H_5O_7$ was found to be the next better choice to partition the ACN to the bottom phase when compared to the ATPS of Na_2SO_3, $MgSO_4$ (Table 4.2). The ATPS formed with K_2HPO_4 and 1-proponal failed to partition the ACN in the bottom phase. However, more than 90% of HCA was partitioned in the bottom phase of all the ATPS studied other than the K_2HPO_4 - 1-proponal system. The lowest partitioning coefficient (<0.042) with the highest extraction efficiency of HCA (>96%) in the bottom phase was observed for the ATPSs formed with $Na_3C_6H_5O_7$ and $(NH_4)_2SO_4$. HCA strongly interacts with the salts and hence the solubility of HCA in the free water of the bottom phase increases. The sulphate salts were found to be more hydrophilic than the phosphate and citrate salts and hence the hydrophilic components like ACN and HCA are retained in the bottom phase. However, a considerable amount of ACN was expelled into the top phase in the ATPSs formed by Na_2SO_3 and $MgSO_4$ due to the lesser volume of free water and a larger amount of salts present in the bottom phase.

The 1-propanol-$(NH_4)_2SO_4$ ATPS showed the partition coefficient of GL (18.61) and IGL (10.57) with a yield of 90.28% and 80.89%, respectively. The 1-propanol-MgSO4 (or) ZnSO4 (or) Na_2SO_3 ATPSs also showed a promising partitioning coefficient and extraction efficiency of both compounds in the top phase. Conversely, the 1-propanol-$Na_3C_6H_5O_7$ and 1-propanol- K_2HPO_4 ATPSs failed to partition the GL and IGL into the top phase effectively. The hydrophobic nature and insolubility of the GL and IGL in water resulted in the better interaction and affinity with alcohol through hydrogen bonds promoted the partitioning coefficient of GL and IGL in the top phase. Further, the lower concentration of propanol in the bottom phase for the dissolution of GL and IGL also helped to improve the partitioning coefficient and yield in the top phase. The screening of ATPS using 1-proponal with different salts revealing that the ATPS formed with sulphate salts are better for the differential partitioning of ACN and HCA. These systems may be further optimized for the better

yield and partitioning coefficients of ACN, HCA, GL, and IGL. However, the detailed studies on the implementation of second step ATPS may be required to fractionate the ACN and HCA separately from the bottom phase obtained in the present ATPE.

When the ethanol is used as a phase forming component with different salts, ethanol- $(NH_4)_2SO_4$ showed a yield of 67.54% ACN in the bottom phase with the partition coefficient of 0.72 and partitioned about nearly 90% of the GL and IGL into the top phase. However, both the ATPSs formed by phosphate salts (NaH_2PO_4 and K_2HPO_4) were efficient to partition the hydrophobic molecules, GL and IGL, with a yield of more than 90% (Table 4.2). But the salt phase of these systems showed a poor affinity towards the hydrophilic molecules and caused a poor partitioning for ACN and HCA in the bottom. The lesser availability of free water in the bottom phase of the phosphate salts ATPSs might be the major reason for the lesser partitioning coefficient of the hydrophilic molecules (ACN and HCA). The stronger affinity of GL and IGL to the ethanol leads to the partitioning of GL and IGL into the ethanol rich top phase. Among the three salts studied, ethanol- $(NH_4)_2SO_4$ could able to partition all the four molecules simultaneously into different phases. Meanwhile, ethanol- K_2HPO_4 ATPS was found to be a promising system to partition the hydrophobic compounds towards the top phase, but the ACN and HCA partitioning need to be improved in the bottom with further optimisation of the system. The present studies reveal that GL, IGL can be possibly separated to top phase easily but HCA and ACNs present in the bottom phases may be removed/ fractionated at later stages using different purification strategies including ATPS. From the screening analysis, it was concluded that the sulphate salts like $(NH_4)_2SO_4$ and $MgSO_4$ in combination with ethanol and 1-propanol could be of a better choice when considering the partitioning ability and pH maintenance are considered. The three ATPS, Ethanol- $(NH_4)_2SO_4$, 1-Propanol- $MgSO_4$, and 1-propanol- $(NH_4)_2SO_4$ were considered for the detailed experimental study to extract and separate the four bioactive compounds efficiently.

4.2 THE SELECTIVE SEPARATION AND DIFFERENTIAL PARTITIONING STUDIES OF BIOACTIVE COMPOUNDS IN ALCOHOL-BASED ATPS

The initial screening studies revealed that the three different ATPS composed of ethanol - $(NH_4)_2SO_4$, 1-propanol-$MgSO_4$, and 1-propanol-$(NH_4)_2SO_4$ have the potential to separate all the bioactive compounds within the system. Ethanol being a safe solvent/cost-effective and used in food industries and good salting-out ability of the $(NH_4)_2SO_4$ salt (Khayati and Shahriari 2016), the partition characteristics at different concentrations were studied by considering the phase diagram of the ATPS. Ethanol-$(NH4)_2SO_4$ system was well studied in the alcohol-salt ATPS category for ACN partitioning (Hua et al. 2013; Liu et al. 2013a; Sang et al. 2018; Wu et al. 2011, 2014). However, the system should be prepared at different combinations within the $(NH4)_2SO_4$ concentrations ranging from 15 to 26% and ethanol from 20 to 30 % due to the narrow two-phase area in the phase diagram (Qin et al. 2017). The ATPS formed with 1-propanol- $MgSO_4$/ $(NH_4)_2SO_4$ were considered for the partitioning studies because of the stronger phase-forming ability of 1- propanol with salts when compared to other alcohols (1- propanol > 2-propanol > ethanol > methanol) (Khayati and Shahriari 2016; Wang et al. 2010c) and the good solubility of both the salts in 1-propanol.. The 1-propanol is less polar compared to ethanol, which may be beneficial to partition hydrophobic GL, IGL to the top phase. Moreover, these ATPS has a favourable native pH (<5.5) to specifically maintain the stability of ACNs, as the ACNs are highly unstable and undergo structural changes at the pH beyond 6 and 7 (Castañeda-Ovando et al. 2009). The organic acid, HCA get neutralized and degraded at the basic pH, while the insignificant effect was noticed on the GL and IGL. The preliminary experiments on the effect of pH at extreme acidity and alkalinity resulted in the degradation of ACNs. Hence the experiments are conducted without altering the system pH.

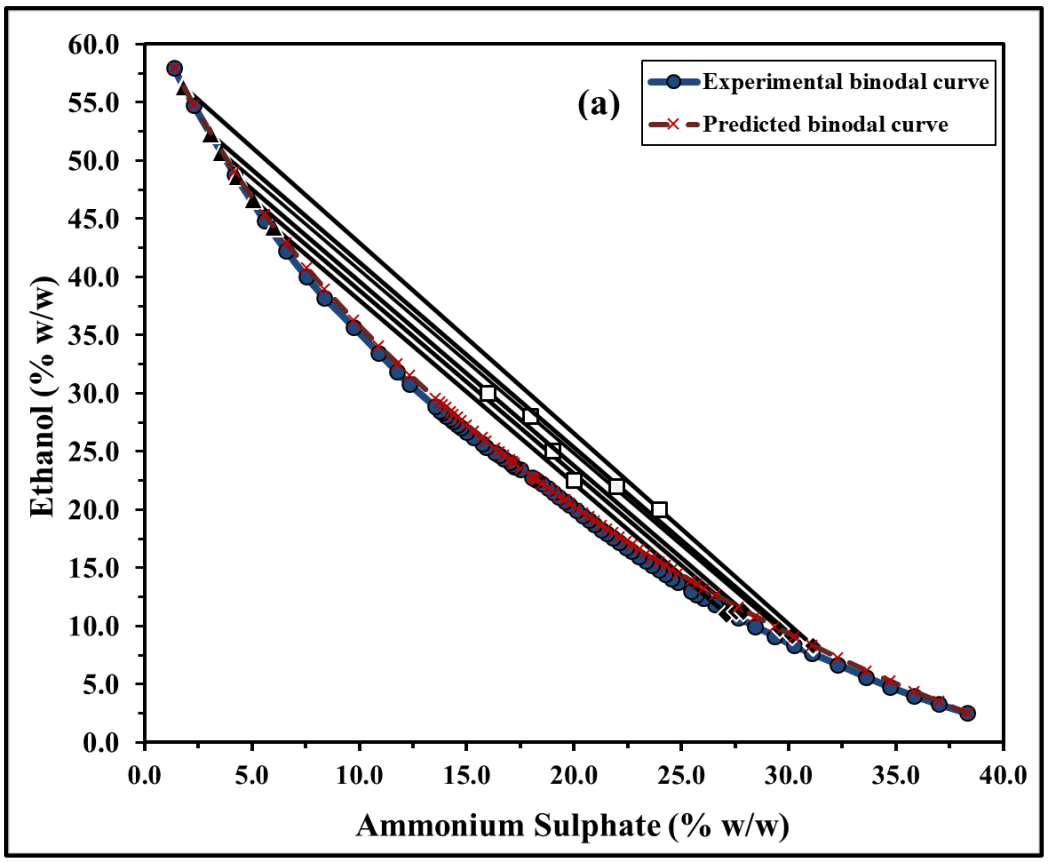
In general, the equilibrium characteristics of the ATPS will be represented through tie-line length (TLL). The total concentration of the system laying at different points on a specific tie line results in a constant equilibrium concentration of the components (salt and alcohol) in the equilibrated top and bottom phases (nodes of the tie line). The partitioning characteristics of the solutes in the ATPS hence depends on

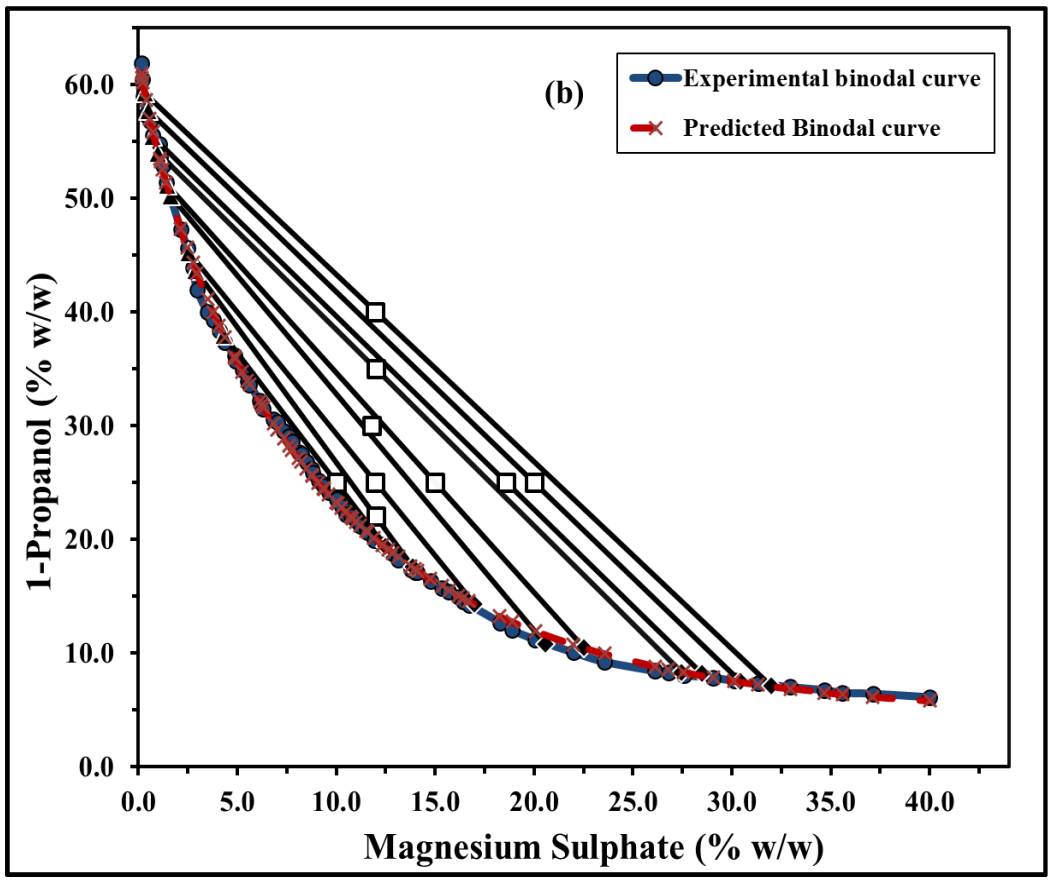
the equilibrium concentration of the phase forming components, which offer a specific level of different attractive forces for the partitioning. The effect of phase components on the simultaneous partitioning characteristics of these four bioactive compounds in these systems was studied and optimized by performing the experiments within the two-phase region of ATPS. The effect of phase forming components concentration was studied by varying the TLL. The crude extract obtained using 80 % aqueous ethanol as solvent was used for the partitioning studies in the ethanol - $(NH_4)_2SO_4$ system. However, the crude extract of bioactive compounds from GI rinds obtained using the aqueous 1-propanol (55 % (v/v) as a solvent, which was identified as a suitable solvent to extract the ACNs, HCA, GL, and IGL at higher concentrations used for the ATPE consist of 1-propanol. The composition of bioactive compounds present in the crude was analysed in the HPLC and found to be 306.92 mg/L of ACNs, 24909.55 mg/L of HCA, 4537.65, and 787.33 mg/L of GL and IGL, respectively. It also contains 16692.50 mg/L of carbohydrates and 2849.31 mg/L of proteins.

The net effective forces required to simultaneously partition the bioactive compounds to either of the phases of ATPS at the phase equilibrium conditions solely depend on the concentration of phase forming components. The results showed that the GL with IGL partition towards the top phase with a K value of greater than 1, whereas ACN with HCA partition in the bottom phase in all the three systems. The hydrophilicity of ACN and HCA and their solubility characteristic in the salt solution lead to the partitioning of these molecules in the salt-rich bottom phase. Since the ACNs are stable only in the acidic region, the HCA and ACNs are partitioned together in the salt-rich bottom phase. However, GL and IGL molecules are partitioned into the top phase. As these molecules are hydrophobic, they preferred to partition in the 1-propanol- rich top phase similar to the lutein esters partition in ethanol rich phase (Fu et al. 2018).

4.2.1 Phase diagram of the ATPSs

The phase diagram of the ATPS consists of binodal curve and tie-lines. The binodal curves of the selected system were constructed using the cloud point titration method at 30 °C (303.15 K) and the binodal experimental data in weight percent is shown in Figure 4.5 (a-c). The binodal points obtained for the systems ethanol + $(NH_4)_2SO_4$ + H_2O and 1-propanol + $(NH_4)_2SO_4$ + H_2O are correlated successfully with an empirical non-linear equation as applied by wang and others for hydrophilic alcohol-salt systems (Eq. 3.5) (Wang et al. 2010c). Similarly, the binodal data of 1-propanol + $MgSO_4$ + H_2O system was correlated successfully with an empirical non-linear equation (Eqs 3.4 – 3.6) to predict the binodal curve. The equation (3.4) proposed by Merchuk *et al*., (Merchuk et al. 1998) have often been used to correlate binodal data of polymer-based ATPSs (Zhao et al. 2011) and ionic liquid-based ATPSs (Han et al. 2012; Zafarani-Moattar and Hamzehzadeh 2011). However, the equation (3.6) proposed by Mistry et al., (Mistry et al. 1996)) has been applied mainly for alcohol-based systems (Guo et al. 2012a; Wang et al. 2010c; b) and found a relatively limited application for non-alcoholic systems. The coefficients or fitting parameters of the equation (3.4) were found through regression analysis. The fitting parameters, corresponding correlation coefficient (R^2), standard mean square error, and standard deviations (sd) for the utilised system are given in Table 3.1 in the methods section. It was observed that Eqns. (3.5) and (3.6) can fit the binodal data of all the selected systems at higher accuracy based on R^2 values which is greater than 99 %. Hence, Eqs. (3.5) and (3.6) and their fitting parameters were further used for the calculation of equilibration concentrations of the phases. The experimental and predicted phase diagrams are shown in Figure (4.5 a-c) and those are used to decide the global system composition in the two-phase region to study the effect of phase forming components on the partitioning characteristics and phase equilibrium.


(a)
Experimental binodal curve
Predicted binodal curve
Ethanol (% w/w)
Ammonium Sulphate (% w/w)
60.0
55.0
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0


(b)
Experimental binodal curve
Predicted Binodal curve
1-Propanol (% w/w)
Magnesium Sulphate (% w/w)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0

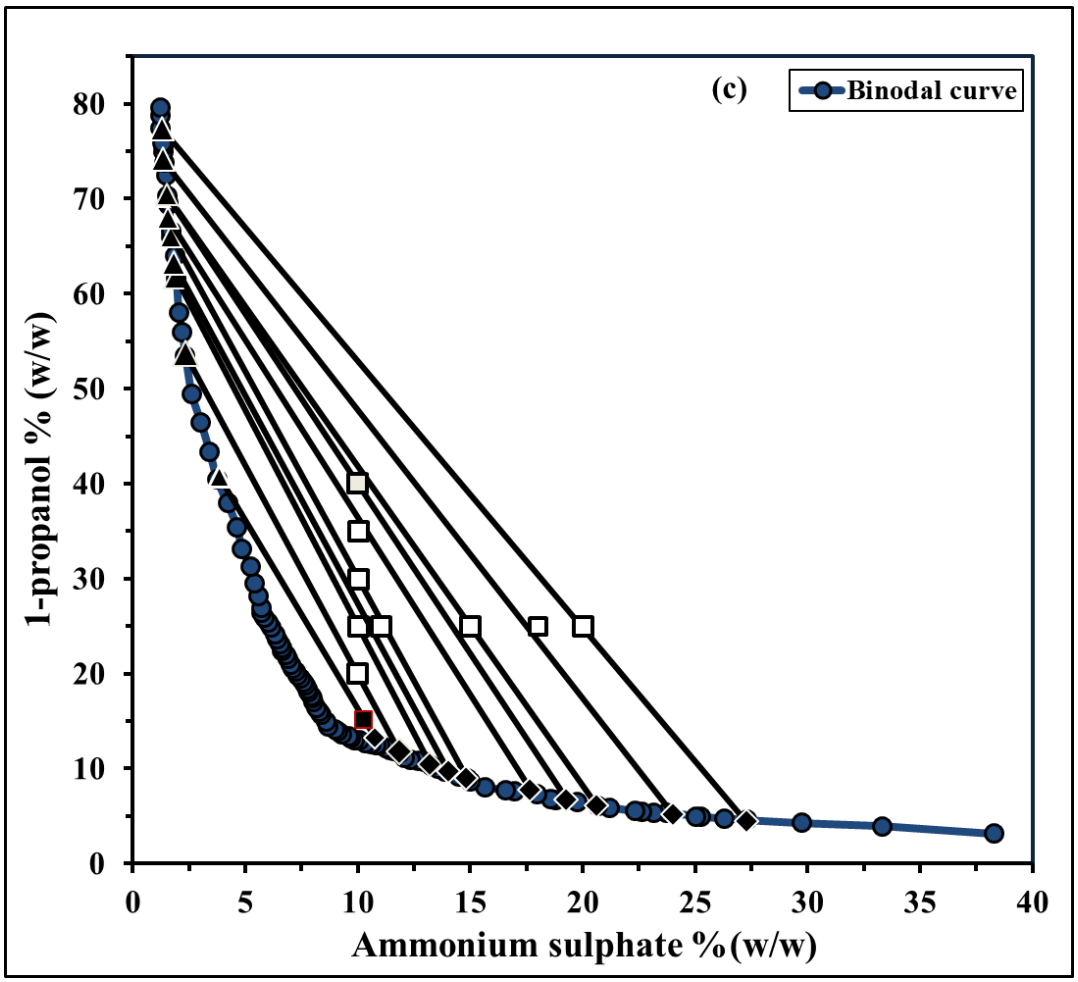


Figure 4.5 Binodal curve of (a) ethanol and ammonium sulphate (b) 1-propanol and magnesium sulphate and (c) 1-propanol and ammonium sulphate systems at 303.15 K with tie-lines. (●) represents experimental binodal curve; (--x--) predicted binodal curve; (▲) equilibrium concentrations in the top phase; (♦) equilibrium concentrations in the bottom phase and (□), total composition of the ATP system and (■), is the optimised system through RSM respectively.

4.2.2 Liquid-liquid equilibrium data and tie-lines

The ATPS of different compositions formed during the partitioning studies of bioactive compounds with selected systems were used to determine TLL and construct tie lines to know the effect of TLL on partitioning. The data of total composition and mass of each phase after phase separation and fitting parameters (as mentioned in section 3.2.4) are used to calculate equilibrium concentrations (w_1^t, w_1^b, w_2^t, and w_2^b) using lever rule and solving four derived equations obtained from each

of these equations (Eqs 3.5 and 3.6) using MATLAB (Eqs 3.7 to 3.14) (Wang et al. 2010c, 2011). A similar method of calculation of LLE was successfully performed for the alcohol–salt ATPSs (Caldeira et al. 2019; Guo et al. 2012a; Wang et al. 2010a) and ionic liquid–salt (Han et al. 2012). These equations are helpful to calculate LLE data quickly. The tie-lines were connected within the binodal curves and represented in Figure 4.5 (a-c).

As explained for alcohol-salt systems (Guo et al. 2012a), it was observed that the increasing global concentration of alcohol and salt increases the equilibrium concentration of alcohol in the top phases and salt concentration in the salt-rich bottom phases. The alcohol easily gets expel towards the alcohol-rich top phase with increasing alcohol concentration due to their lower solubility in water and the greater hydrophobicity associated with the alkyl chain (Pimentel et al. 2017). The alcohols with a lower affinity for water are easily excluded from the salt-rich aqueous phase due to the salting-out effect of the inorganic salts.

It can be observed that an increase in the amounts of alcohol led to an increase of the TLL when the concentrations of salts are minimum/constant, similarly, an increase of the amounts of salts led to an increase of the TLL. Similar observations were reported by other authors as well (Guo et al. 2012a). When total amounts of phase forming components present in the ATPSs are considered, increasing both amounts of alcohol and salts can result in the increase of the TLL, which can promote the phase-separation. In any alcohol-salt system, the alcohol has higher hydrophobicity compared to that of salts and resulting in lower solubility in water which eases the alcohol to expel/retain towards the upper (alcohol-rich) phase. This is also due to the salting-out effect of inorganic salts in which the alcohols with a lower affinity for water are easily excluded from the salt-rich aqueous phase. The greater hydrophobicity associated with the alkyl chain length also ultimately improved/ facilitated the faster two phase formation and separation (Pimentel et al. 2017). Similar behaviour was observed for all the alcohol - $MgSO_4$/ $(NH_4)_2SO_4$ ATPSs studied in the present study. Besides, as the alcohol amount in the upper phase increases, the water is transferred to the lower phase and increasing their interaction with hygroscopic salts like $MgSO_4$ and $(NH_4)_2SO_4$. The upper phases were

predominantly composed of 1-propanol or ethanol, while in the lower phases, salt was prevalent, with water being the major component of both phases. This nature of alcohol-salt systems that render hydrophobic top phase and hydrophilic to moderately hydrophobic bottom phase is utilised to separate moderately hydrophilic ACN, hydrophilic HCA into the salt-rich bottom phase, whereas the hydrophobic GL, as well as IGL, were selectively extracted towards the alcohol rich top phase.

4.2.3 Differential partitioning behaviour of bioactive compounds in Ethanol-ammonium sulphate system

In general, the equilibrium characteristics of the ATPS will be represented through tie-line length (TLL). The total concentration of the system laying at different points on a specific tie line results in a constant equilibrium concentration of the components (salt and alcohol) in the equilibrated top and bottom phases (nodes of the tie line). The partitioning characteristics of the solutes in the ATPS hence depends on the equilibrium concentration of the phase forming components, which offer a specific level of different attractive forces for the partitioning. Hence, the effect of phase forming components ethanol-$(NH_4)_2SO_4$) concentration on the partitioning of ACN, HCA, GL, and IGL were studied by varying the TLL. The total of six different global concentrations of the system was examined for the partitioning behaviour of bioactive compounds. The resulted TLL of these systems is from 38.60 to 56.29 wt. % which are reported in Table 4.3. As seen from the table, the ethanol concentration in the top phase is prominent and $(NH_4)_2SO_4$ in the bottom phase with increasing TLL. The affinity of GL, IGL could retain or separate towards the top phase from crude extract because of their hydrophobic nature and higher solubility in ethanol. The ACN, HCA molecules intended to retain towards the bottom because of high water content and a low quantity of ethanol in the bottom phase. All the experiments were conducted in triplicate and average values were used for calculations. The error in the analysis was calculated as standard deviation which are within ± 1.

The partition coefficient of all the four components found to increases with increasing TLL till 48 (Figure 4.6) and remained constant with a further increase in TLL to 50 and 56. However, the K_{GL} and K_{IGL} were found to increase up to the TLL of 43.28 and remain constant (Figure 4.6) which indicates that the higher ethanol

concentration in the top phase may not require to partition the GL and IGL into the top phase.

Table 4.3 Phase Equilibrium Compositions for the Ethanol + $(NH_4)_2SO_4$+ H_2O System at 30 °C

$(C_2H_5OH$ / $(NH_4)_2SO_4)$ (wt. %)	TLL (%)	Top phase (wt. %)		Bottom phase (wt. %)		Slope
		w_1^t	w_2^t	w_1^b	w_2^b	
23/20	38.60	44.32	6.00	12.02	27.14	-1.581
25/19	41.37	46.61	5.08	11.69	27.27	-1.576
30/16	43.28	48.61	4.33	11.94	27.32	-1.574
28/18	48.55	50.71	3.59	9.72	29.6	-1.528
22/22	50.44	52.33	3.05	9.55	29.78	-1.600
20/24	56.29	56.36	1.81	8.31	31.14	-1.638

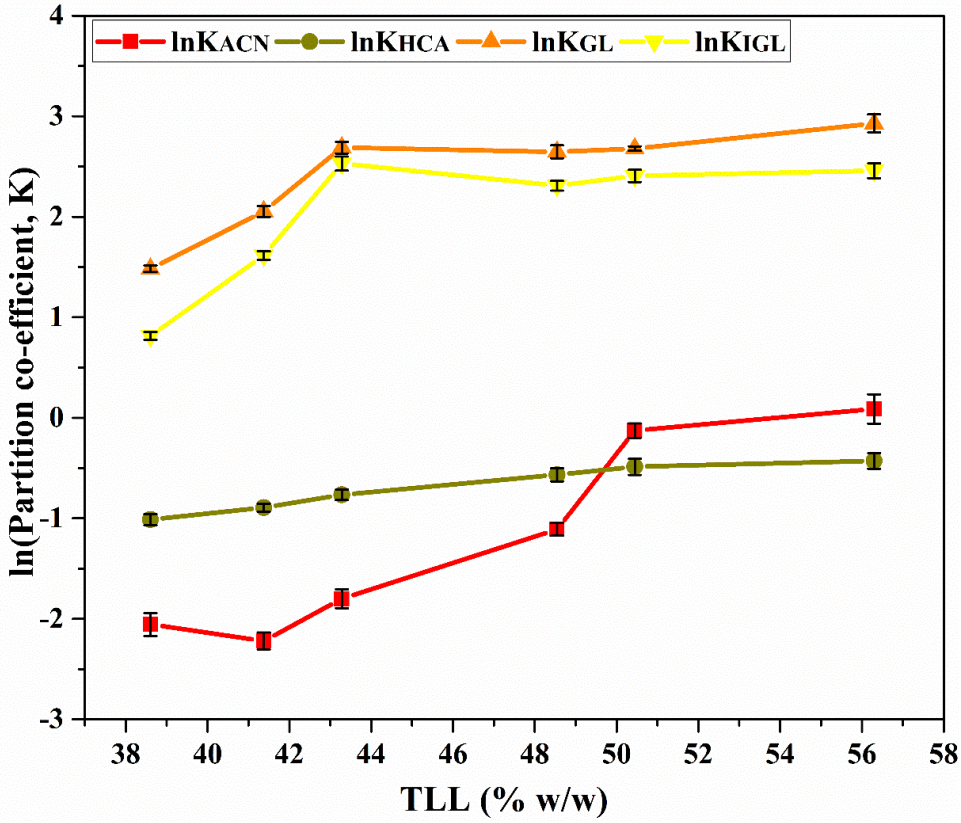


Figure 4.6 Effect of TLL of ethanol and ammonium sulphate ATPS on the partition coefficient of ACN (■) and HCL (●) in bottom phase and GL (▲) and IGL (▼) in top phase.

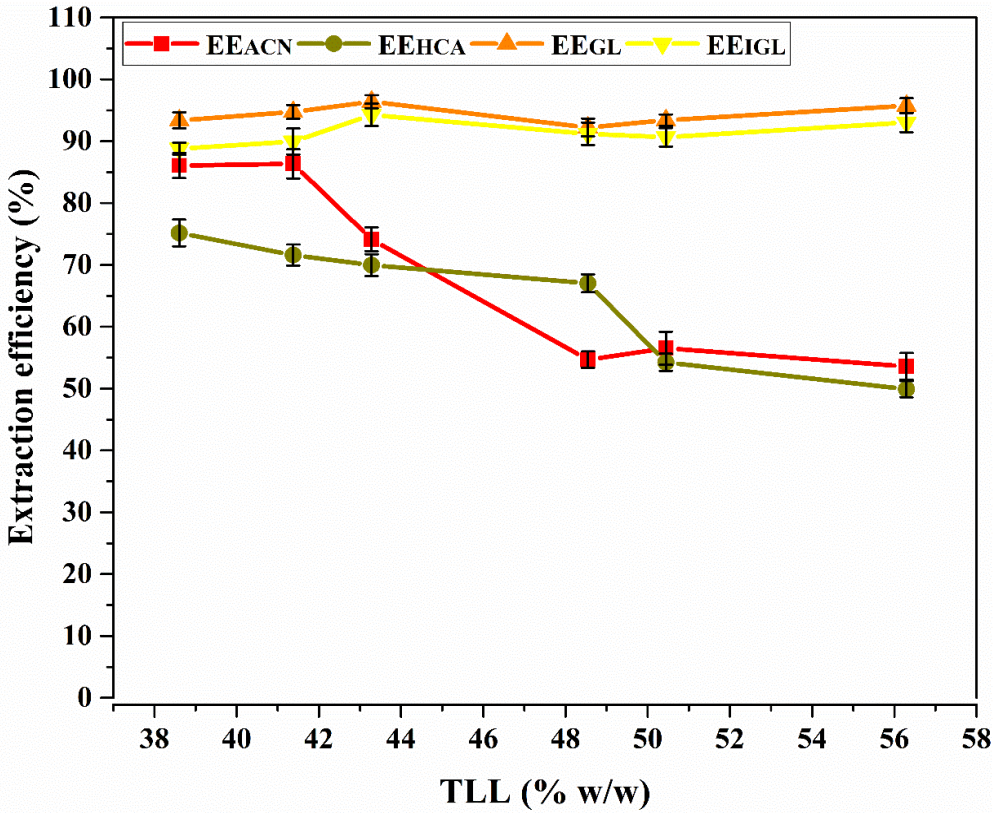


Figure 4.7 Effect of TLL of ethanol - ammonium sulphate ATPS on the % extraction efficiency of ACN (■) and HCL (●) in bottom phase and GL (▲) and IGL (▼) in top phase.

The lower TLL is enough to partition ACN and HCA compared to GL and IGL. The K_{ACN} and K_{HCA} also found to increase with increasing TLL due to the rejection of ACN and HCA from the bottom phase to the top phase. The GL and IGL extraction efficiency were not influenced by the TLL due to the increased solubility of GL and IGL into the ethanol, however, the ACN and HCA extraction efficiency in the bottom phase was gradually decreased with increasing TLL (Figure 4.7), since the higher salt concentration in the bottom phase at higher TLL excluded the solutes, ACN and HCA, to the top phase. Further, the bottom phase exhibits the lower solute equilibrium concentrations due to the non-availability of the free water solvent. The solvent water was utilized for the dissolution of higher concentration of phase forming a salt, $(NH_4)_2SO_4$. The higher extraction efficiency of ACN (86.33 %) and HCA (75.17%) in bottom phase with a partition coefficient of K_{ACN} = 0.109 and K_{HCA} = 0.363 and GL (96.39 %), IGL (94.26 %) yield in the top phase were observed between the TLL of 38.60 and 43.28 (Figure 4.6 and 4.7).

4.2.4 Differential partitioning behaviour of bioactive compounds in 1-propanol-magnesium sulphate ATPS

The ATPS formed with 1-propanol- $MgSO_4$ was considered for the partitioning studies because of their stronger phase-forming ability of 1- propanol with salts when compared to other alcohols (1- propanol > 2-propanol > ethanol > methanol)) (Khayati and Shahriari 2016; Wang et al. 2010c) and the good solubility of $MgSO_4$. The ATPS has a favourable native pH (<5.5) to specifically maintain the stability of ACNs, as the ACNs are highly unstable and undergo structural changes at the pH beyond 6 and 7 (Castañeda-Ovando et al. 2009). The organic acid, HCA get neutralized and degraded at the basic pH, while the insignificant effect was noticed on the GL and IGL. The partitioning experiments were carried out in the two-phase region of ATPS. The results showed that the GL with IGL partition towards the top phase with a K value of greater than 1, whereas ACN with HCA partition in the bottom phase. The effect of individual phase forming components concentration and their combined effect in the form of the effect of TLL on the partition coefficient and extraction efficiency were studied. All the experiments were conducted in triplicate and average values were used. The error as standard deviation in the analysis was within ± 1.

4.2.4.1 Effect of $MgSO_4$ concentration

The $MgSO_4$ concentration was varied between 10 to 20 (%, w/w) at a constant 1-propanol concentration of 25 (%, w/w) to deduce the effect of $MgSO_4$ concentration on the partitioning characteristics. The insolubility and precipitation of $MgSO_4$ were observed at the salt concentration higher than 20%. The partition coefficient of GL and IGL found to increase with increasing salt concentration till 15 % w/w and reached a maximum of 28.376 and 11.856, respectively (Figure 4.8). The hydrophobic GL and IGL were transferred to the top phase at lower concentrations of salt due to their strong hydrophobic interaction, which was found to reduce as the $MgSO_4$ concentration increases. Similar findings were observed for anthraquinones derivatives in 1-propanol rich top phase and are dependent on the hydrophobic interaction, the intensity of hydrogen bonds, and the salting-out effect in ATPS (Tan et al. 2013). The ‘‘ion-dipole” interactions between water molecules and salts may be

the primary cause (Wang et al. 2010a). As the phase-forming salt dissolves in the lower phases because of the hydration of ions, the decrease of free water molecules in the lower phase causing the increase of alcohol as well as hydrophobic compounds in the upper phase (Cheng et al. 2016). Further, both the partitioning coefficients were found to decrease with increasing $MgSO_4$ concentration beyond 15% due to the saturation of GL and IGL in the top phase at the limited concentration of 1-propanol and free volume available to accommodate GL and IGL (Guo et al. 2012b).

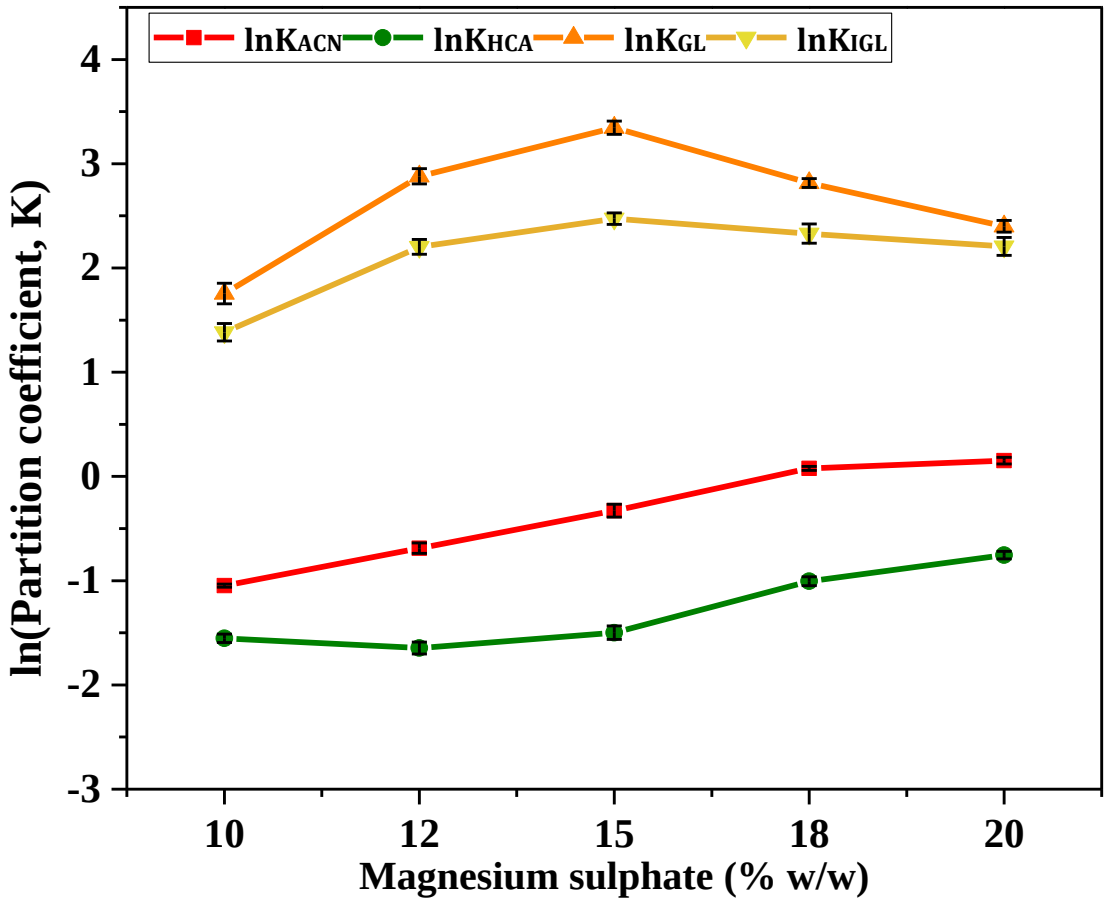


Figure 4.8 Effect of $MgSO_4$ concentration on the partition coefficient of bioactive compounds in a 25 (%, w/w) 1-propanol– X (%, w/w) $MgSO_4$ ATPS at 30 °C. (■), K_{ACN} and (●), K_{HCA} in bottom phase and (▲), K_{GL} and (▼), K_{IGL} in top phase.

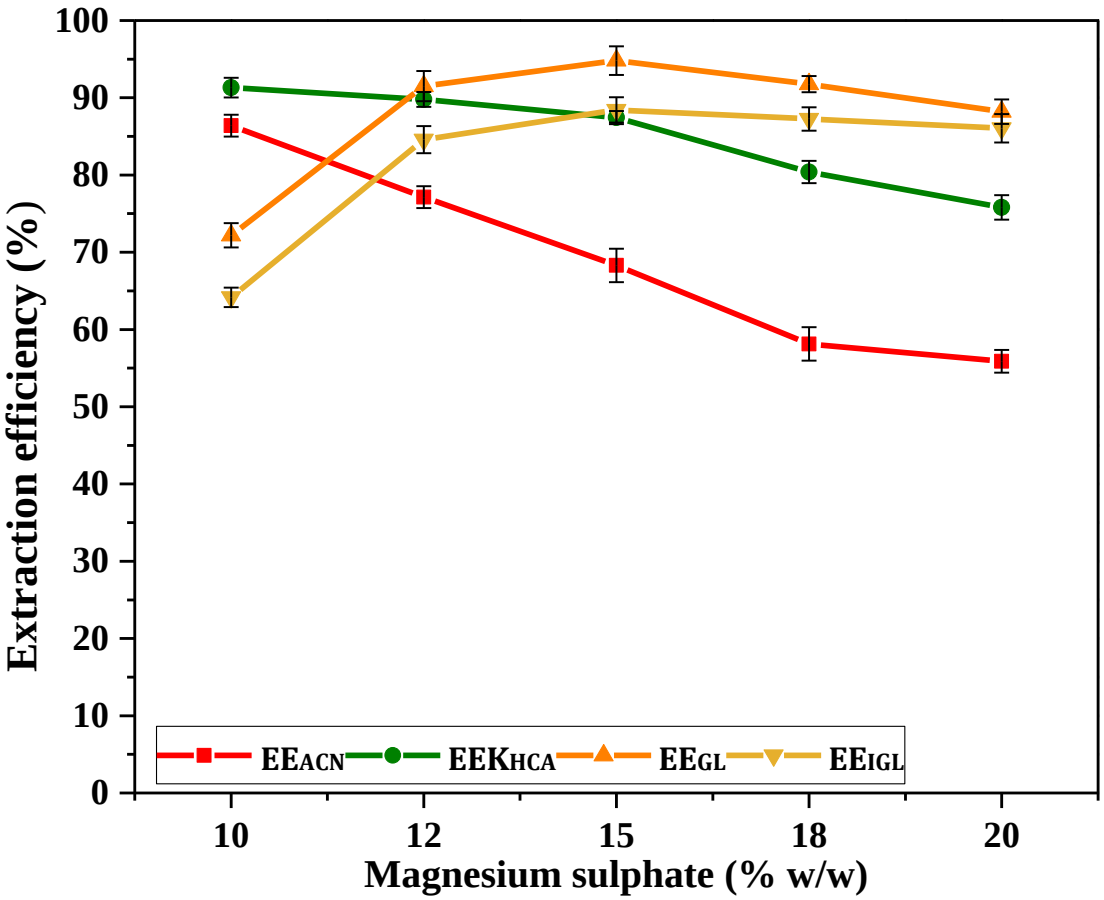


Figure 4.9 Effect of $MgSO_4$ concentration on the extraction efficiency of bioactive compounds in a 25 (%, w/w) 1-propanol–X (%, w/w) $MgSO_4$ ATPS at 30 °C. (■), EE_{ACN} and (●), EE_{HCA} in bottom phase and (▲), EE_{GL} and (▼), EE_{IGL} in top phase).

The salt concentration also reaches saturation in the top phase at some point, and hence more water may be pulled towards the salt-rich phase, which probably made the alcohol-rich phase to reject the GL as well as IGL (Li et al. 2017; Wang et al. 2010a). However, the maximum extraction efficiency of 94.81 % and 88.42 % was observed for GL and IGL, respectively, at 15 % of $MgSO_4$ (Figure 4.9). The partitioning of ACN found to reduce towards the bottom phase with increasing salt concentration. As the salt concentration increases, the equilibrium concentration of $MgSO_4$ also increases in the bottom phase. Consequently, the salting-out characteristics of the salt phase transfer the ACN to the top phase. Hence, the maximum extraction efficiency of 86.39 % was observed at a $MgSO_4$ concentration of 10%. Further, the selectivity of the top phase towards the GL and IGL was also lost

after the salt concentration of 15% due to the interaction of ACN to 1-propanol. As the HCA exhibits strong electrostatic interaction with the salt phase and it leads to the salting in effect, relatively less effect was felt on K_{HCA} with increasing salt concentration.

Hence, a lower concentration of $MgSO_4$ is good enough to hold 91.31 % of HCA in the bottom phase (Figure 4.8 and 4.9). However, a slight increase in K_{HCA} was observed at the higher salt concentration (>15%) due to the salting-out effect of the bottom phase. Similar observations were found for α-lactalbumin in PEG 1000–tri-potassium citrate system (Kalaivani and Regupathi 2013). ACNs exhibit relatively lesser hydrophobicity than GL and IGL and slightly higher than the HCA. Hence, the ACNs start to migrate to the top phase at higher $MgSO_4$ concentration than the HCA due to the salting-out ability of $MgSO_4$ and the hydrophobic attraction of the top phase. The required hydrophobic balance between the phases for the simultaneous partitioning of all the components was achieved at the lower $MgSO_4$ concentration of 12 % w/w (Figure 4.8 and 4.9).

4.2.4.2 Effect of 1-propanol concentration

The effect of 1-propanol concentration on the partitioning characteristic of the biomolecules was studied at a different 1-propanol concentration between 20% to 40% (w/w) at a fixed 12% (w/w) $MgSO_4$ concentration. The partitioning coefficient and extraction efficiency of GL and IGL were found to increase gradually to 44.901 and 9.531, respectively, with increasing 1-propanol concentration until 35 % (w/w). The maximum extraction efficiency of 98.81 % and 94.65 % was achieved for GL and IGL, respectively, at this condition (Figure 4.10 and 4.11). The increasing concentration of 1-propanol will increase the hydrophobicity of the top phase and their interaction with water molecules, which can increase the solubility of hydrophobic biomolecules and their partitioning efficiency in the alcohol-rich top phase (Li et al. 2017; Wang et al. 2010a). Similar observations were reported for the extraction of allicin in ethanol and ammonium sulphate system (Li et al. 2017). The interaction of salt ions with water molecules leads to the hydration of ions and dissolve more salts in the bottom phase, which will also decrease the number of free

water molecules in the bottom phase and these factors improve the exclusion of alcohol and biomolecule to top phase (Wang et al. 2010a). The partition coefficient of IGL was not found to significantly vary with 1-propanol concentration; however, the extraction efficiency of IGL was improved from 69.20 to 94.65 % (Figure 4.10 and 4.11) due to the increase in the top phase volume.

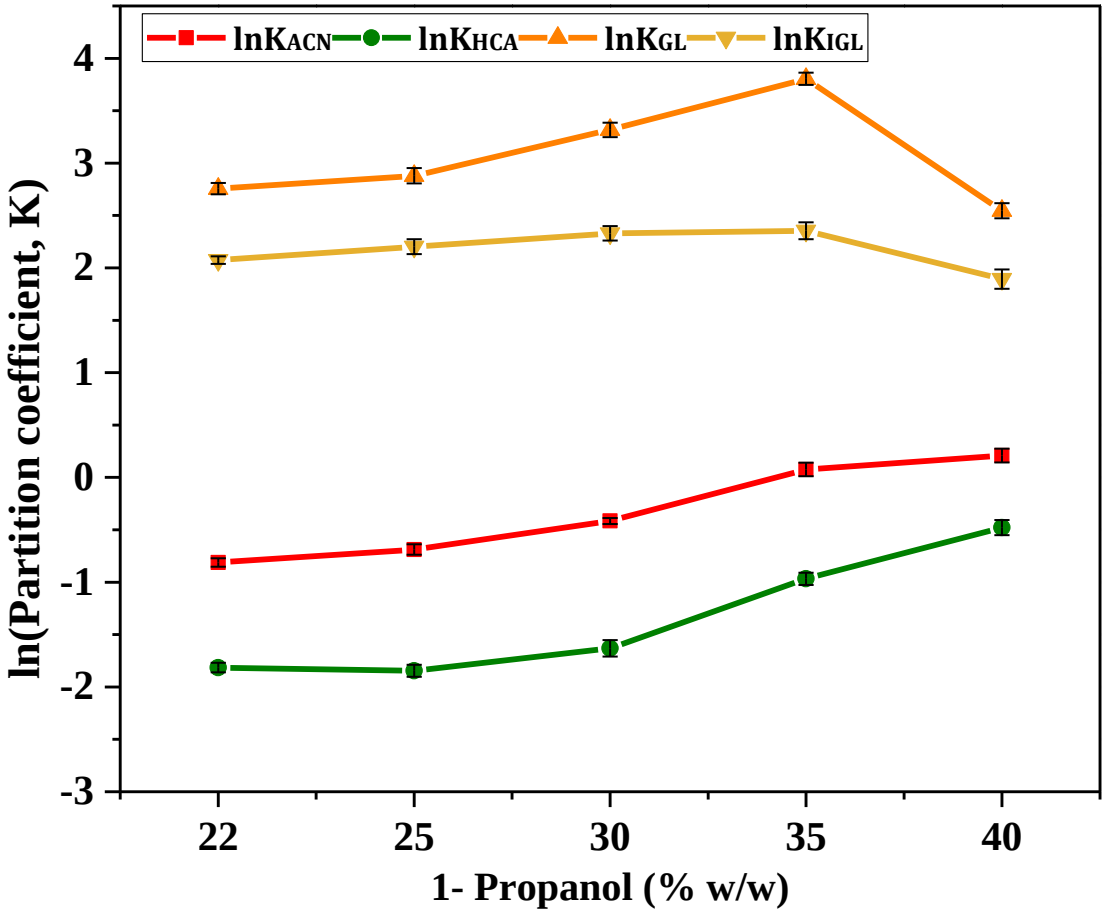


Figure 4.10 Effect of 1-propanol concentration on the partition coefficient of bioactive compounds in $MgSO_4$ (12 (%, w/w) – X (%, w/w) 1-propanol ATPS at 30 °C. (■), K_{ACN} and (●), K_{HCA} in bottom phase and (▲), K_{GL} and (▼), K_{IGL} in top phase.

The ACNs and HCA were partitioned in the salt-rich bottom phase even at lower 1-propanol concentration, which is sufficient to partition the hydrophilic ACNs and HCA in the bottom phase. As the 1-propanol concentration increases, the K_{ACN} and K_{HCA} gradually increased. The minimum K_{ACN} of 0.444 and K_{HCA} of 0.163 with higher extraction efficiency of ACN (90.69 %) and HCA (96.37 %) resulted in a

lower 1-propanol concentration of 22-25 % (w/w) (Figure 4.10 and 4.11). The increased phase volume and water content in the top phase at higher 1-propanol concentration are resulted in attracting the ACNs as well as HCA molecules towards the top phase, and consequently, the extraction efficiency of ACNs and HCA found to decrease sharply. Also, the salting-out behaviour of the salt-rich bottom phase contributed to the poor partitioning of ACNs and HCA. By considering the differential partitioning of four bioactive compounds in a single system, the system containing 25 % 1-propanol with 12 % w/w $MgSO_4$ may be a suitable ATPS and used for further studies.

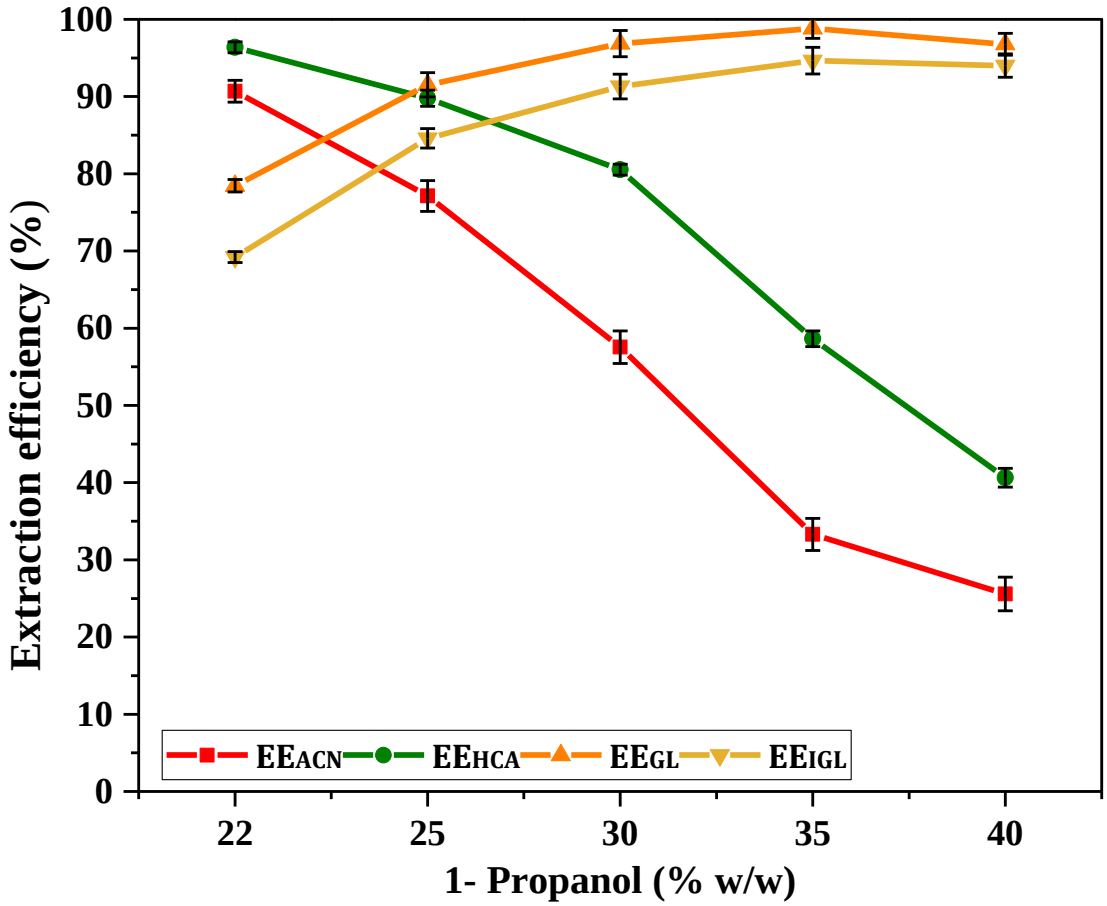


Figure 4.11 Effect of 1-propanol concentration on the extraction efficiency of bioactive compounds in $MgSO_4$ (12 (%, w/w) – X (%, w/w) 1-propanol ATPS at 30 °C. (■), EE_{ACN} and (●), EE_{HCA} in bottom phase and (▲), EE_{GL} and (▼), EE_{IGL} in top phase.

4.2.4.3 Effect of TLL on the partitioning of bioactive compounds

The net effective forces required to simultaneously partition the bioactive molecules to either of the phases of ATPS at the phase equilibrium conditions solely depend on the concentration of phase forming components. The equilibrium concentrations of phase forming components found to increase in the top and bottom phase as the TLL increases. The physical properties and free volume of the phases also tend to change with the TLL. The effect of TLL from 20.378 to 61.004 (%, w/w) on the partitioning of bioactives was studied (Figure 4.12). The phase equilibrium composition in each of the phases was obtained (Table 4.4) and the resulting binodal curve and with the tie lines were shown in Figure 4.5b.

Table 4.4 Phase Equilibrium Compositions for the 1-Propanol + $MgSO_4$ + H_2O System at 30 ^oC

Composition of ATPS (wt. %)		Top phase (wt. %)		Bottom phase (wt. %)		TLL (%)	Slope
1-propanol	$MgSO_4$	w_1^t	w_2^t	w_1^b	w_2^b		
25	10	37.97	4.31	19.31	12.50	20.378	-2.2784
25	12	45.22	2.56	14.29	17.00	34.134	-2.1420
25	15	51.20	1.44	10.47	22.52	45.861	-1.9322
25	18	55.42	0.72	8.19	28.49	54.789	-1.7008
25	20	57.71	0.50	7.45	30.46	58.512	-1.6776
22	12	43.64	2.9	17.51	13.89	28.347	-2.3776
30	12	50.24	1.61	10.77	20.59	43.796	-2.0796
35	12	53.98	1.00	8.29	27.48	52.808	-1.7255
40	12	59.22	0.33	7.08	32.00	61.004	-1.6464

As the TLL increases 20.378 to 61.004 (%, w/w), the equilibrium 1-propanol and $MgSO_4$ concentrations found to increase from 37.97 to 59.22 wt% and 12.50 to 32.00 wt%, respectively in top phase. It was observed from the table that the TLL and slope of the TLL were found to increases with increasing the global concentration of 1-propanol and salt in the ATPS. The increasing trend of TLL and its slope with increasing phase forming components concentration was similar to that of alcohol-salt systems as observed earlier (Guo et al. 2012a; Zhao et al. 2011). From Figure 4.8, it

was observed that the partition coefficients of GL and IGL were increased with increasing TLL and maximum partition coefficient of GL, and IGL was observed between the TLL of 34.13 to 52.80 (%, w/w). However, the increasing TLL does not favour the partitioning of GL and IGL beyond the 1-propanol equilibrium concentration of 55 wt% in the top phase. The higher hydrophobicity of the top phase at elevated TLLs may not be suitable to retain GL, IGL as it becomes not a good solvent. It was also confirmed during the preparation of crude extract as the 55-60 % (v/v) 1-propanol- aqueous mixture could extract maximum amounts of GL and IGL and above which the extractability declines. Meanwhile, the salt concentration also reaches saturation in the top phase at some point, and hence more water may be pulled towards the salt-rich phase, which probably made the alcohol-rich phase reject the GL as well as IGL. Similar observations can be found for antibiotics partitioning in ethanol/2-propanol-ammonium sulphate ATPS (Wang et al. 2010a).

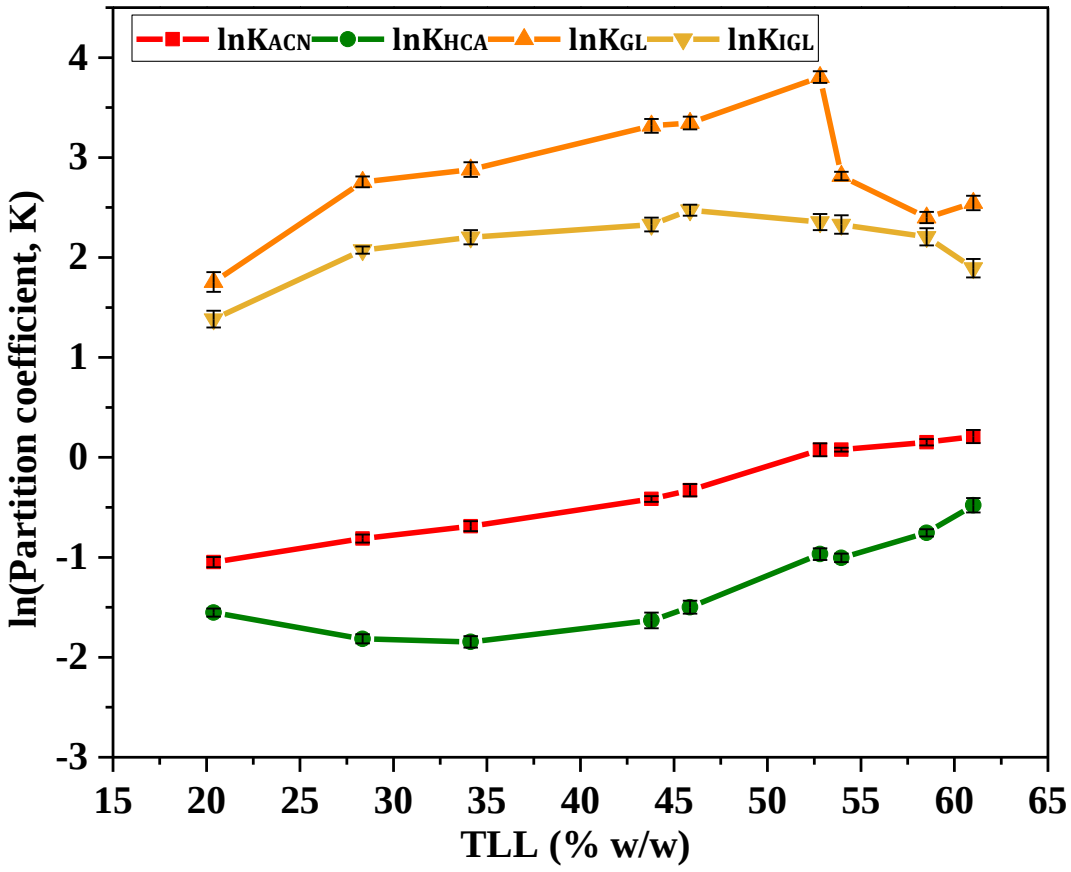


Figure 4.12 Effect of TLL on partition coefficient of bioactive compounds in various 1-propanol – $MgSO_4$ ATPS at 30 °C. (■), K_{ACN} and (●), K_{HCA} in bottom phase and (▲), K_{GL} and (▼), K_{IGL} in top phase)

As the TLL increases the partitioning of ACN decreases gradually and HCA found to partition to the bottom till 45.86 % and further decreases in the bottom phase, which was visualized with K_{ACN} and K_{HCA} values (Figure 4.8). The significant change of salt concentration in the bottom phase was observed when compared to the change of 1-propanol concentration in the top phase with increasing TLL (Table 4.2). The lower equilibrium salt content of 12.50 wt. % with a higher free volume of water in the bottom phase could accommodate more ACNs with mild hydrophobicity offered by the 1-propanol concentration of 4.31 wt. % at the TLL of 20 (wt. %). However, the $MgSO_4$ molecules try to interact with the free water at higher TLL and lead to the lesser partitioning of ACN in the bottom due to the salting-out of ACNs (Li et al. 2017; Wang et al. 2010a). But the HCA strongly interacts with the salt at higher TLL up to 43.796 % (salt equilibrium concentration from 12.50 to 20.59 wt%) which showed higher extraction efficiency more than 96% in the bottom phase, beyond this efficiency further decreases. These combined effects led to the decreased extraction efficiency of ACNs and HCA in the bottom phase at higher TLLs, as the ACN and HCA were migrated to the top phase because of reduced free volume and salting-out effect. However, decent simultaneous partition characteristics of GL and IGL in the top phase and ACN and HCA in the bottom phase can be observed at TLLs between 20.378 and 43.796 (%, w/w).

4.2.5 Differential partitioning behaviour of bioactive compounds in 1-propanol-ammonium sulphate ATPS

The ATPS formed with 1-propanol and $(NH_4)_2SO_4$ ATPS has been identified as a suitable system for the simultaneous partitioning of relatively hydrophobic biomolecules (GL and IGL) to alcohol rich phase and hydrophilic biomolecules (ACNs and HCA) to the salt-rich bottom phase from the extract of GI fruits during the screening of suitable ATPS by considering various alcohols, different molecular weight of PEG with different salts as phase forming components. The effect of phase components on the simultaneous partitioning characteristics of these four bioactive compounds in 1-propanol and $(NH_4)_2SO_4$ system was studied and optimized by performing the experiments within the two-phase region of ATPS. The binodal curve of the system was constructed and shown including tie-lines in Figure 4.5c.

ATP systems containing different concentrations of 1-propanol and ammonium sulphate were prepared to know the partition behaviour of bioactive components. The concentrations of bioactive compounds were analysed and the partition coefficient (K), extraction efficiency (EE) of all the biomolecules were calculated and reported in Figure 4.13 and 4.14. All the experiments were conducted in triplicate and average values were used. The error as standard deviation in the analysis was within ± 1 which is included in the graphs.

The K values less than one for ACN and HCA indicate the partitioning of these molecules towards the bottom phase because of their hydrophilicity and higher solubility in the salt phase. Lesser solubility of ACN in the bottom phase was observed compared to HCA, as they contain aromatic ring which makes them less hydrophilic compared to HCA. Sang et al. showed that the K values of monomer anthocyanins increased with increased hydrophobicity and lesser polarity (Sang et al. 2018). It has been found that the partitioning behaviour of compounds in ATPS was related to their molecular structures and the hydrophobicity was found to increases with increasing distribution ratio of five different small organic molecules (Rogers et al. 1998). Acylated anthocyanins are more hydrophobic compared than the non-acylated ACN and prefer alcohol rich top phase (Sang et al. 2018). The hydrophilic non-acylated ACN present in the GI preferred to partition into the bottom phase until the presence of free volume in accordance with the literature findings. The other hydrophobic molecules like GL and IGL partitioned in the top phase with the partition coefficient greater than one. GL and IGL are hydrophobic in nature preferred the 1-propanol rich top phase, which is similar to that of lutein esters enriched in ethanol rich phase (Fu et al. 2018). However, the attraction of GL and IGL to 1-propanol top phase also limits the ACN and HCA to enter top phase and try to expel them to bottom. Also, Wang and others suggested that hydrophobic substances are easier to be excluded from the bottom phase than the top phase in ATPS (Wang et al. 2010a).

4.2.5.1 Effect of $(NH_4)_2SO_4$

Partitioning of bioactive compounds depends on the type and concentration of salt that promotes or disturbs the hydrophobic interaction between the phase forming components and a biomolecule. The effect of $(NH_4)_2SO_4$ concentration was analysed

at a varied salt concentration between 10 (%, w/w) to 20 (%, w/w) at a constant 1-propanol concentration of 20 (%, w/w). The $(NH_4)_2SO_4$ exhibits the insolubility and shown the precipitation above 20 % (w/w) salt within the systems. The partition coefficient of GL and IGL found to increase with increasing salt concentration till 12 % w/w and found to decrease with the further increase, whereas the larger amount of ACNs got partitioned to the bottom phase at lower $(NH_4)_2SO_4$ concentration till 15% w/w and further decreased with increasing the salt concentration (Figure 4.13a). GL and IGL are hydrophobic and their affinity towards solvent readily dissolves them in the 1-propanol-rich phase. The increase of $(NH_4)_2SO_4$ concentration in the ATPS lead to the partitioning of higher amounts of GL and IGL in the 1-propanol rich phase due to the increase in the concentration of 1-propanol in the upper phase (Guo et al. 2012b). The partitioning coefficient of HCA was observed to increase gradually as salt increases (Figure 4.13a) due to the salting-in effect and increase of bottom phase volume. A similar observation was found for α-lactalbumin in PEG 1000–tri-potassium citrate system (Kalaivani and Regupathi 2013). Comparatively higher interaction of HCA with slat was observed in the bottom phase when compared to ACNs, accordingly, the HCA extraction efficiency also found to increase with increasing $(NH_4)_2SO_4$ concentration (up to 96.57% at 20 % (w/w)) as seen in Figure 4.13b. However, limited free volume in bottom phase beyond 12 % (w/w) $(NH_4)_2SO_4$ restrict the partitioning of ACNs and could able to retain a maximum of 84.47 % ACNs in bottom phase (Figure 4.13b). Meanwhile, ACNs compounds start to migrate to the top phase with increasing concentrations of salt because of the salting-out ability of $(NH_4)_2SO_4$; further the top phase act as not a good solvent for ACNs at higher salt concentrations due to less free space and increased hydrophobicity (Wang et al. 2010a). The maximum extraction efficiency of 95.16 % and 92.03 % were observed for GL and IGL, respectively in the 1-propanol rich top phase. However, the extraction efficiency of GL and IGL in the top phase decreased with increasing salt concentration due to the reduction of free space available to dissolve the GL and IGL (Figure 4.13b).

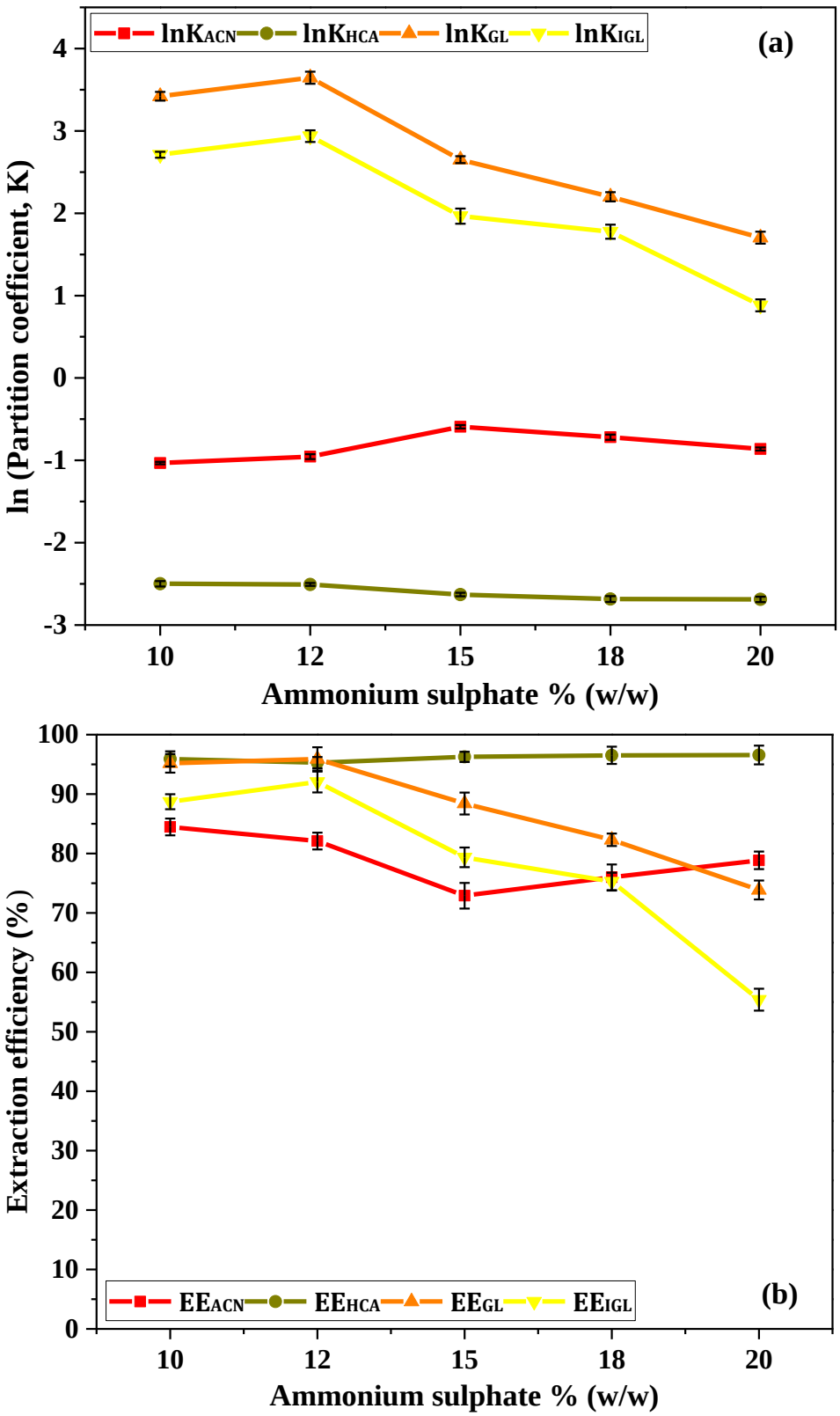


Figure 4.13 Effect of ammonium sulphate concentration on (a) partition coefficient and (b) extraction efficiency of bioactive compounds in a system of 25 (%, w/w) 1-propanol–X (%, w/w) ammonium sulphate at 30 °C. (■), ACN and (●), HCA in bottom phase (▲), GL and (▼), IGL in top phase

It was reported that the aqueous 1-propanol (55 to 65 %, v/v) and aqueous ethanol (80%, v/v) were suitable to extract maximum GL and IGL and the increasing concentration of alcohols leads to the lesser extraction because of the higher hydrophobicity of medium which becomes highly nonpolar to interact with these compounds and may also degrade the GL and IGL (Nainegali et al. 2019). The increase in salt concentration attracts the water molecules towards the salt-rich bottom phase and leading to the rejection of GL as well as IGL from the 1-propanol rich top phase (Li et al. 2017; Wang et al. 2010a). The ATPS with lower $(NH_4)_2SO_4$ concentration (about 10 %, w/w) could be the better system for the simultaneous partitioning of all the four bioactive compounds, which was further considered to study the other variables to maximize the extraction efficiency of bioactive components.

4.2.5.2 Effect of 1-propanol

The hydrophobic interaction of the system may be altered by the type of PEG/alcohol and their concentrations in the ATPS, specifically the partition characteristics of the molecules into the top phase (Kalaivani and Regupathi 2013). Hence, the experiments were conducted to study the effect of 1-propanol concentration on the partitioning characteristics of the biomolecules in the range of 20% to 40% (w/w) at a fixed $(NH_4)_2SO_4$ concentration of 10% (w/w). The extraction efficiency and K values of GL and IGL were found to increase with increasing 1-propanol concentration up to 35 % (w/w) and the extraction efficiency was found to decrease at a higher concentration of 1-propanol (Figure 4.14 a and b). The increasing hydrophobicity in the top phase with increasing 1-propanol concentration may interact with water molecules and improve the partitioning of GL and IGL towards the top phase (Li et al. 2017; Wang et al. 2010a). In addition, the exclusion of alcohol and biomolecule to top phase from the bottom phase is significantly improved due to the reduction of free water molecules in the bottom phase by the hydration of phase-forming salts (Wang et al. 2010a). GL and IGL content in the top phase was increased with increasing propanol concentration but meanwhile decreased partitioning co-efficient of ACNs and HCA were observed with increasing 1-propanol concentration (Figure 4.14a).

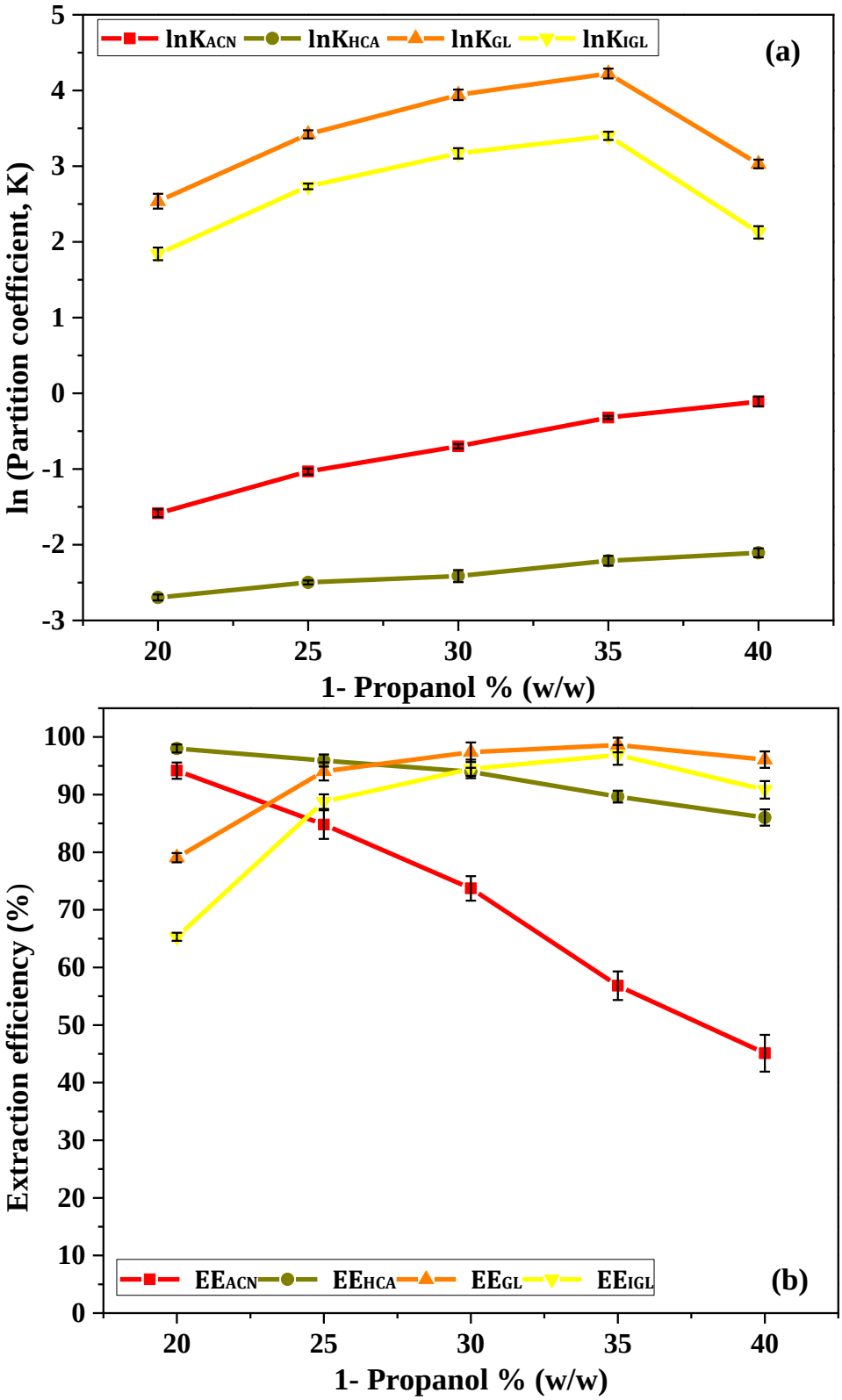


Figure 4.14 Effect of 1-propanol concentration on (a) partition coefficient and (b) extraction efficiency of bioactive compounds in a systems of 10 (%, w/w) ammonium sulphate–X (%, w/w) 1-propanol at 30 °C. (■), ACN and (●), HCA in bottom phase (▲), GL and (▼), IGL in top phase

The increasing amount of 1-propanol and water in the top phase attract the ACNs towards the top phase and hence the extraction efficiency sharply decreased in the bottom phase. But HCA showed a gradual decrease in K values and extraction efficiency because of its higher solubility due to their hydrophilic interaction with salt (Figure 4.14 a and b). The significant change in extraction efficiency was not observed between the 1-propanol concentration of 30 to 35 %, yet, 97.36 % (K_{GL} = 51.536) and 94.47 % (K_{IGL} = 23.792) extraction efficiency were achieved at 30 % 1-propanol concentration itself. The 20 % (w/w) 1-propanol resulted in extraction efficiency of 94.17 % (K_{ACN} = 0.205) of ACNs and 98.01 % (K_{HCA} = 0.0675) of HCA in bottom phase. The observed trend is similar to that of the partition characteristics of allicin in ethanol and ammonium sulphate ATPS (Li et al. 2017).

4.2.5.3 Effect of TLL on the partitioning of bioactive compounds

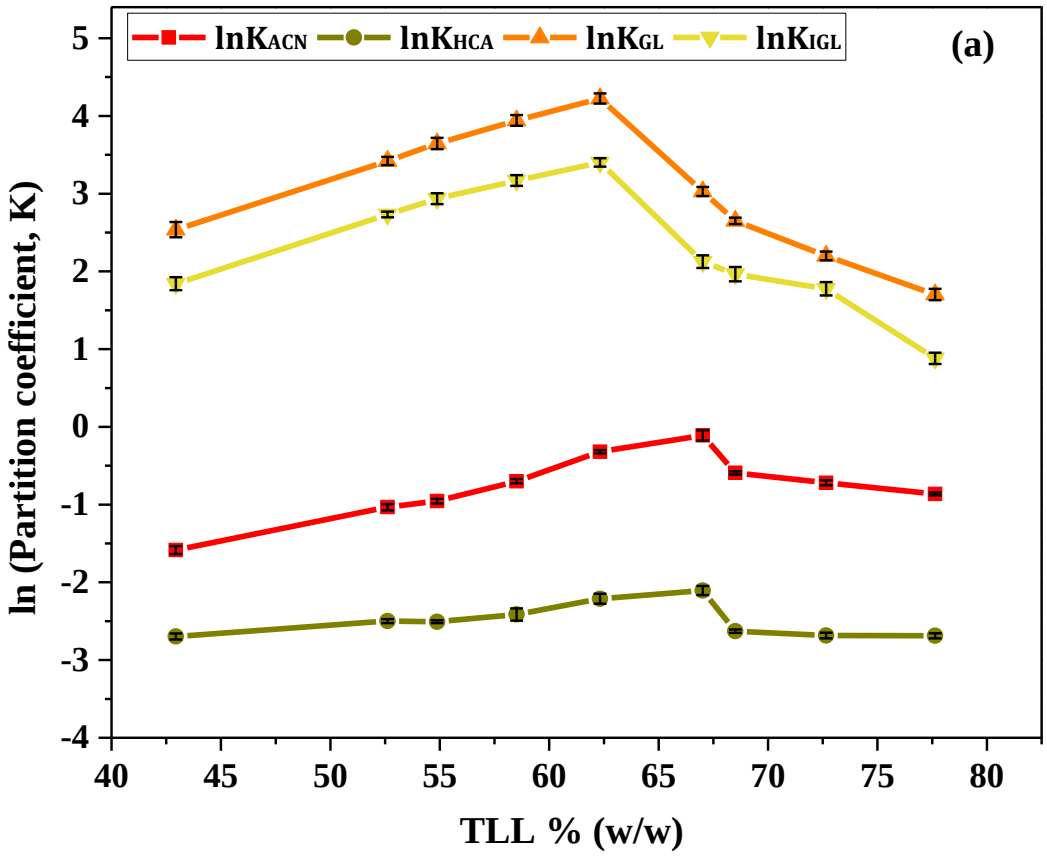
The increasing TLL is associated with the increase of top phase solvent and bottom phase salt concentrations at phase equilibrium and the consequent variation of physical properties and the available free volume of the phases in the system, which are responsible for the net partition of biomolecules. The equilibrium compositions in the top and bottom phases of the studied systems and corresponding TLL, STL values were reported in Table 4.5. The partitioning characteristics and extraction efficiency were analysed between the TLL of 42.93 to 77.63 (%, w/w)) and presented in Figure 4.15 a and b. The partition coefficient and extraction efficiency of GL and IGL were found to increase with increasing TLL due to the hydrophobic forces offered by increasing 1-propanol concentration in the top phase at higher TLL (Figure 4.15a).

However, the maximum extraction efficiency of GL and IGL was observed at 62.31 (%, w/w) TLL and a further increase in TLL does not favour the partitioning of these compounds to the top phase due to the saturation of top phase with GL and IGL. A similar trend was observed for the partitioning of α-Lactalbumin into the top phase in PEG-1000– trisodium citrate system (Kalaivani and Regupathi 2015), wherein the partitioning was increased till the saturation of the PEG rich top phase. The extraction efficiency of ACNs and HCA in the bottom phase were found to decrease with increasing TLL till 67%, however, the higher TLL above 67% favours the extraction of ACNs and HCA by shifting them from top to bottom phases and the extraction of

GL and IGL found to decrease at these range of TLL (Figure 4.15b). The simultaneous partitioning of GL and IGL into the top phase and ACNs and HCA to the bottom phase was significant between the TLLs of 42.93 and 58.51 (%, w/w).

Table 4.5 Phase Equilibrium Compositions for the 1-Propanol + $(NH_4)_2SO_4$+ H_2O System at 30 °C

Composition of ATPS (wt %)		Top phase (wt %)		Bottom phase (wt %)		TLL (% w/w)	Slope STL
1-propanol	$(NH_4)_2SO_4$)	w_1^t	w_2^t	w_1^b	w_2^b		
25	10	61.74	1.91	10.36	13.23	52.612	-4.538
25	12	63.21	1.82	9.69	14.04	54.897	-4.379
25	15	70.25	1.53	6.02	20.65	67.015	-3.359
25	18	74.12	1.34	5.10	24.04	72.657	-3.040
25	20	77.39	1.27	4.41	27.29	77.479	-2.804
20	10	53.67	2.34	11.8	11.86	42.939	-4.398
30	10	66.01	1.71	8.99	14.84	58.512	-4.342
35	10	67.97	1.57	7.77	17.68	62.318	-3.736
40	10	70.40	1.51	6.72	19.28	66.112	-3.583



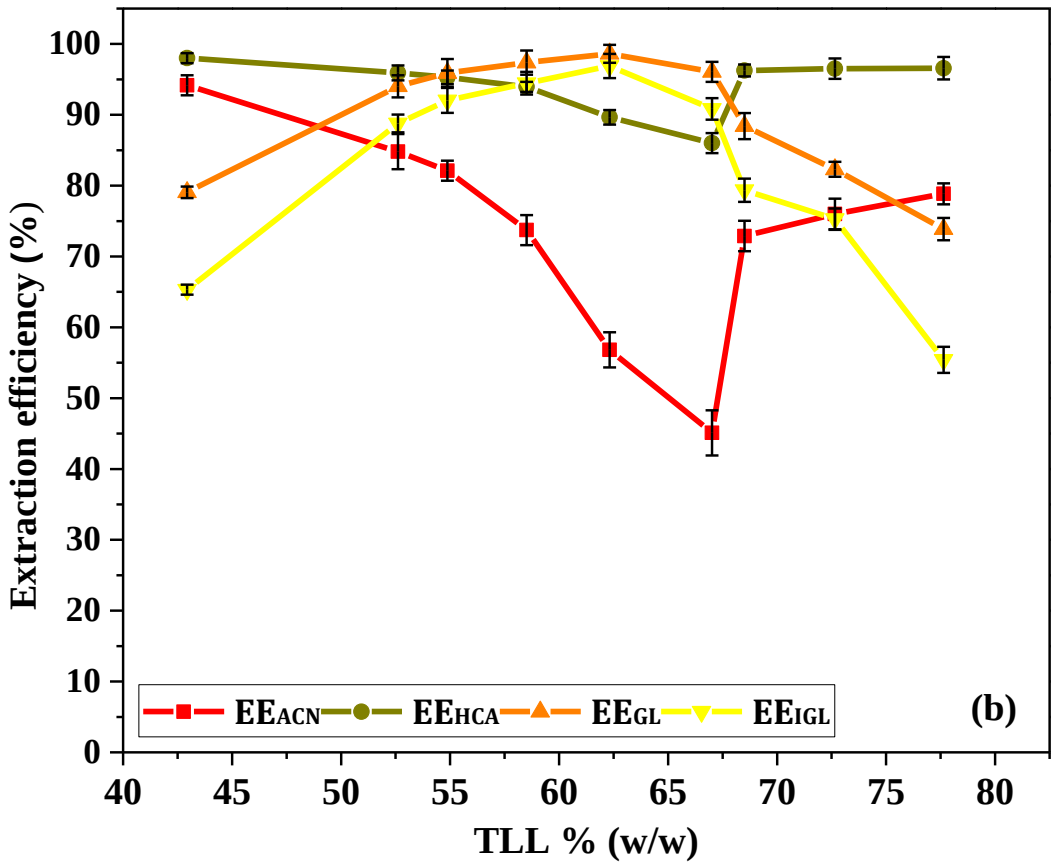


Figure 4.15 Effect of TLL on (a) partition coefficient and (b) extraction efficiency of bioactive compounds in various 1-propanol – ammonium sulphate systems at 30 °C. (■), ACN and (●), HCA in bottom phase (▲), GL and (▼), IGL in top phase

4.2.6 Optimization of process variables of ATP by using RSM in 1-propanol-ammonium sulphate system

The simultaneous extraction and separation of multiple compounds from the crude in a single step ATPE process are very complex and the analysis of synergistic interaction effects between the influencing factors on the partitioning coefficient may help to understand such extractions. Table 4.6 shows the global system composition of all the three alcohol-based ATPSs used for the partitioning of bioactive compounds and provide the maximum yield from the crude extract of GI by studying the one variable at a time experiment. The 1-propanol- ammonium sulphate system was found to be a suitable system to simultaneously partition the hydrophobic and hydrophilic components to the top and bottom phases, respectively. The minimum global

concentrations of 1-propanol (20% w/w) and $(NH_4)_2SO_4$ (10% w/w) showed the better extraction efficiency at the TLL of 42.93 when compared to other systems. For the successful implementation of the ATPE, it is necessary to study the interactive effect of significant variables and optimise them for the simultaneous extraction of ACNs, HCA, GL, and IGL at different goals of the operation. The significant factors and their range of values on the simultaneous partitioning were obtained from the results of one factor at a time experiment, which was used for the RSM studies. The statistical tool ‘‘Minitab, version 17.0’’ was used to design the extraction experiments and analyse the responses to obtain the maximum simultaneous extraction of bioactive compounds. The following experiments for RSM studies were conducted in triplicate and average values were used for analysis. The error in the analysis were within ± 1%.

Table 4.6 The suitable global phase composition obtained for alcohol-based ATPSs used for the partitioning of bioactive compounds

ATP Systems	Variables			Extraction efficiency, EE (%)			
				Bottom		Top	
	Alcohol (% w/w)	Salt (% w/w)	TLL %	EE_{ACN}	EE_{HCA}	EE_{GL}	EE_{IGL}
Ethanol- $(NH_4)_2SO_4$	25	19	41.37	86.33	75.17	96.39	94.26
1- Propanol $MgSO_4$	25	12	34.13	90.51	96.31	94.20	89.44
1- Propanol - $(NH_4)_2SO_4$	20	10	42.93	94.17	98.01	95.16	92.03

4.2.6.1 Analysis of Responses

The three independent process variables namely, 1-propanol concentration, $(NH_4)_2SO_4$ concentration, and the crude load was considered as significant variables. These phase components have wider and diverse effect on separation of these diversified compounds, so there is need to arrive at an optimised conditions to get maximum yields for all the compounds. pH, time of extraction and temperature are not considered in the optimisation. This is because, the presence of multiple compounds and phase components within the system where further addition of acid or base will increase the complexity of the system. Anthocyanins being pH sensitive needs acidic medium which is supported by both 1-propanol and HCA compounds.

As alcohol- salts ATPs showed instantaneous phase separation and attained equilibrium faster, the time of incubation and temperature are not considered as major variables in the present study.

The number of experiments with a different combination of process variable's value was obtained through the central composite design (CCD), which is one of the efficient design of experiments method for complex systems. The lower, upper and nominal values of the variables in 5 different levels used for the experimental design are listed in Table 3.3. The partition coefficient and extraction efficiency of each of the four compounds (ACN, HCA, GL, and IGL) were determined at each design point as 8 responses (4 direct responses and 4 supporting responses) and reported in Table 4.7. The individual responses were correlated to the significant variables of the process by developing the quadratic Eqs. (4.1) to (4.8) through regression analysis.

$$K_{ACN} = 0.521- 0.02637\ A- 0.0741\ B + 0.1674\ C + 0.001602\ A^2 + 0.002857\ B^2 + 0.08317\ C^2 + 0.000276\ AB - 0.01283\ AC - 0.00418\ BC \quad (4.1)$$

$$K_{HCA} = -0.0011 + 0.010169\ A - 0.01492\ B+ 0.03830\ C - 0.000024\ A^2 + 0.000766\ B^2 + 0.004012\ C^2 - 0.000365\ AB - 0.000798\ AC - 0.001876\ BC \quad (4.2)$$

$$K_{GL} = 93.4 + 2.76\ A - 24.89\ B + 74.5\ C + 0.1365\ A^2 + 1.1887\ B^2 + 3.54\ C^2 - 0.3234\ AB - 2.390\ AC - 1.997\ BC \quad (4.3)$$

$$K_{IGL} = 49.1 + 0.001\ A - 13.39\ B + 64.10\ C + 0.12790\ A^2 + 0.6696\ B^2+ 2.611\ C^2 - 0.1646\ AB - 1.971\ AC - 1.845\ BC \quad (4.4)$$

$$EE_{ACN} = -14.2 + 6.699\ A + 7.34\ B + 0.07\ C - 0.1273\ A^2 - 0.1690\ B^2 - 1.47\ C^2 - 0.1676\ AB - 0.165\ AC + 0.505\ BC \quad (4.5)$$

$$EE_{HCA} = 103.43 - 0.299\ A + 0.285\ B - 1.99\ C - 0.01301\ A^2- 0.0423\ B^2 - 0.322\ C^2+ 0.03962\ AB + 0.0475\ AC + 0.1069\ BC \quad (4.6)$$

$$EE_{GL} = -124.6 + 14.28\ A - 13.74\ B + 126.6\ C - 0.0865\ A^2 + 0.804\ B^2 + 3.77\ C^2 - 0.1866\ AB - 3.414\ AC - 3.336\ BC \quad (4.7)$$

$$EE_{IGL} = -214.9 + 17.61\ A - 15.16\ B + 180.3\ C - 0.0803\ A^2+ 1.020\ B^2 - 0.89\ C^2 - 0.279\ AB- 4.192\ AC - 5.08\ BC \quad (4.8)$$

Table 4.7 Experimental Design points and corresponding experimental responses

Run order	Factors			Responses							
				Partitioning co-efficient (K)				% Extraction efficiency (EE)			
	1-propanol (A) (%w/w)	$(NH_4)_2SO_4$ (B) (%w/w)	Crude Load (C) (g)	K_{ACN}	K_{HCA}	K_{GL}	K_{IGL}	EE_{ACN}	EE_{HCA}	EE_{GL}	EE_{IGL}
1	25	14	1.5	0.361	0.050	6.820	4.268	84.27	97.26	78.13	69.09
2	20	16	2.0	0.286	0.034	10.858	5.883	92.27	98.53	80.36	63.91
3	35	14	1.5	0.897	0.073	37.973	29.715	51.62	93.25	94.34	98.63
4	25	14	1.5	0.351	0.049	10.566	6.254	86.79	97.87	82.50	73.62
5	25	14	1.5	0.351	0.048	6.560	4.883	86.79	97.79	75.50	69.64
6	20	12	2.0	0.274	0.053	18.069	12.142	91.10	98.26	86.58	74.26
7	25	14	1.5	0.341	0.048	6.820	4.268	85.15	97.79	78.13	69.09
8	30	12	1.0	0.614	0.079	35.540	25.749	74.26	94.80	95.15	96.35
9	30	16	1.0	0.654	0.053	23.383	20.286	66.71	96.23	94.81	95.16
10	30	16	2.0	0.606	0.050	5.136	3.166	65.12	96.49	78.58	69.34
11	25	14	1.5	0.361	0.049	6.820	4.268	86.79	97.87	78.13	69.09
12	20	16	1.0	0.203	0.030	4.946	3.019	92.00	99.06	61.20	49.05
13	20	12	1.0	0.177	0.040	4.422	2.176	93.05	98.90	55.32	37.86
14	25	14	2.5	0.483	0.056	8.196	4.505	84.09	97.28	85.61	67.91
15	15	14	1.5	0.124	0.020	4.598	5.409	96.31	99.73	44.92	25.03
16	25	10	1.5	0.369	0.082	38.358	21.681	87.34	96.10	94.42	90.54
17	25	14	1.5	0.341	0.049	6.820	4.268	86.79	97.87	78.13	69.09
18	25	14	0.5	0.384	0.049	14.145	10.262	86.36	97.66	78.49	70.03
19	30	12	2.0	0.586	0.083	25.538	16.287	70.85	94.31	93.51	89.61
20	25	18	1.5	0.424	0.039	14.949	9.289	80.64	98.12	87.87	81.82

where, K_i and EE_i are the predicted responses like partition co-efficient and extraction efficiency for the components ACNs, HCA, GL and IGL. A and B are concentration of 1-propanol % (w/w) and $(NH_4)_2SO_4$ % (w/w), respectively in un-coded units. C is crude load (g).

The significance of the eight quadratic equations was analysed using the statistical method 'Analysis of variance (ANOVA)''. The statistical parameters are estimated to determine the impact of independent variables on the responses. The coefficient of determination (R^2), adjusted R^2 and the predicted R^2 were estimated to test the global fitness of the model. The greater coefficient of determination (R^2 > 0.99) and model F-value for all the models indicated that the models had adequately represented the real relationship between the parameters chosen and authenticates the significance of the proposed equations (Table 4.8). The predicted R^2 values with adjusted R^2 for all the equations show the good agreement of variables to their respective responses (Eqs.4.1 – 4.8) (Table 4.8) i.e. predicted R^2 (0.9913, 0.9929, 0.9239, 0.9772, 0.9554, 0.9702, 0.8711, 0.9152, respectively) values show good agreement with adjusted R^2 values (0.9970, 0.9972, 0.9751, 0.9910, 0.9853, 0.9841, 0.9589, 0.9768, respectively). The model P-values ($p < 0.0001$) of all the equations shows that there is less than 0.01% of the effect of the variable is not accounted by the model. Significantly higher 'F-value' also indicates that the regression model is highly significant to predict the responses (Kalaivani and Regupathi 2015).

The lack of fit test compares residual error to the pure error which was obtained by repeating the central point six times and P-value of lack of fit test with greater than 0.05 for all the models (shows non-significant) and lesser F-value indicates that all the models indicate that these equations may able to predict the corresponding responses satisfactorily (Table 4.8). Low values of coefficient of variance designated that the models were reliable and precise (Alara et al. 2018). All the linear terms of the independent variables (A, B, C) have a significant effect ($p < 0.05$) on the responses like partition coefficient and extraction efficiency of bioactive compounds except for the crude load (C) ($p = 0.232$) on the extraction efficiency of IGL (Table A4.1 and 4.2 (Appendix)). The squared terms (A^2, B^2, C^2) of all the variables for the partition coefficient (Eq. 4.1 to 4.4) were significant ($p < 0.05$),

however all the product terms of Eq. 4.2 to 4.4 (AB, AC, and BC) for the partition coefficient of HCA, GL, IGL were significant ($P < 0.05$) and propanol/load (AC) term alone significant among the product terms of the equation 4.1 for ACNs (Table A4.1 and 4.2 (Appendix)). The square terms of 1-propanol (A^2) and $(NH_4)_2SO_4$ (B^2) were significant for the extraction efficiency equation of all bioactive components (Eq. 4.5 – 4.8). All three product terms of Eq. 4.7 and 4.8 (AB, AC, BC) were found to be significant for the extraction efficiency of GL and IGL. However, the terms associated with crude loading (C) (i.e., AC and BC) (Eq. 4.5 and 4.6) were found to be insignificant ($P > 0.15$) to predict the extraction efficiency of ACNs and HCA (Table A4.1 and 4.2 (Appendix)) (Agcam et al. 2017; Alara et al. 2018).

Table 4.8 ANOVA for equations of partition coefficient/extraction efficiency (Eqs. 4.1- 4.8).

Responses	R^2	Adj. R^2	Pred. R^2	Std. Dev.	Model F-value	Model P-value	Lack of fit	
							F-value	P-value
K_{ACN}	99.84%	99.70%	99.13%	0.01006	712.47	<0.0001	1.52	0.328
K_{HCA}	99.85%	99.72%	99.29%	0.00087	763.45	<0.0001	1.12	0.450
K_{GL}	98.69%	97.51%	92.39%	1.81769	83.73	<0.0001	1.74	0.280
K_{IGL}	99.53%	99.10%	97.72%	0.79149	233.00	<0.0001	0.96	0.516
EE_{ACN}	99.22%	98.53%	95.54%	1.36372	142.15	<0.0001	2.03	0.228
EE_{HCA}	99.16%	98.41%	97.02%	0.20670	131.72	<0.0001	0.53	0.748
EE_{GL}	97.84%	95.89%	87.11%	2.71934	50.27	<0.0001	1.90	0.249
EE_{IGL}	98.78%	97.68%	91.52%	2.84127	90.02	<0.0001	3.90	0.081

4.2.6.2 Interactive effects of variables

The interactive effects between the independent variables on the responses were analysed using the contour plots generated for partition coefficient (Figure 4.16 (a–f) and 4.17 (a–f)) and extraction efficiency (Figure 4.18 (a–f) and 4.19 (a–f)) using the quadratic equations (Eqs. 4.1 – 4.8). The graphical contour plots represent the effect of two independent variables on the responses while maintaining the other variable at a constant level. The combined effect of 1-propanol (A) and $(NH_4)_2SO_4$

(B) on partition coefficients of ACNs (Figure 4.16a) and HCA (Figure 4.16d) was obtained when the crude load (C) was kept at 1.5 g. The lowest K_{ACN} value of 0.12 was achieved at a lower 1-propanol concentration of 15 % (w/w) and 12 to 15 % (w/w) of $(NH_4)_2SO_4$. Further increase in both the concentrations tends to increase the partition coefficient due to the increased interaction of moderately hydrophilic ACN molecules to the 1-propanol rich top phase and limited free volume in the bottom phase (Sang et al. 2018). However, the optimum K_{ACN} (lower than 0.2) was observed between the 1-propanol concentration of 15 to 20% (w/w) and 10 to 14 % (w/w) of $(NH_4)_2SO_4$ with the crude load between 1.0 to 2.0g (Figure 4.16b and c). These results suggest that lower concentrations of 1-propanol (15 %) and 10 to 13 % (w/w) of $(NH_4)_2SO_4$ with crude load lesser than 2.0 g may be suitable to retain the ACNs in the bottom phase.

Minimum K_{HCA} of less than 0.03 was obtained in the system formed with 15 % 1-propanol concentrations and 13 to 15% (w/w) $(NH_4)_2SO_4$ at the crude loading of about 1.5g (Figure 4.16d and e). A gradual decrease in the partition coefficient (K_{HCA}) was observed with increasing $(NH_4)_2SO_4$ concentration and crude load from 1.5 to 2.5g (Figure 4.16f). The hydrophilic HCA tends to partition in the salt-rich phase and K_{HCA} found to decrease accordingly with increasing salt concentration with lower 1-propanol concentrations due to the substantial interaction of HCA caused by the salting-in effect and free volume of bottom phase. The interactive effects of independent variables on the extraction efficiency of the ACNs and HCA were shown in Figure 4.17 (a–f). 90 % of the ACNs (Figure 4.17a) and 99 % of the HCA were yielded in the bottom phase of the system formed at lower concentrations of 1-propanol (15 to 20 %, w/w) and 12 to 16 % (w/w) of $(NH_4)_2SO_4$ when the crude load (C) was kept at 1.5 g or less than 2.0 g (Figure 4.17f).

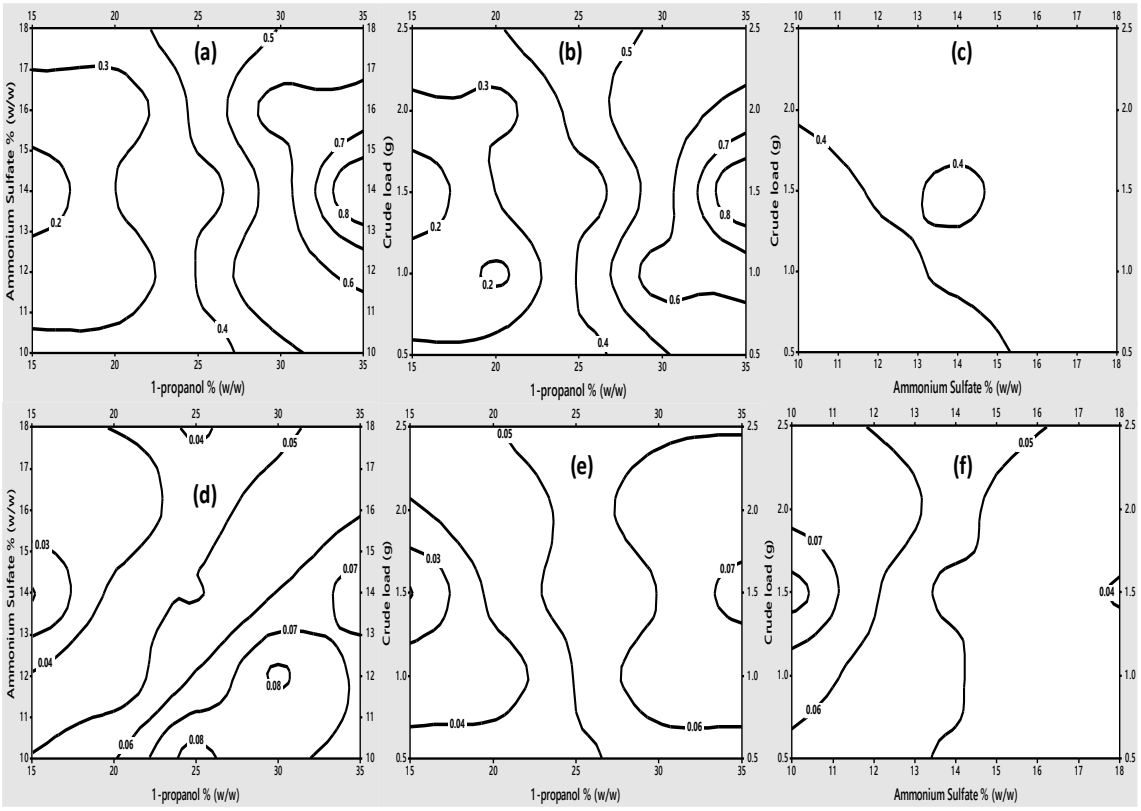


Figure 4.16 (a-f) Contour plots of the variables on the partitioning coefficients of ACN (a-c) and HCA (d-f)

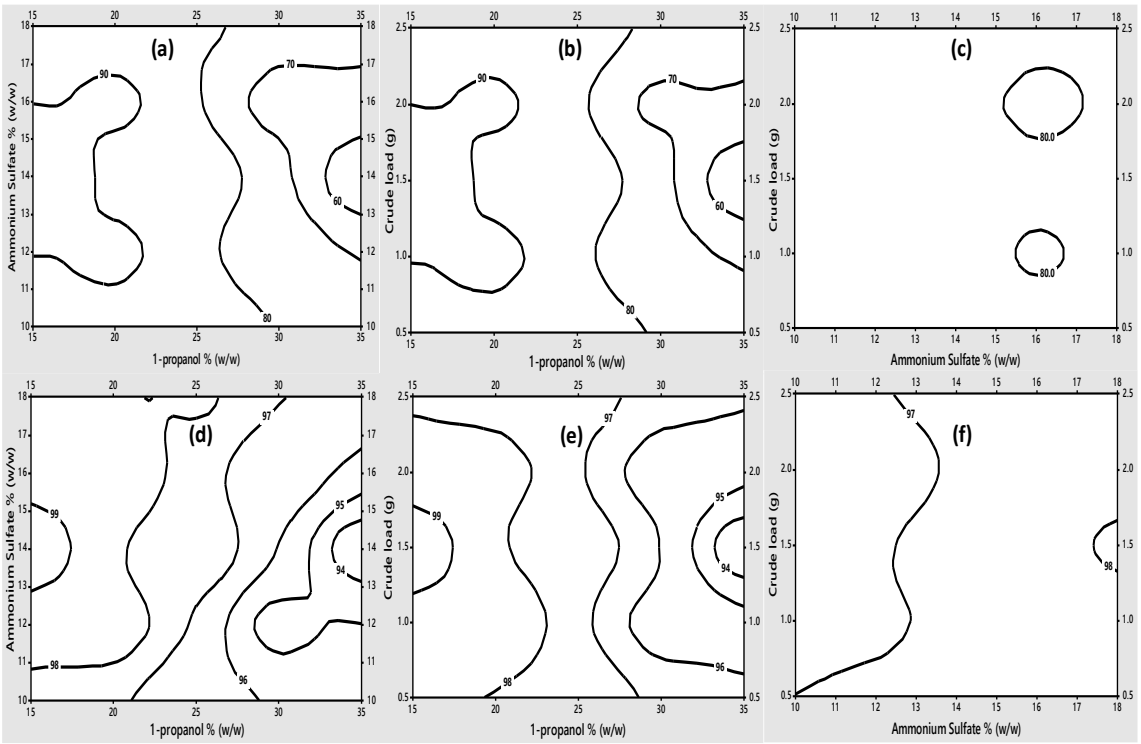


Figure 4.17 (a-f) Contour plots of the variables on the extraction efficiency of ACN (a-c) and HCA (d-f)

The aqueous insoluble hydrophobic GL and IGL compounds are preferred the solvent rich top phase in the 1-propanol-$(NH_4)_2SO_4$ system (Li et al. 2017; Wang et al. 2010a). Accordingly, the optimal K_{GL} and K_{IGL} values higher than 35 and 25, respectively were obtained at higher 1-propanol concentrations (25 to 35 % w/w) with a minimum salt concentration of around 10 % (w/w) and lower crude load about 1 – 2 g (Figure 4.18a - f). The interactive effect between $(NH_4)_2SO_4$ and crude load (C) at 25% 1-propanol reviled that the lower salt concentration of 10 % w/w is enough for higher partition coefficient even for the higher crude load of 2 to 2.5g. The GL and IGL interact with the solvent 1-propanol due to their hydrophobicity and salting-out effect, which lead to a larger free volume of 1-propanol in the top phase. The abundant 1-propanol in the top phase able to accommodate a larger quantity of GL and IGL at increased crude load. From Figure 4.19 (a) and (d), it was observed that the extraction efficiency was <90 % for GL as well as IGL between the concentration of 10 to 15 % (w/w) for 1-propanol and $(NH_4)_2SO_4$ (Li et al. 2017; Wang et al. 2010a).

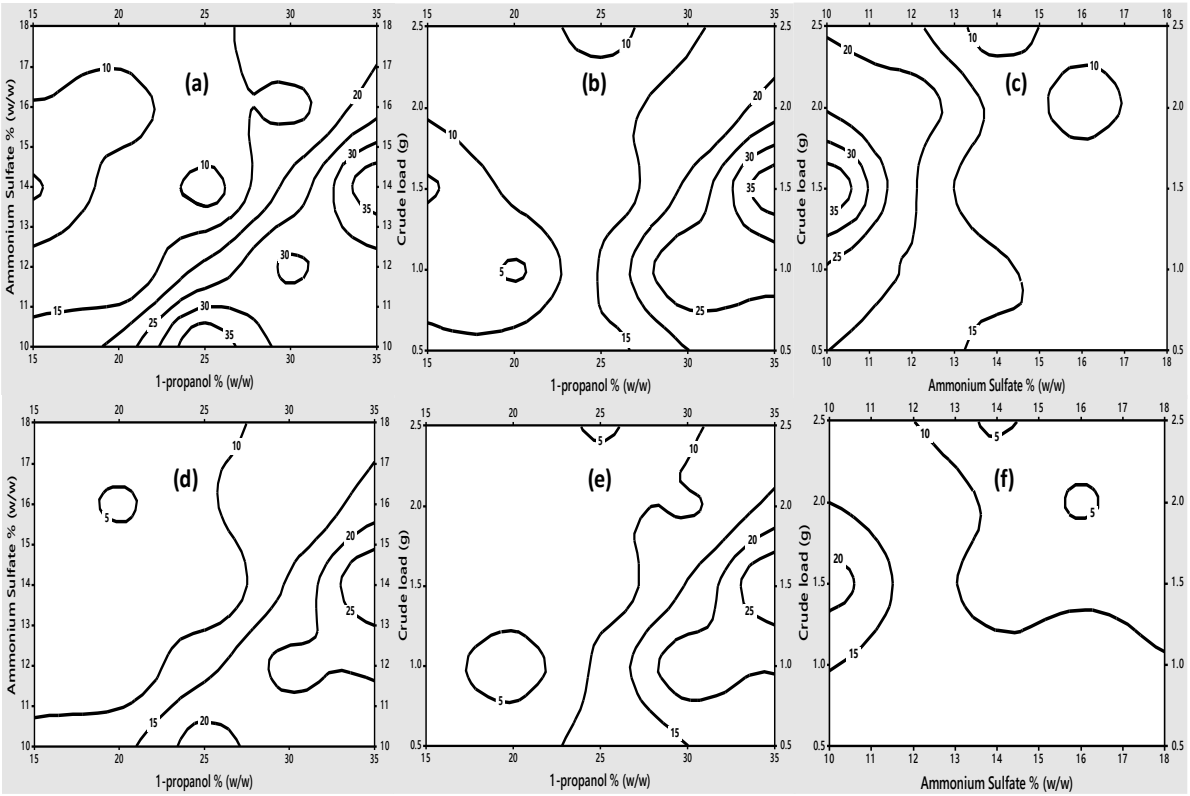


Figure 4.18 (a-f) Contour plots of the variables on the partitioning coefficients of GL (a-c) and IGL (d-e)

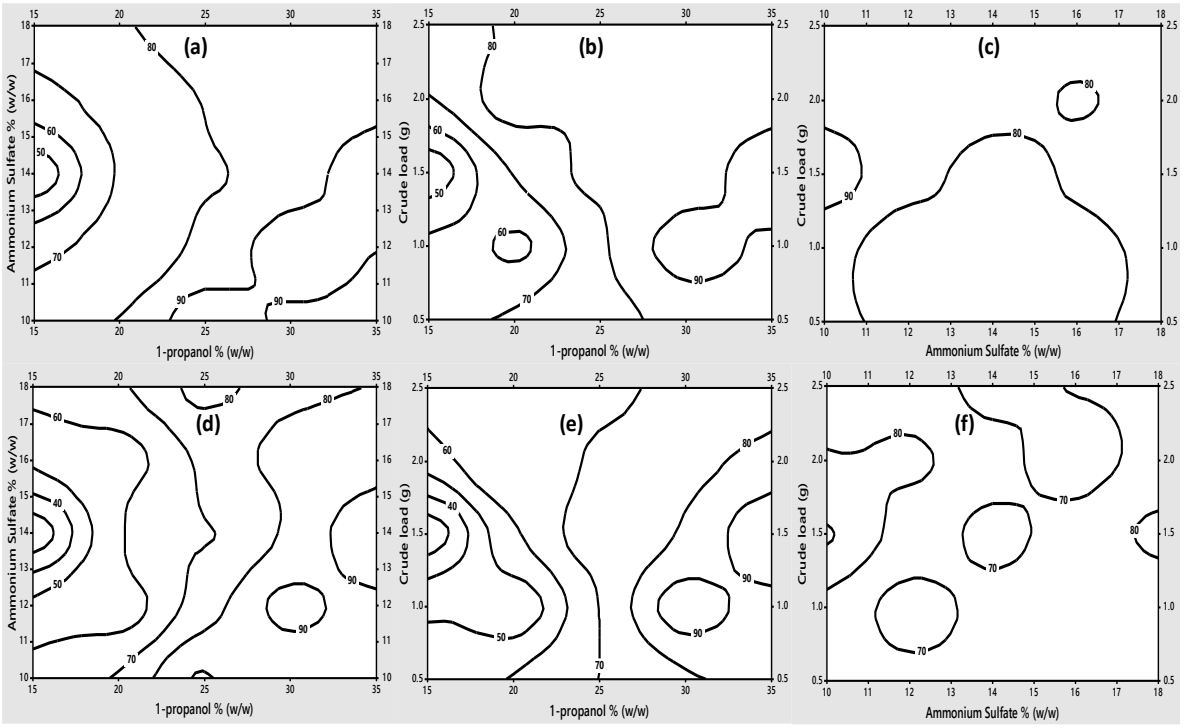


Figure 4.19 (a-f) Contour plots of the variables on the extraction efficiency of GL (a-c) and IGL (d-e)

The lower salt concentration of 10 % (w/w) and 20 to 35 % (w/w) 1-propanol at higher crude loading can accommodate more than 95 % of GL and IGL in the top phase (Figure 4.19 a-f). By considering the simultaneous extraction of all four bioactive compounds, the system consisting of lower 1-propanol (15 to 20 %, w/w) and around 10 % (w/w) $(NH_4)_2SO_4$ with maximum crude loading may able to accommodate significant hydrophobic compounds (< 90 %GL and IGL) in top phase, when retaining most of the ACNs and HCA in the bottom phase. The indifferent process conditions obtained for the maximum extraction efficiency of hydrophilic (ACNs and HCA) and hydrophobic compounds (GL and IGL) to the top and bottom phases of ATPS, respectively demand the optimum process conditions for the different goal of purification.

4.2.6.3 Multiple-response optimization

The optimized process condition for any one of the responses often varies disproportionately from the optimal conditions of other responses, when multiple responses are dependent on the same set of variables. The simultaneous optimization

of multiple responses for different goals of separation and purification of different biomolecules may be a conceivable approach, where the responses are interdependent. It is mandatory to find the single feasible optimal condition for the desired goal to implement it in the practical application. Hence, the desirability objective function based multi-response optimization was performed through a numerical optimization approach as reported earlier reports (Agcam et al. 2017; Jamshidi et al. 2018; Kalaivani and Regupathi 2015; Porto and Natolino 2018). This involves the simultaneous optimization of quadratic models obtained for individual responses by the transformation of multiple responses to a weighted mean average of individual desirability function. The inputs are the model equations, weightage, target value or the upper and the lower bounds. The desirability (D) ranges from zero to one decides the least to most desirable optimal conditions. As ACNs, HCA, GL, and IGL interact differently with phase components such as 1-propanol-$(NH_4)_2SO_4$ system, the desirability approach for multi-response optimisation was applied to find out optimal conditions. The partitioning of GL and IGL to alcohol rich phase and ACNs and HCA to salt-rich phase were targeted and accordingly five different goals were considered for the optimization as shown in Table 4.9. The optimum conditions and the desirability for different goals were obtained using the statistical software and reported in Table 4.9.

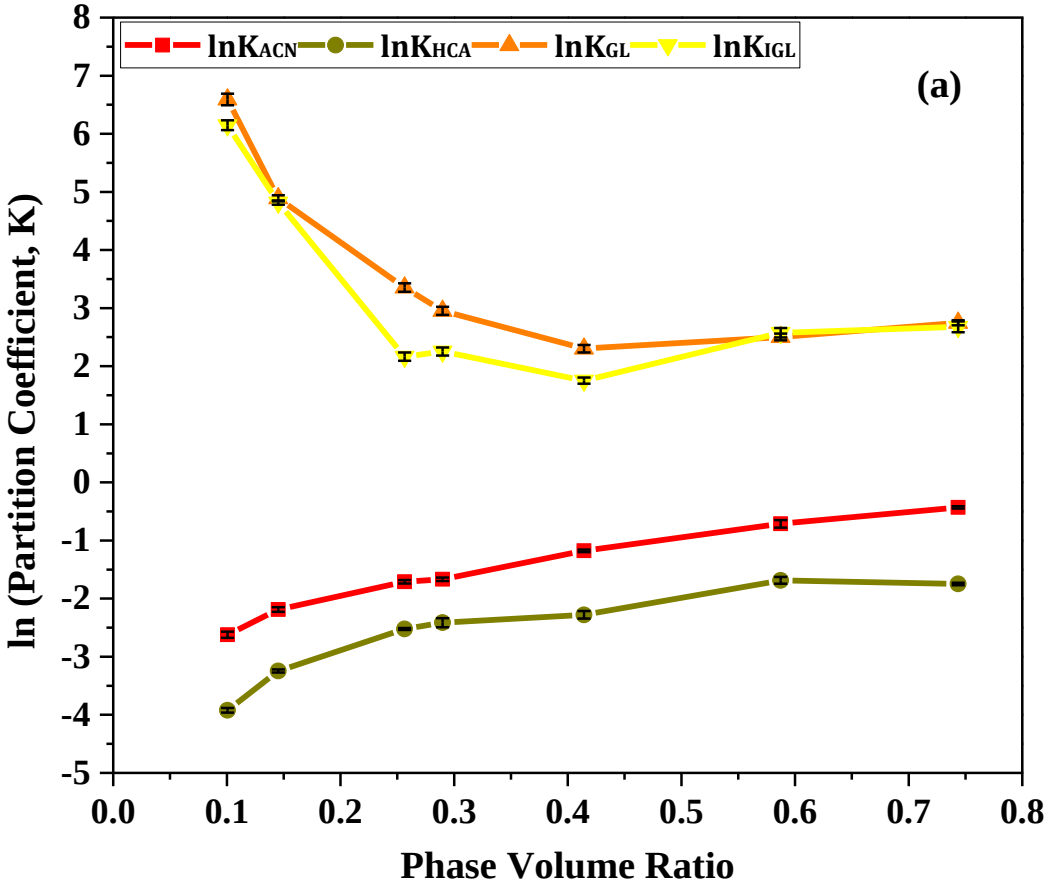
The desirability for three different goals was achieved as 0.842, 0.853, 0.877, 0.823 and 0.823 respectively for (i) Maximising the K value of GL, IGL in the top phase and minimise the K values of ACNs, HCA, (ii) Maximise the extraction efficiency of GL, IGL in the top phase and ACNs, HCA in the bottom phase and (iii) Maximise K values of GL, IGL and minimise for ACNs, HCA along with maximising the extraction efficiency of all the components to their respective phases. The predicted responses at the optimized condition for all the three goals were compared with the experimental responses obtained by conducting the experiments at the optimized values of variables (A, B, C). The experimental extraction efficiency and K values of ACNs and HCA for all three goals are better than the predicted values by the response model and nearly the same for GL and IGL. The composition of ATPS predicted for the third goal (15.202 % w/w 1-propanol, 10.242 % w/w $(NH_4)_2SO_4$ and 2.5g crude load) was further considered for the enrichment and purification studies.

Table 4.9 Optimum conditions obtained for different goals and the experimentally determined values

Goals	Solutions	Factors			Partition co-efficient (K) and Extraction efficiency (% EE)					Desirability
		A	B	C	Results	ACN	HCA	GL	IGL	
Maximise K value of GL, IGL Minimise the K values of ACN, HCA	1	15	18	2.197	Predicted (K)	0.333	0.030	38.393	24.953	0.842
					Experimental (K/EE)	0.262/ 94.25	0.026/ 99.38	36.450 /89.79	20.589 /83.25	
Maximise EE values of GL, IGL, ACN, HCA	1	15.912	12.113	2.41	Predicted (EE)	91.90	98.54	95.15	77.95	0.853
					Experimental (K/EE)	0.129/ 98.13	0.020/ 99.75	135.79 /95.34	37.219 /84.86	
	2	**15.202**	**10.242**	**2.5**	**Predicted (EE)**	**87.13**	**98.09**	**99.91**	**98.55**	**0.877**
					Experimental (K/EE)	**0.080/ 99.19**	**0.016/ 99.83**	**370.77 /97.39**	**120.58 /92.38**	
Maximise K values of GL, IGL, Minimise for ACN, HCA and Maximise EE for all	1	15	12.062	2.5	Predicted (K)	0.388	0.045	38.324	31.108	0.823
					Experimental (K)	0.146	0.021	96.912	33.907	
					Predicted (EE)	91.19	98.50	99.02	81.27	
					Experimental (EE)	97.88	99.74	94.09	84.80	
	2	15	18	2.5	Predicted (K)	0.421	0.033	44.253	29.075	0.823
					Experimental (K)	0.242	0.021	45.309	32.236	
					Predicted (EE)	97.15	97.77	94.81	73.10	
					Experimental (EE)	95.44	99.53	90.85	87.60	

4.2.6.4 Effect of Phase Volume Ratio

The phase volume ratio of the system alters the free volume within the phases which can change the partitioning/extraction efficiency of the biomolecules (Kalaivani and Regupathi 2015). The effect of phase volume ratio was studied at 15.202 % (w/w) 1-propanol, 10.242 % (w/w) $(NH_4)_2SO_4$ having the TLL of 28.505 % (w/w) at a crude load of 25 wt.% (Figure 4.20). The experiments were conducted in triplicate and average values were utilised. The error in the analysis were within ± 1%.



From Figure 4.20a, it was observed that the partitioning coefficient of GL and IGL was less until the volume ratio of 0.3 and became stable at a higher volume ratio. The lower volume of top phase is sufficient to hold all the GL and IGL molecules in the top phase, however, the increased top phase volume would reduce the partitioning characteristics due to the decrease in hydrophobicity of phase with the increasing water content of top phase which started accommodating ACN molecules. Accordingly, the

extraction efficiency of ACNs, HCA in the bottom phase gradually decreases with increasing phase volume ratio. However, the higher volumes of either of the phases resulted in instability to retain the respective molecules in their phases due to the dilution of phase components.

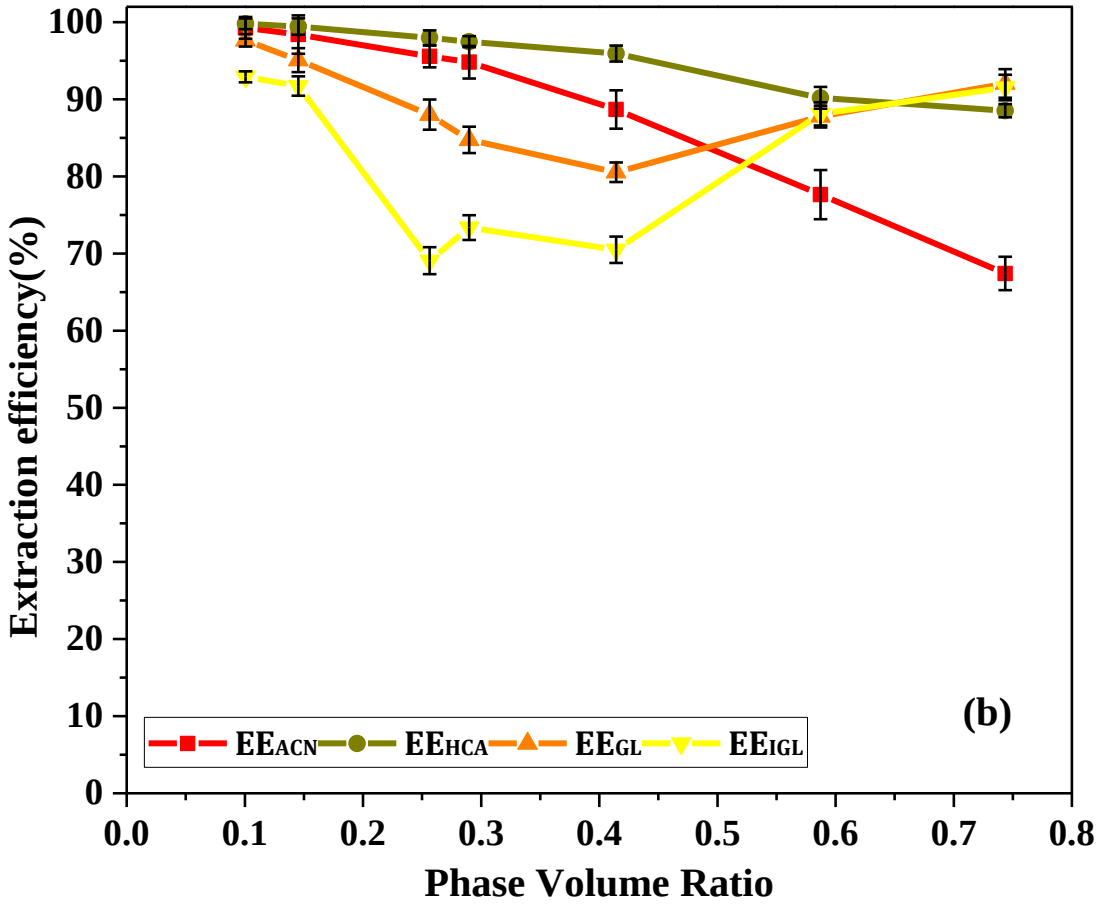


Fig. 4.20 Effect of phase volume ratio on the (a) partitioning coefficient and (b) extraction efficiency of bioactive compounds. (■), ACN and (●), HCA in bottom phase (▲), GL and (▼), IGL in top phase.

The lower phase volume of 0.1 is optimum to maintain 99.27 %, 99.80 % of ACNs and HCA molecules in the bottom phase and 97.65 %, 92.91 % of GL and IGL to top phase respectively (Figure 4.20b).

4.3 THE ENRICHMENT OF THE BIO-ACTIVE COMPOUNDS EMPLOYING SECOND STAGE ATPE WITH OPTIMISED SYSTEMS

The second step Aqueous Two-Phase Extraction (ATPE) strategy is reported in the literature for the recovery of the partitioned molecule from the ATPS phases. The present study is the first of its kind to discuss the application of second step ATPE to improve the partitioning and purity of a specific group of molecules in a particular phase in a multi-component ATPE process. The purity of the compounds partitioned in specific phases of ATPS is further improved by implementing the second sage ATPE to remove the non-targeted biomolecules. In the first step ATPE, the ACN and HCA are portioned to the bottom phase and GL, IGL to the top phase in the 1-propanol- $(NH_4)_2SO_4$ system. However, some quantity of GL and IGL are still present in the bottom phase and similarly, traces of ACN and HCA are left in the top phase. To remove that, the prepared new top and bottom phases (of the same system without crude loading) and mixed with the bottom and top phases, respectively. This step is named as enrichment step ATPE where bioactive compounds were enriched in terms of extraction efficiency, recovery, and purity. The steps followed for initial extraction and separation of bioactive compounds involving first and second stage or enrichment step ATPE is represented in Figure 4.21.

4.3.1 Second stage ATPE in 1-propanol- magnesium sulphate system

The second stage ATPE ($ATPE^2$) was implemented to recover the traces of ACNs and HCA present along with GL and IGL in the top phase of first stage ATPE ($ATPE^1$) and traces of GL and IGL present in the bottom phase of $ATPE^1$. A fresh ATPS (second ATPS) was formed at the same TLL of 34.134 of $ATPE^1$, without the addition of crude and the equilibrated phases were carefully separated and used for the $ATPE^2$. The fresh bottom phase was mixed with the top phase of $ATPE^1$ to recover (R_{ACN2} and R_{HCA2}) the left out ACNs and HCA. Similarly, the fresh top phase was mixed with the bottom phase of $ATPE^1$ to recover the traces of GL and IGL (R_{GL2} and R_{IGL2}). The recovery of each biomolecule at $ATPE^1$ and $ATPE^2$ was listed in Table 4.9. Further, the overall recovery

of the components (R_{Total}) in the individual phases were obtained by considering both the top phases of $ATPE^1$ and $ATPE^2$ and bottom phases of $ATPE^1$ and $ATPE^2$ (Table 4.10).

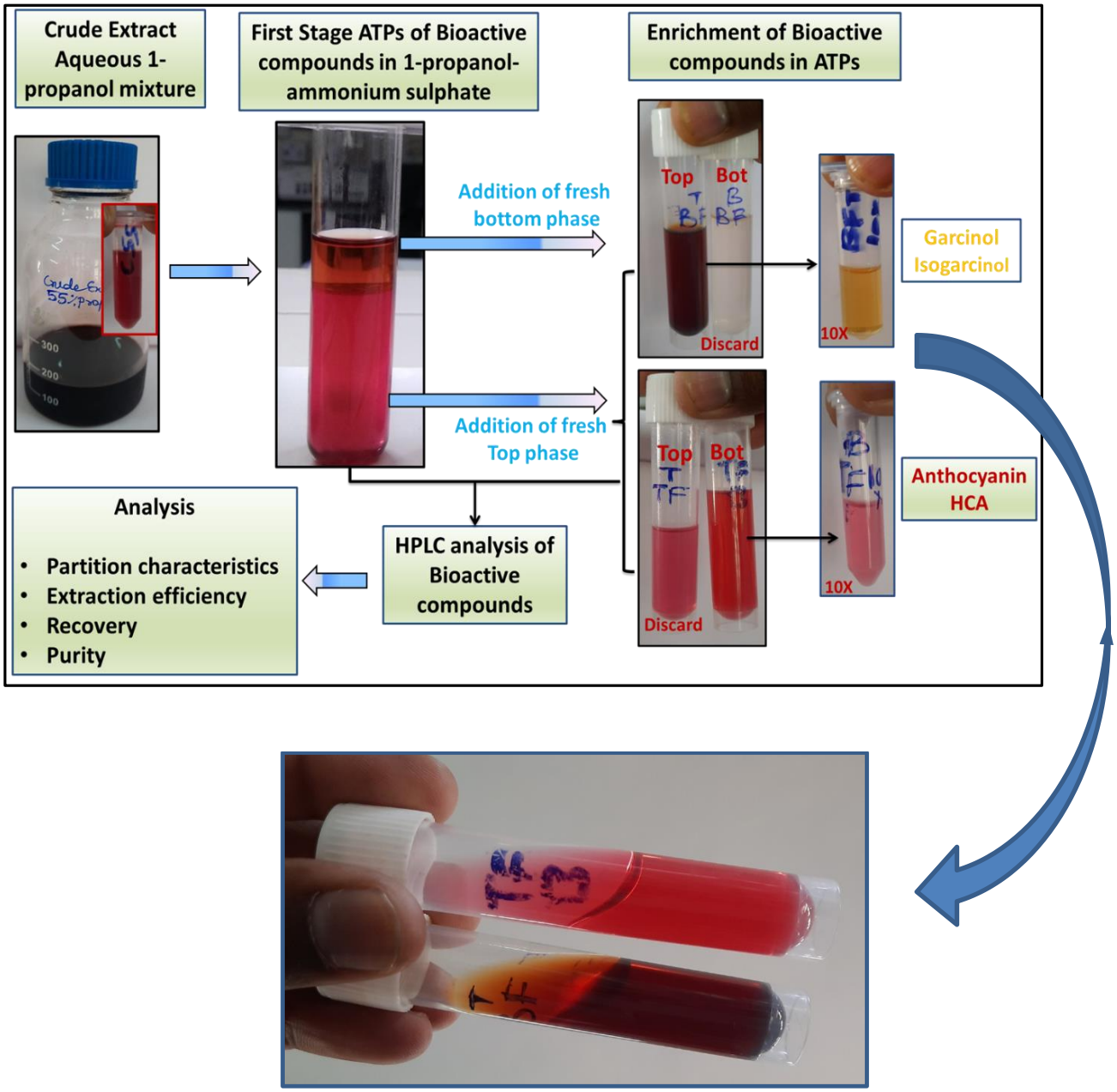


Figure 4.21 The pictorial representation of the separation of bioactive compounds from initial extraction to second stage ATPE

The R_{ACN1} and R_{HCA1} in the $ATPE^1$ were 69.25 % (extraction efficiency-90.68 %) and 85.34 % (extraction efficiency-96.31 %), respectively. The addition of fresh bottom phase would recover 65.08 % (R_{ACNs2}) and 90.56 % (R_{HCA1}) from the top phase of $ATPE^1$ and the least amount of GL and IGL (approx. 5 to 8 %) was lost by their migration to fresh bottom. The overall recovery (R_{Total}) of ACNs and HCA increased to 77.55 % and 89.72 % with a maximum purity of 99.08 % of ACN and HCA in the combined bottom phases of $ATPE^1$ and $ATPE^2$ with respect to GL and IGL (Table 2). Similarly, when the fresh top phase added to the bottom phase of $ATPE^1$, the traces of GL and IGL migrate from the bottom phase of $ATPE^1$ to the fresh top phase ($ATPE^2$). However, a marginal improvement in the partitioning of GL and IGL was observed in the $ATPE^2$ with the improved recovery of GL from 94.02 % ($ATPE^1$) to 95.38 % ($ATPE^2$) and for IGL from 90.66 % to 91.66 %. The overall purity of GL and IGL in the top phase was observed as 60.38 % due to the presence of a considerable quantity of HCA in the top phase. Even though 89.72% of HCA was recovered during the process, due to the higher content of HCA compared to other molecules, specifically GL and IGL, is responsible for the lower purity (Table 4.10). The purity of all components in their respective phases may be further improved by fine-tuning the partition parameters in the $ATPE^2$ process, which is not focused in this present work. The phases of $ATPE^2$ can be subjected to subsequent separation processes to attain the high purity of biomolecules.

The added crude extract into the ATPE contains the impurities like proteins (2849.31 mg/L) and carbohydrates (16692.50 mg/L). The top and bottom phases of the $ATPE^2$ was analysed to find out the concentration of these impurities. The alcohol-based ATPSs are known to partition the proteins and carbohydrates to the salt-rich bottom phase (Cheng et al. 2016; Hua et al. 2013; Wu et al. 2011). Most of the proteins (78%) and carbohydrates (94%) were partitioned in the salt-rich bottom phase of $ATPE^2$ by enriching the top phase with GL and IGL. However, the impurities may be removed by introducing the additional phases of ATPS and fine-tune the partitioning parameters to enrich the ACNs and HCA in the bottom phase by eliminating these impurities or employing other purification steps.

Table 4.10 First and Second stage ATPS results showing concentrations, partitioning coefficients and recovery in either phase after each step of ATPS.

Stages	Phases	Bioactive compounds											
		ACN			HCA			GL			IGL		
		(mg/L)	K_{ACN}	EE_{ACN} (%)	(mg/L)	K_{HCA}	EE_{HCA} (%)	(mg/L)	K_{GL}	EE_{GL} (%)	(mg/L)	K_{IGL}	EE_{IGL} (%)
ATPE[1]	**Top**	79.91	0.453	90.51	2857.40	0.166	96.31	2884.58	15.737	94.02	285.48	9.717	90.66
	Bottom	176.32			17258.49			183.30			29.38		
ATPE[2]	**Top-ATPE[1]**	26.09	0.502	89.61	215.96	0.083	98.11	2754.87	21.238	95.50	262.81	11.597	92.05
	Fresh Bottom - ATPE[2]	52.01			2587.56			129.71			22.66		
	Fresh Top-ATPE[2]	29.43	0.210	95.36	1837.48	0.120	97.31	171.31	14.291	93.45	25.80	7.195	87.81
	Bottom-ATPE[1]	139.86			15344.03			11.99			3.59		
Total Recovery (R_{Total})		191.88		**77.55**	17931.59		**89.72**	2926.18		**95.38**	288.61		**91.66**
Purity (I)		**Bottom phase purity for ACNs and HCA = 99.08%**						**Top phase purity for GL and IGL = 60.38**					

The experiments were conducted in triplicate and average values were utilised. The error in the analysis were within ± 1%.

4.3.2 Enrichment step ATPE in 1-propanol- ammonium sulphate system

The bottom phase from the optimized process ($ATPE^1$) contains the concentration (mg/ L) of ACNs: 564.71 and HCA 49419.24 with minor concentration of GL: 22.61 and IGL: 11.839 and the top phase contains the concentration (mg/ L) of GL: 8382.70, and IGL: 957.55 with minor concentration of ACNs: 45.60, HCA: 804.17. The co-existing trace compounds GL and IGL were eliminated from the bottom phase by introducing the fresh top phase ($ATPE^2$) prepared based on the equilibrium concentration according to the tie line. Similarly, the GL and IGL were enriched by removing the traces of ACNs and HCA from the top phase by introducing the fresh bottom phase ($ATPE^2$). The equilibrium compositions of 1-propanol with $(NH_4)_2SO_4$ in the top phase calculated were 40.81 and 3.82 % w/w, in the bottom phase were 13.17 and 10.79 % w/w giving TLL 28.505 ($ATPE^2$). These were prepared accordingly and introduced to the first step top and bottom phases ($ATPE^1$) respectively as explained in methods section 3.2.10.1. The extraction efficiency, EE (Eq. 3.2 and 3.3) and recovery, R (Eq. 3.18 and 3.19) of all the components in their respective phases with respect to their concentration in the initial crude and their purity, P (Eq. 3.20 and 3.21) after the enrichment step partitioning were reported in Table 4.11.

Table 4.11 Enrichment of bioactive compounds using second stage ATPE

Stages	Type of Phases	Bioactive compounds	Extraction efficiency %	Recovery %	Purity %
Optimized system (First stage)	Top (I)	GL	**97.39**	**99.73**	**91.78**
		IGL	**92.38**	**99.17**	**91.78**
	Bottom (I)	ACN	**99.19**	**92.40**	**99.93**
		HCA	**99.83**	**98.41**	**99.93**
ATPE performed with Top (I) and Fresh Bottom phases	Top	GL	**94.65**	**99.17**	**99.45**
		IGL	**89.05**	**97.96**	**99.45**
	Bottom	To be discarded with the minor concentration of ACNs and HCA			
ATPE performed with fresh Top and Bottom (I) phases	Top	To be discarded with the minor concentration of GL and IGL			
	Bottom	ACN	**98.94**	**83.27**	**99.99**
		HCA	**99.81**	**96.26**	**99.99**

The experiments were conducted in triplicate and average values were utilised. The error in the analysis were within ± 1%. The removal of traces of GL and IGL is not significant since a higher purity of ACNs and HCA (>99%) was obtained in the ATPE[1] itself. However, the finding may be helpful to understand the loss of yield due to the partitioning characteristic of ACNs and HCA while trying to increase the purity to extreme value. However, almost all the GL and IGL were partitioned with the purity of > 99 % in the top phase (Table 4.10). As the IGL is the isomeric form of GL, both the components have similar chemical and biological properties (Schobert and Biersack 2019) and hence both of them may be used together for antioxidant properties. The GL and IGL further separated from 1-propanol rich-top phase (Boiling point of propanol: 97 °C and GL, IGL: 710.8 °C) in the vacuum rotary evaporator and the recovered 1-propanol can be recycled in the ATPE process.

Similarly, The ACNs are stable in the pH range of less than 6 and hence the ACNs are portioned together with HCA in the salt-rich bottom phase by considering the stability of ACNs. However, few studies are available to separate the HCN from the fruit extract in free form using ion-chromatography (Moffett et al. 1997, 2001) U.S. patent no. 5,536,516) and osmotic membrane distillation (OMD) (Ramakrishnan et al. 2008) (US patent no 20070154578 A1) and the sodium salt of HCA using NaOH and acetone in a large-scale isolation process (Ibnusaud et al. 2000a; b) (U.S. Patent No. 6,147,228). These methodologies may be implemented to separate the HCA from the bottom phases of ATPE, which contains ACNs and phase forming components.

4.3.3 Fractionation of ACN and HCA in 1-propanol-Ammonium sulphate ATPS

An attempt has been made to separate out ACN and HCA from the enriched bottom phase (ATPE[2]). The separation ability and extraction efficiency of ACN in top and HCA in bottom phases were analysed. The composition of the bottom phase has been changed from 15 to 40 % w/w of 1-propanol at constant $(NH_4)_2SO_4$ concentration of 15 % w/w. The mass of each system was kept constant to maintain the desired concentration within the system. As seen in Figure 4.22, the increase in 1-propanol concentration resulted in the partition coefficient as well as the extraction efficiency of ACN increases in the top phase up to 35 % w/w and beyond that, it

decreased. Even though HCA prefers the bottom phase, a gradual transfer of HCA molecules was observed towards the top phase with increasing 1-propanol concentration. All the experiments were conducted in triplicate and average values were utilised for analysis. The error in the analysis were within ± 1%.

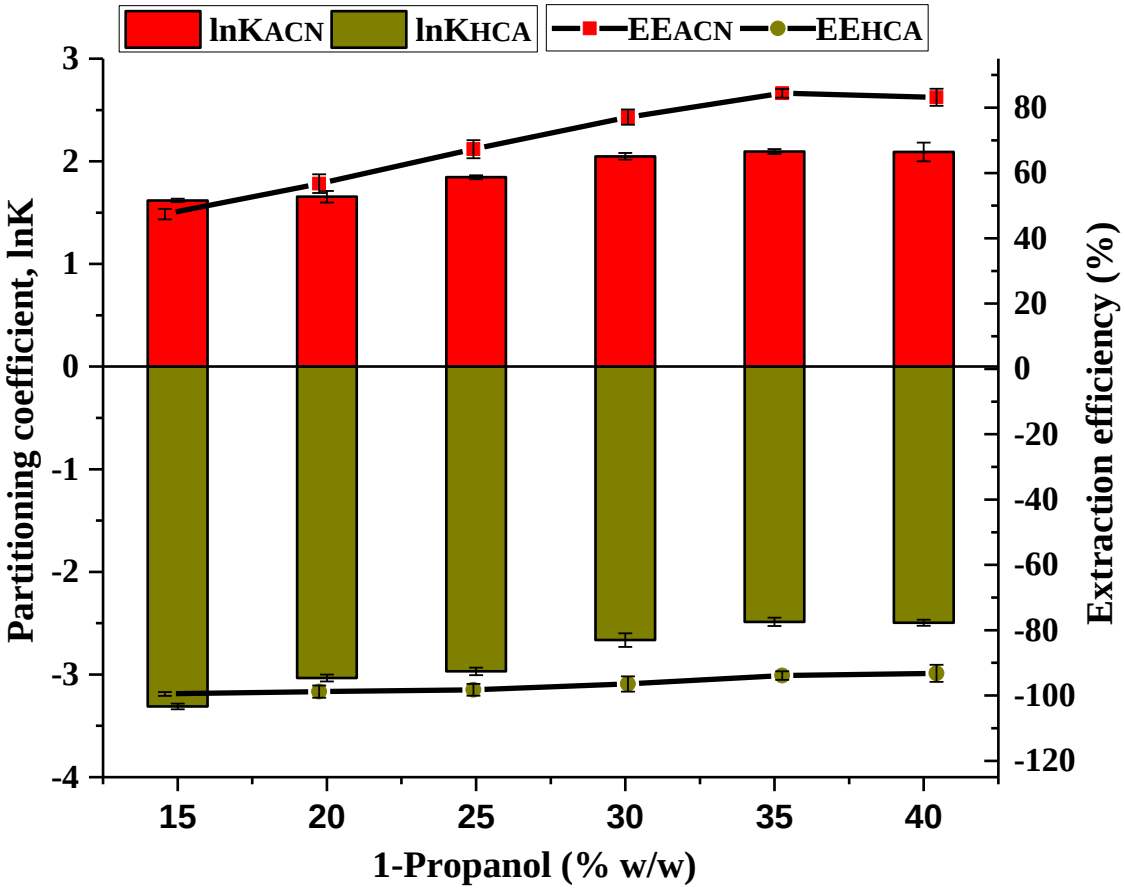


Figure 4.22 Fractionation of ACN in top phase and HCA in bottom phase from enriched bottom phase at different concentrations of 1-propanol and at constant $(NH_4)_2SO_4$ in terms of partition coefficient and extraction efficiency

As HCA molecules are hydrophilic, always seem to prefer the salt rich phase with higher interaction with salts present. The increase in 1-propanol concentration increases the hydrophobicity and volume of the top phase, which is proved to be suitable for ACNs up to some extent (Hua et al. 2013; Nainegali et al. 2017; Sang et al. 2018; Wu et al. 2014). Moreover, this condition could accommodate more amounts of ACN molecules but further increase in hydrophobicity reduced the extraction efficiency (Figure 4.22). Meanwhile, due to available free volume in the top phase

enhance the partitioning of HCA towards top phase which decreased the extraction efficiency at higher 1-propanol concentrations. In view of maximum separation of ACN and HCA mid-levels of 1-propanol concentration could be beneficial as seen in Figure 4.22. The 30% w/w propanol is considered a better system to extract a good amount of ACNs which reached about 77.11 % (K_{ACN} = 7.756) in the top phase whereas HCA extraction efficiency was lowered from 99.51 to 96.46 % (K_{HCA} = 0.069).

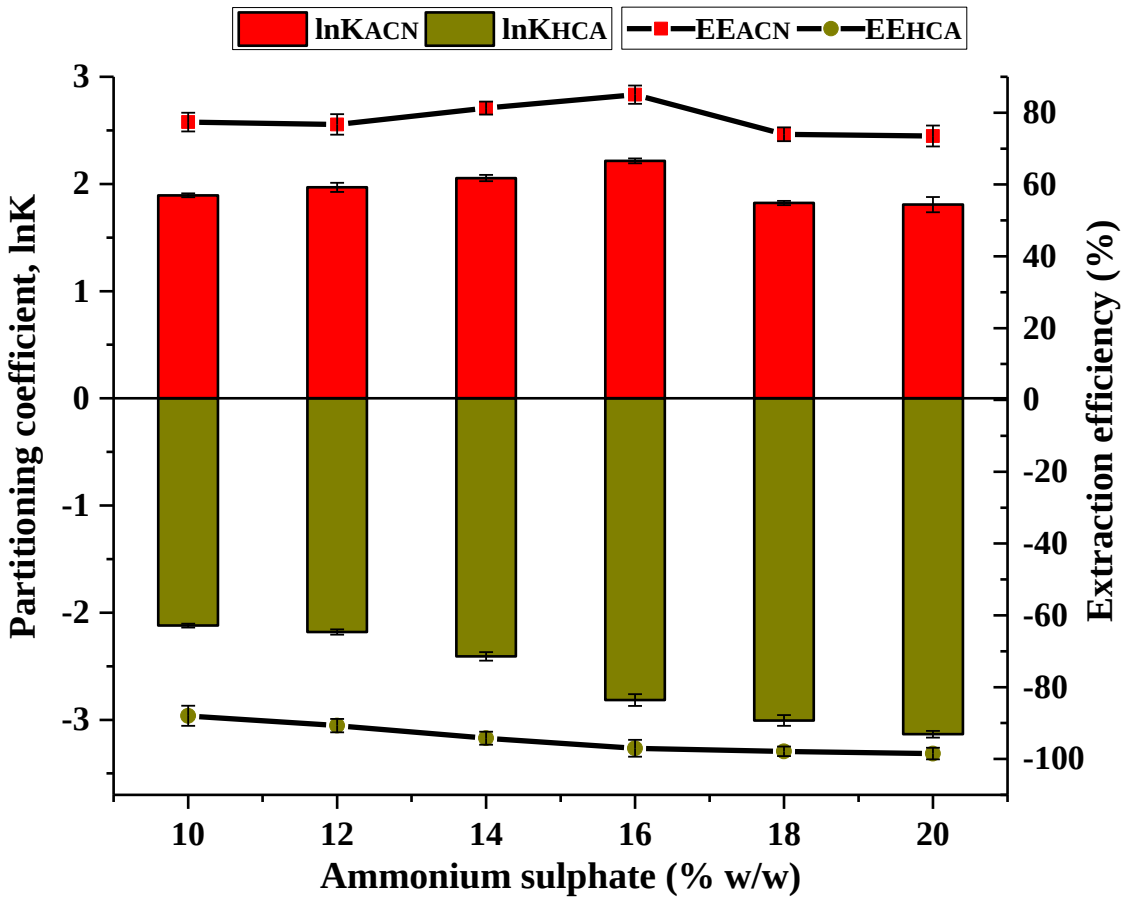


Fig. 4.23 Fractionation of ACN in top phase and HCA in bottom phase from enriched bottom phase at different concentrations of $(NH_4)_2SO_4$ and at constant 1-propanol in terms of partition coefficient and extraction efficiency

Further, 30% w/w 1-propanol is kept constant and salt is varied from 10 to 20 % w/w to study the effect of salt and enhance the separation of these compounds. The composition of enriched bottom phase has been varied from 10 to 20 % w/w of salt at a constant 1-propanol concentration of 30 % w/w. The results are shown in Figure

4.22. It can be observed that with an increase in salt concentration the extraction of ACN towards the top phase increased from 77.40 to 85.04 % from 10 to 16 % w/w salt respectively. Further, the trend in partitioning and extraction of ACN decreased as salt amount increases as seen in Figure 4.22. However, the HCA proved to retain in the salt phase and showed a gradual increase in extraction efficiency as salt concentration increases. The maximum efficiency of HCA (98.50 %) in the bottom phase was observed at a maximum concentration of salt i.e. 20 % w/w. From the obtained results it was observed that the shift in ACN molecules in the top phase is because of the salting-out effect (Tan et al. 2013; Wang et al. 2010a). Also, water and salt interaction in the bottom phase retrieves water molecules from the top phase that decreases the polarity of the top phase which can accommodate more ACNs (Cheng et al. 2016). Further increase in salt makes the top phase unsuitable for ACNs hence the extraction efficiency decreases (Guo et al. 2012b; Li et al. 2017; Sang et al. 2018). Hence, the 30% w/w 1-propanol with 16 % $NH_4(SO_4)$ may effectively partition and separate ACNs to top phase with an efficiency of 85.04 % (K_{ACN} = 9.168) and HCA in bottom phase with an efficiency of 97.12 % (K_{HCA} = 0.058).

4.3.4 Removal and Recycling of phase components of 1-propanol-Ammonium sulphate ATPS

After the second step extraction or enrichment step, the enriched 1-propanol and $(NH_4)_2SO_4$ rich top and bottom phases were collected and stored at 4 °C, to precipitate out small amounts of salts. Further, more salts were allowed to crystallize with the addition of 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 3 volumes of methanol to the respective phases utilising solvent dilution crystallization method. Similar procedures have been followed by other authors which are found in the literature (Li et al. 2017; Tan et al. 2013). As seen for Figure 4.23, the 0.5 and 0.75 volumes of methanol mixed with enriched bottom phase could not precipitate out $(NH_4)_2SO_4$ whereas 1:1 ratio can initiate the precipitation. Further increase in the ratio of methanol increased the salt precipitation and reached 87 % at 1.75 volumes of methanol (Figure 4.23). Two volumes of methanol could be sufficient to precipitate out almost salts (nearly 91.50 %) in both the phases. Further addition of methanol didn't show any increase in salt precipitation amount but the phase became completely turbid. This may be due to the

degradation of bioactive compounds within the system. Methanol has a low boiling point and can be easily recovered from other alcohols as it cannot form an azeotrope with water and lower the energy cost. After filtration, rotary evaporation was employed to recycle 1-propanol and the extract containing GL, IGL and ACN, HCA which were collected separately are stored (Li et al. 2017; Tan et al. 2013). Hence, the phase forming components were recovered from the phases and could be recycled for other experiments.

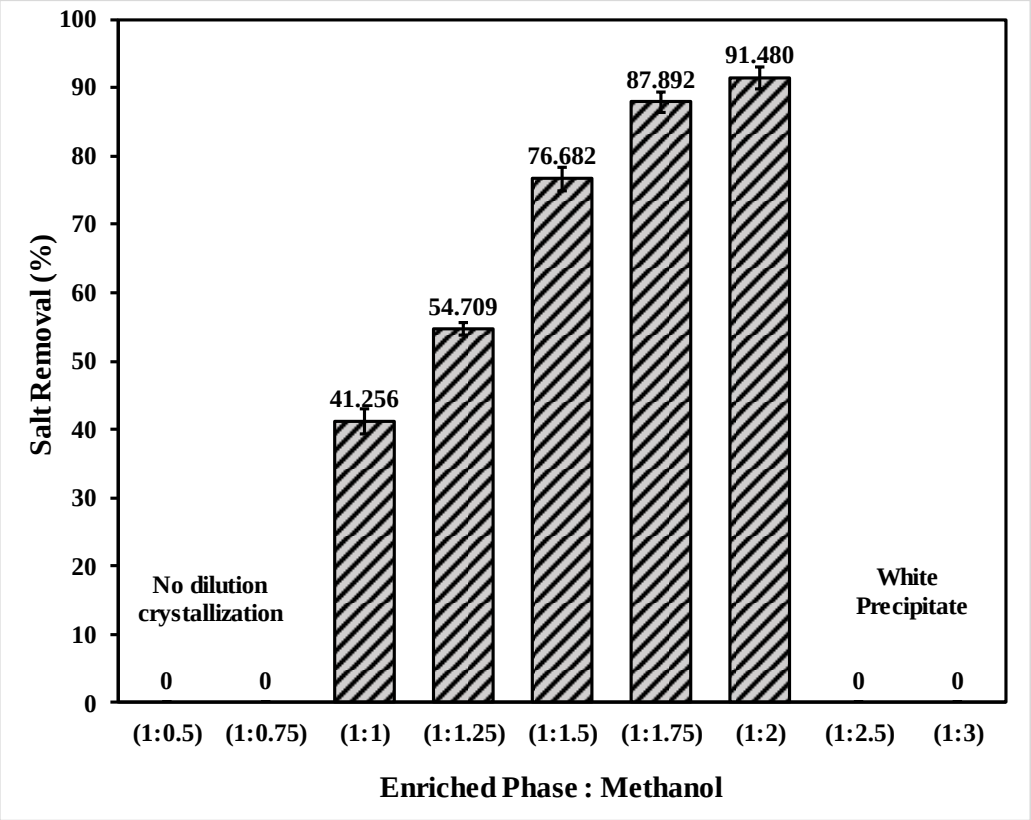


Figure 4.24 Removal of salts using solvent dilution crystallisation method

4.3.5 HPLC analysis of bioactive compounds before and after ATPE

The stability of the bioactive components during the ATPEs are analysed based on the retention time of the bioactive components by comparing the samples of standards like cyanidin 3-O glucoside, garcinia acid as HCA, and GL and IGL with crude extract and the resulting phases of $ATPE^2$ (Figure 4.25a, b, c). All the individual components (ACNs, HCA, GL, and IGL) shows the identical retention time for all the samples before (crude extract) and after $ATPE^2$. The two types of ACNs were observed as reported elsewhere in crude extract and both the phases of ATP (Nayak et al. 2010b) (Figure 4.25a). The pH-dependent, instable, ACNs also found to be intact during the extraction process, since the HCA and ACNs are partitioned together in the bottom phases of ATPE to maintain the acidity lesser than pH 5, in which the ACNs are generally found to be stable (Chandrasekhar and Raghavarao 2015). The isomers of HCA, HCA lactone, and free HCA were observed with the retention times of 14 and 15 min, respectively, at 210 nm for standards (Figure 4.25b) (Jayaprakasha and Sakariah 2002) and phases of $ATPE^2$ (Figure 4.25b ii & iv). The retention times of 15 min for GL at 250 nm and 9 min for IGL at 276 nm, respectively, were noticed (Figure 4.25c) and hardly any variation was found between the crude extract (Figure 4.25c ii) and phases of $ATPE^2$ (Figure 4.25c iii, iv). The HPLC chromatograms of ACNs, HCA, GL, and IGL confirm that the components are stable during the ATPE and not affected by the process conditions and the phase forming components.

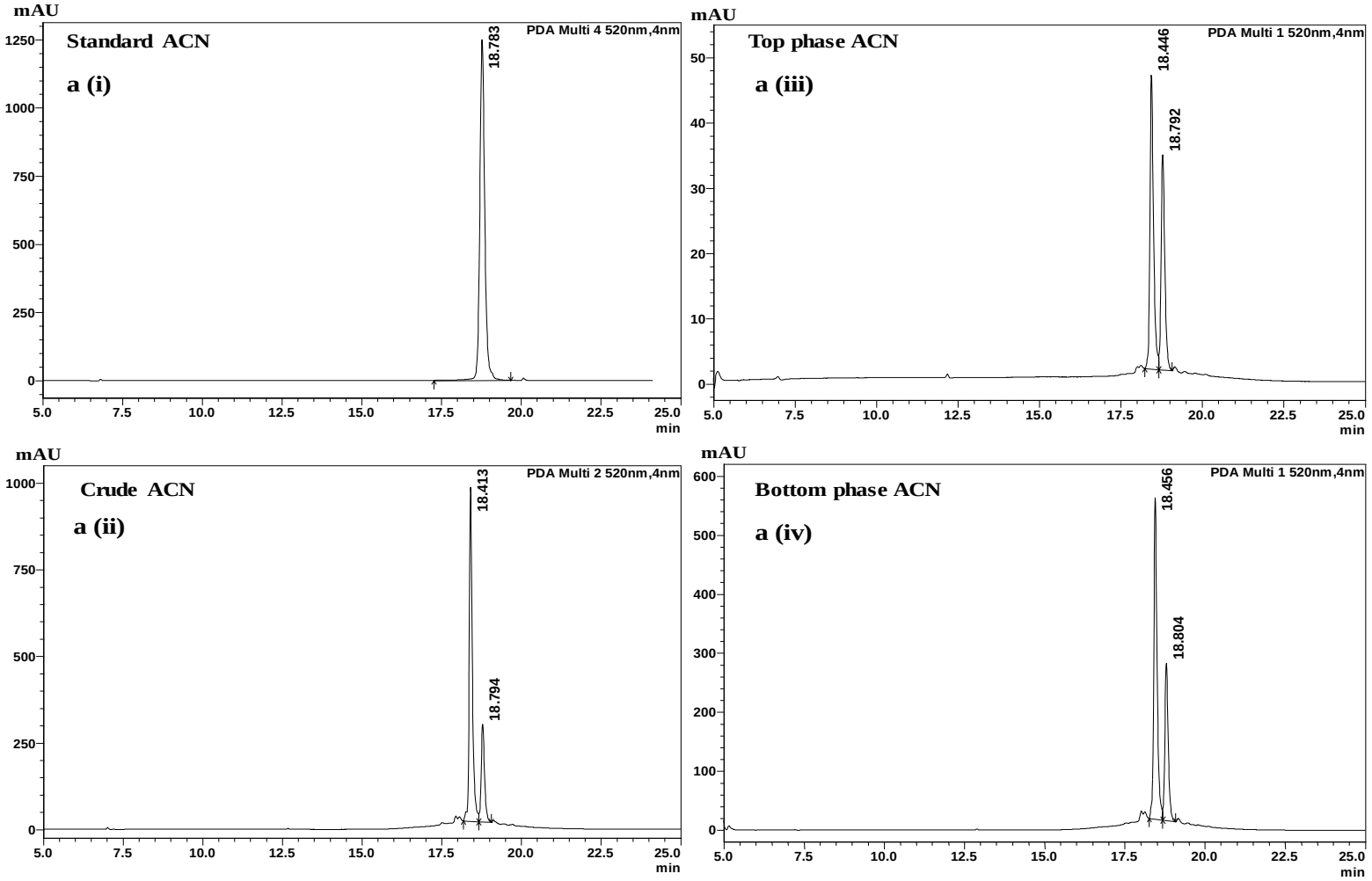


Figure 4.25a HPLC chromatogram of ACNs; a(i) present in standard, a(ii) crude extract and a(iii) top and a(iv) bottom phases of ATPE[2]

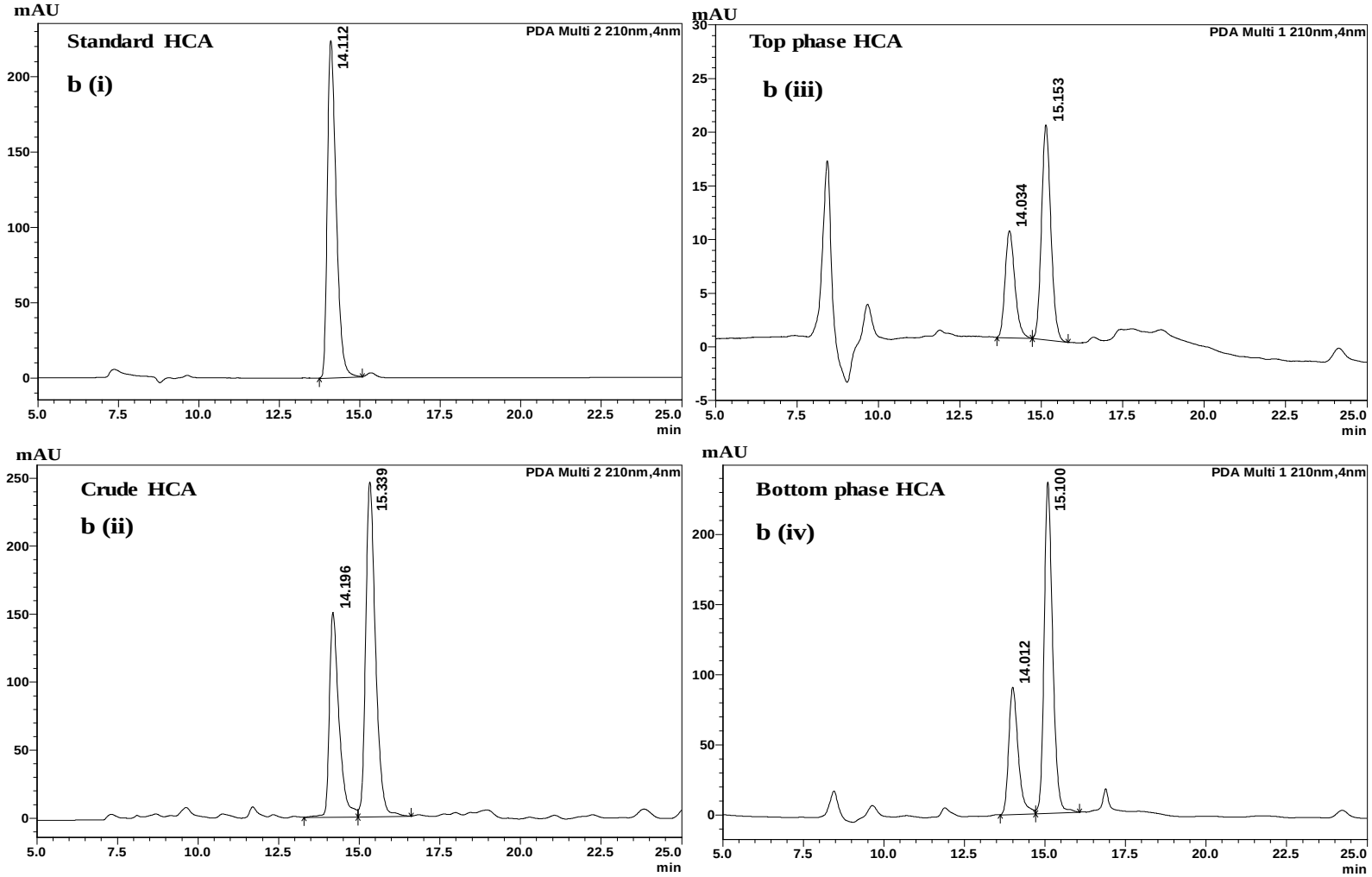


Figure 4.25b HPLC chromatogram of HCA; b (i) present in standard, b (ii) crude extract and b (iii) top and b (iv) bottom phases of ATPE[2].

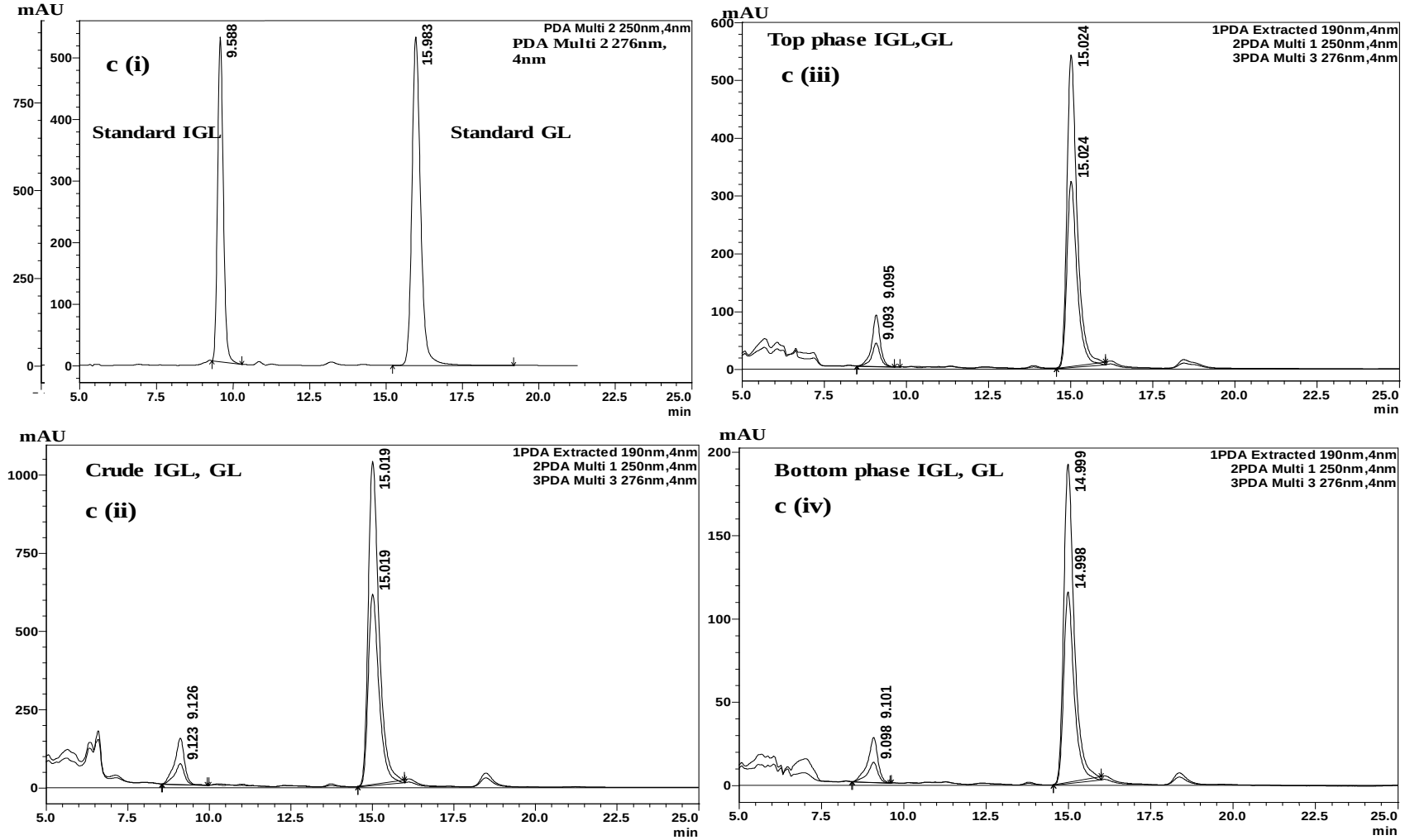


Figure 4.25c HPLC chromatograms of GL and IGL; c (i) present in standards, c (ii) crude extract and c (iii) top and c (iv) bottom phases of ATPE[2]

4.4 STUDIES OF CONTINUOUS ATPE OF BIOACTIVE COMPOUNDS IN ROTATING DISC CONTACTOR (RDC)

The conventional fabricated RDC column, which consists of twenty stator rings and the rotating discs attached with a central shaft and placed at the center of each compartment, was considered as a continuous extractor for the extraction of bioactive compounds from kokum extract in the current study. The performance of the extraction column was evaluated by analysing the hydrodynamic and mass transfer behaviour at different operating conditions. Salt phase (heavier phase) was used as the continuous phase and 1-propanol phase (lighter) is dispersed in the form of droplets in the continuous phase. The dispersed phase holdup is the most important characteristic in hydrodynamic studies. Holdup refers to the amount of dispersed phase available in the column to remove the target compounds from the feed at any instant during the steady-state operation. The holdup and size of the drops were directly proportional to the interfacial area. Correspondingly, the mass transfer efficiency is the function of the drop size as well as the holdup of the dispersed phase. Thus, the holdup measurements of the column under specified conditions were used to evaluate the mass transfer capability of the extractor. The knowledge of holdup is very important as it does not only influence the mass transfer coefficient but also limiting the column operation at flooding condition (Hemmati et al. 2015a). The extent of mixing and separation of the phases governs the rate of partition and recovery of bioactive compounds. The rate of compounds transfer from the continuous phase to the dispersed phase depends on the mass transfer coefficient. The turbulence created by the movement of the rotor discs placed at the center of each compartment enhances the dispersion and thereby increases the mass transfer of the solute from one phase to another phase (Gavhane 2006). For an efficient and reliable extractor design, the information on the dispersed phase holdup, mass transfer coefficient at various conditions, and physical properties of the phase components such as density, viscosity, and interfacial tension are necessary. The physical properties of the phase components used in the present study are given in Table 4.12.

The optimization of the ATPE conditions of four bioactive compounds in batch studies showed a better extraction efficiency and recovery in 65.51 wt% TLL of

1-propanol-ammonium sulphate system with 25% crude load. Hence, the system was chosen to study the continuous extraction of bioactive compounds from the crude extract. The phases were prepared at the equilibrium concentration corresponding to the selected TLL. The crude was mixed with the salt phase while preparing the equilibrated (by phase forming components) phases. In the present study, the effects of dispersed phase velocity, continuous phase velocity, and the rotor speed on the holdup, volumetric mass transfer coefficient, recovery, and separation efficiency were evaluated.

Table 4.12 Physical properties of the 1-propanol – ammonium sulphate system

System composition	**Density (Kg/cm^3)**	**Viscosity (mPa.s)**	**Surface tension (mN/m)**
1-propanol rich phase (1-propanol – 68.63 wt. %, $(NH_4)_2SO_4$ – 3.82 wt. %)	985.09	1.32	43.56
Salt rich phase (1-propanol – 13.17 wt. %, $(NH_4)_2SO_4$ – 10.79 wt. %, crude extract – 25 wt. %)	1107.65	1.96	31.43
Kokum crude extract	1006.82	0.761	29.40

4.4.1 Holdup studies with ATPS in rotating disc contractor

Dispersed phase holdup is defined as the fraction of the column volume occupied by dispersed phase at steady-state operation. Dispersed phase holdup in rotating disc contractor (RDC) is a complex function, which depends on various parameters such as phase flow velocities, rotor speed, drop size, drop velocity, rate of drop coalescence, drop shape, drag and shear forces on drop and geometry of the column. Dispersed phase holdup in RDC is an essential parameter to evaluate the performance, degree of extraction, and mean residence time of the fluid phases. Even though the dispersed phase holdup for aqueous-organic conventional extraction systems was well studied, limited research was done till date to understand the holdup behaviour with ATPS.

Further for the scale-up of the present extraction process, the holdup behaviour with selected ATPS along with the crude extract is highly essential. Hence, in the initial studies, the dispersed phase holdup experiments were carried in 1-propanol/ ammonium sulphate system without crude loading. The experiments were conducted at TLL of 65.514 % w/w to understand the effect of dispersed phase flow velocity (1.0×10^{-3} to 3.24×10^{-3} m/s), continuous phase flow velocity (1.0×10^{-3} to 3.24×10^{-3} m/s), and rotor speeds (0 to 150 rpm) on dispersed phase holdup. All the reported experiments for the RDC studies (dispersed phase holdup experiments) were conducted in triplicates and average values were used for calculations and reported. The error in the analysis are below ± 2%.

4.4.1.1 Effect of phase flow velocities on dispersed phase holdup

To study the effect of phase flow velocities, experiments were conducted at five different dispersed phase flow velocities (1.0×10^{-3}, 1.58×10^{-3}, 2.16×10^{-3}, 2.74×10^{-3} and 3.24×10^{-3} m/s) with different rotor speeds and fixed continuous phase flow velocity and fixed TLL (Figure 4.26). As the dispersed phase velocity increases at constant continuous phase flow velocity. The dispersed phase holdup increases due to the formation of a large number of droplets with smaller diameter due to the increase in the kinetic and buoyancy forces acting on the droplets. The formation of smaller and more number of PEG droplets was seen in PEG-salt systems (Barhate et al. 2004). The smaller droplets had low raising velocity due to the comparatively lesser buoyancy force associated with it and therefore, retains in the column for a longer duration that enhancement of holdup (Kalaivani and Regupathi 2016). The increase in the dispersed phase velocity shows an increase in drop frequency, leading to a decrease in the drop rise velocity and an increase in the drop residence time RDC (Kumar and Hartland 1987). The enhancement of holdup with the dispersed phase flow rate is significant because of the lower interfacial tension and viscosity of the system (Hemmati et al. 2015b). Similar results have been reported in other extraction columns including bubble columns, slurry reactors, airlift loop reactors, spray extraction columns, and perforated rotating disc contactor (Kalaivani and Regupathi 2016; Sarubbo et al. 2003).

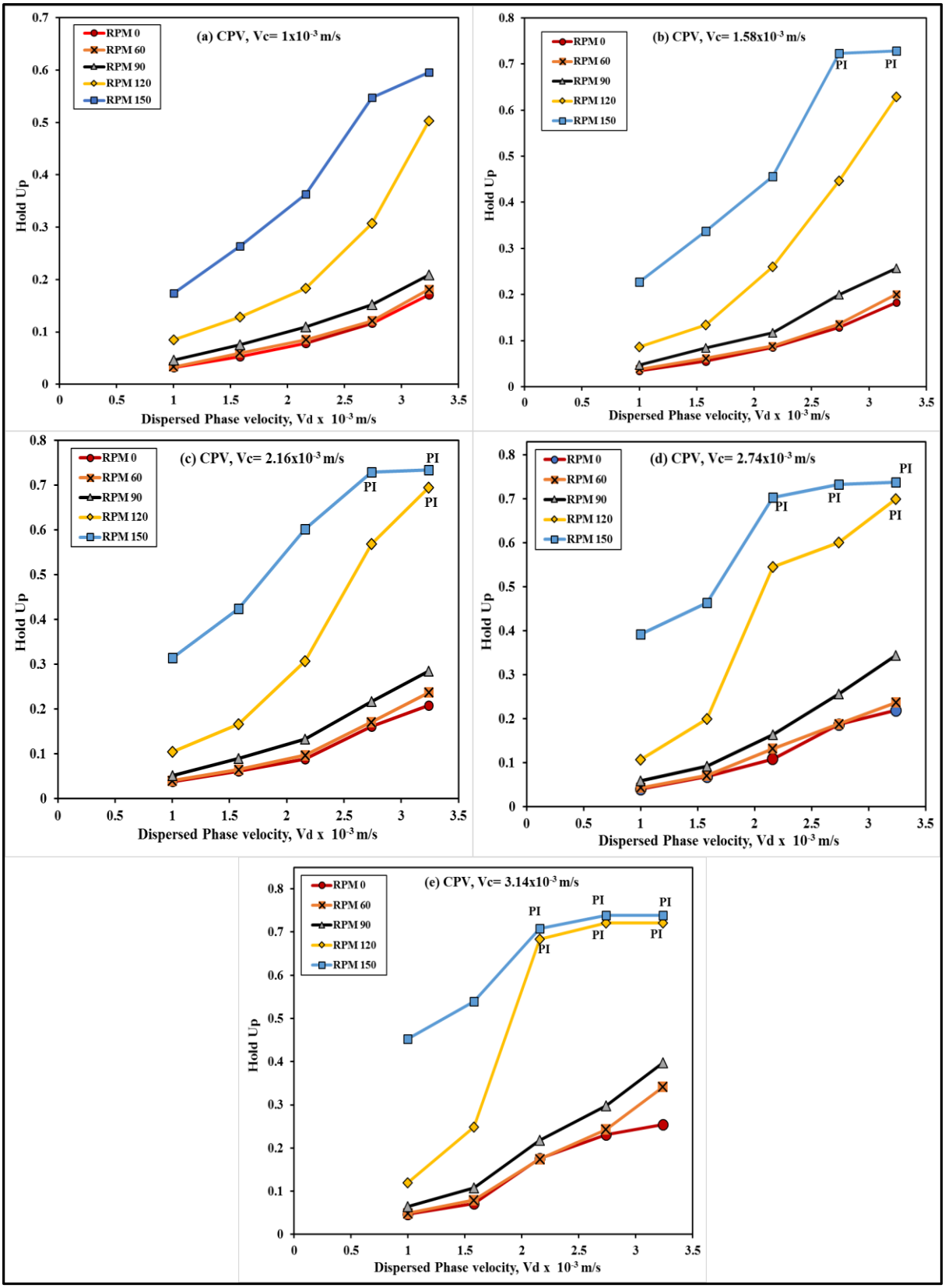


Figure 4.26 Effect of dispersed phase flow velocities on dispersed phase holdup of 1-propanol/ $(NH_4)_2SO_4$ system at various rotor speed. PI is phase inversion.

For example, this trend of the positive effect of dispersed phase velocity on dispersed phase holdup and mass transfer coefficient was noticed in PRDC column (Porto et al. 2000) in the continuous extraction of BSA in PRDC using PEG/phosphate system, continuous extraction of α-toxin in PEG/phosphate system (Cavalcanti et al. 2008) and also in the continuous extraction of α – lactalbumin (Kalaivani and Regupathi et al. 2016). As seen from Figure 4.26, the increase in agitation (rotor speed) gradually increases the hold up in the RDC column because of the increased number of droplets and residence time within the system. The 120 rpm showed a drastic increase in holdup values with an increase in dispersed phase velocities compared to lower rotor speeds. However, it is reported that the drop size distribution has no significant change with the dispersed phase flow rate for a liquid system with a low interfacial tension system consist of butanol–water compared to the toluene–water system (Hemmati et al. 2015b). Hence, the dispersed phase holdup was not increasing much significantly with increasing dispersed phase flow rate compared to that of the effect with increasing rotor speed.

Similarly, the effect of continuous phase velocity on dispersed phase holdup was studied at five different continuous phase flow velocity (1.0×10^{-3}, 1.58×10^{-3}, 2.16×10^{-3}, 2.74×10^{-3} and 3.24×10^{-3} m/s) with different rotors speed at constant dispersed phase flow velocity (Figure 4.27). Irrespective of the dispersed phase flow rate, the holdup was observed to increase slowly with increasing continuous phase flow rate at lower rotor speeds (no much significant change). A similar observation was reported for different liquid-liquid systems in perforated rotating disc contactor (PRDC) (Hemmati et al. 2015a). This means that without agitation or agitation, the increase in continuous phase flow velocity has no impact on drop rice which could not retain them much time within the column. At increased rotor speed above 90 rpm could rise the holdup and this increased along with the increase in continuous phase velocity as seen in Fig 8. This may be due to the higher shear force attributed to the continuous phase velocity and the large difference in the physical properties between the phases. By increasing the continuous phase velocity, the drag force between the dispersed drops and the continuous phase increases, which increases the residence time of the drops in the system due to the restricted movement of the drops.

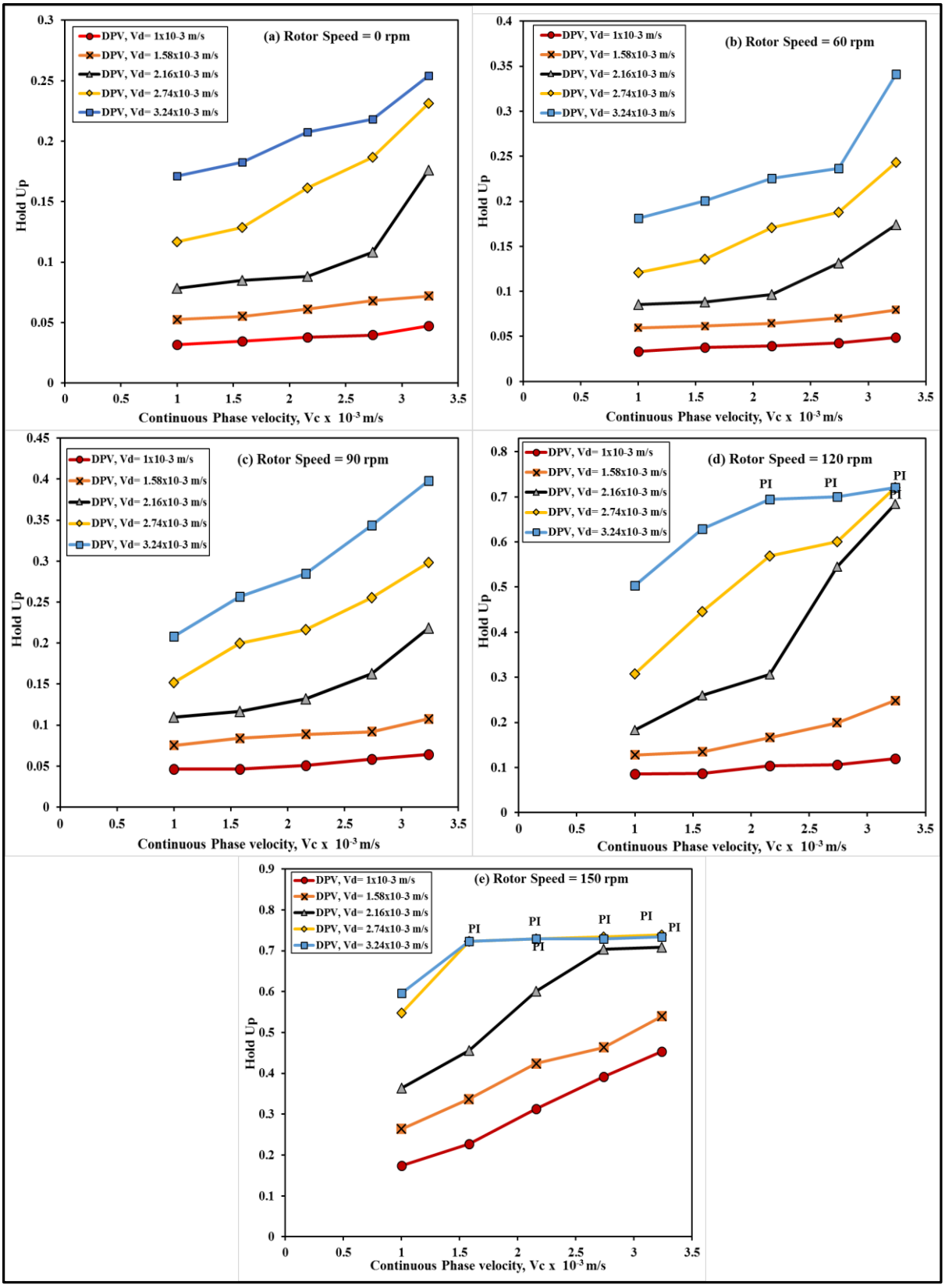


Figure 4.27 Effect of continuous phase flow velocities on dispersed phase holdup on 1-propanol/ $(NH_4)_2SO_4$ system at various dispersed phase velocities.

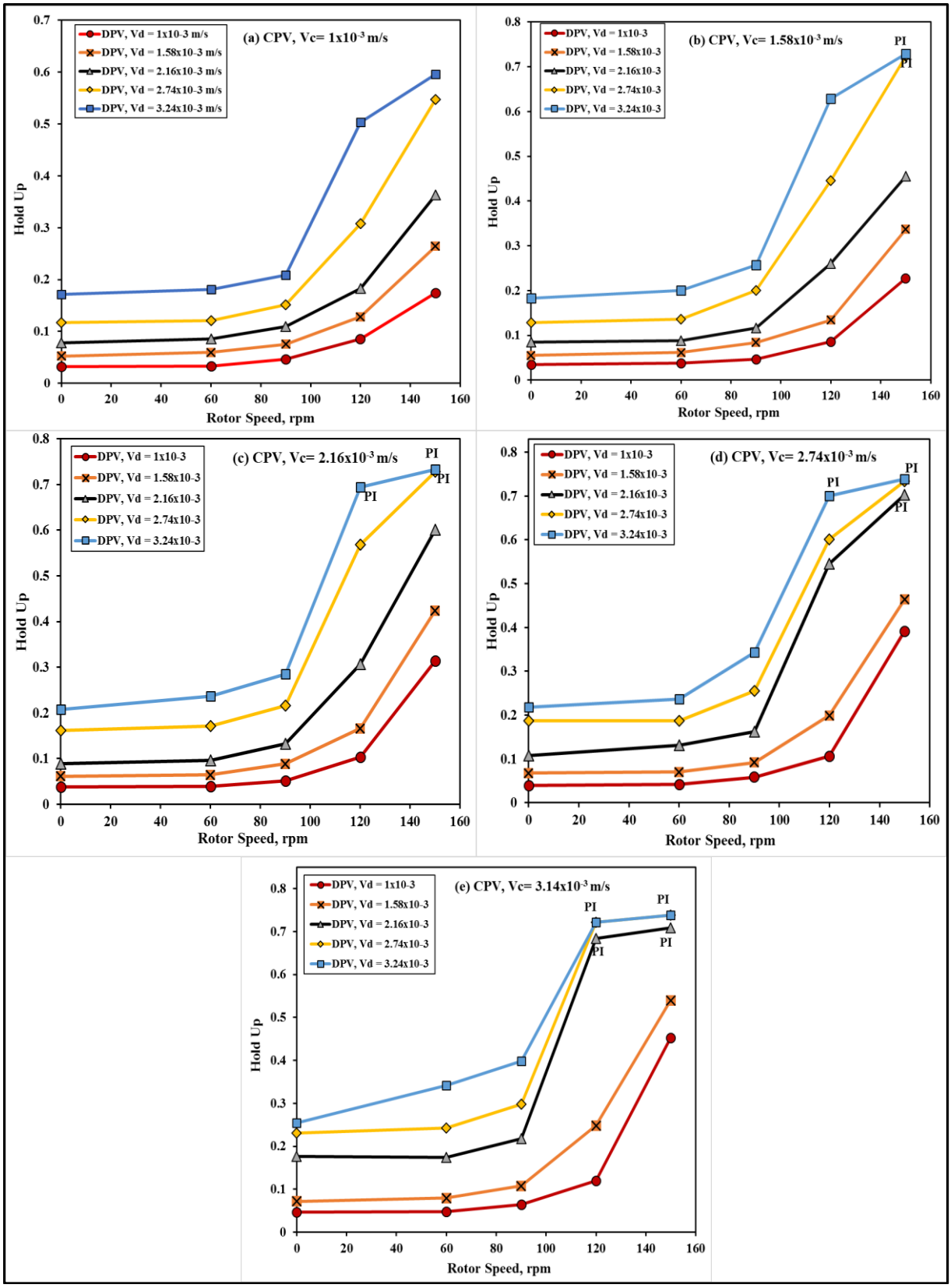


Figure 4.28 Effect of rotor speed on dispersed phase holdup in 1-propanol/ $(NH_4)_2SO_4$ system at varied dispersed phase velocities.

Hence, a greater number of smaller drops were formed and retained for longer duration in the dispersion zone due to the lesser gravitational force on the smaller weight drops at higher continuous phase flow rates. For higher flow rates, the effect of drag force becomes more prominent than the gravitational force on drops and increased holdup. This effect was observed to be more pronounced at higher rotor speeds. The results were similar and in accordance with obtained trends as reported for packed bed and RDC columns (Igarashi et al. 2004a; Kalaichelvi and Murugesan 1998; Kalaivani and Regupathi 2016; Murugesan and Regupathi 2004).

4.4.1.2 Effect of rotor speed on dispersed phase holdup

The performance of the agitated column extractors highly depends on the rotor speed and geometry of the column. The effect of rotor speed on dispersed phase holdup with ATPS was studied by conducting the experiments at different rotor speeds (0, 60, 90, 120, 150 rpm) for tie line 65.14 and each dispersed phase velocities (1.0×10^{-3}, 1.58×10^{-3}, 2.16×10^{-3}, 2.74×10^{-3} and 3.24×10^{-3} m/s) and continuous phase velocities (1.0×10^{-3}, 1.58×10^{-3}, 2.16×10^{-3}, 2.74×10^{-3} and 3.24×10^{-3} m/s). The observed holdup values at different conditions were shown in Figure 4.28.

The dispersed phase hold-up increases by increasing the rotor speed in both the conditions of increasing dispersed phase velocities as well as continuous phase velocities. But, as observed holdup values were found higher with dispersed phase velocities compared to continuous phase velocities. The drops are frequently colliding with each other and coalescing at lower rotor speed, therefore the formed larger droplets. For this reason, lower rotor speeds have smaller hold-up (Moreira et al. 2005). This can be discussed as follows, the dispersed phase holdup was virtually independent of rotor speed at lower phase velocities. At no agitation condition, the droplets of dispersed phase were entrapped beneath the rotor disc and stator ring of RDC and provided the rise in dispersed phase holdup with increasing phase velocities. However, with lower agitation and flow rates of phases the entrapped drops start to release from the beneath of the discs and travelled faster with increased drop terminal velocity towards the interface since the size of the drops is comparatively larger. Although the moving dispersed phase drops were deflected by the rotating discs, the appreciable breakup of drops was not observed due to insufficient dynamic pressure

difference created to overcome the interfacial tension between the phases. Hence, the dispersed phase holdup found to decrease with increasing rotor speed until it reaches the critical rotor speed (60 rpm). Further, irrespective of the dispersed and continuous phase flow rate, the holdup found to increase with agitation higher than the critical rotor speed (Kalaivani and Regupathi 2016).

The RDC setup designed and fabricated for ATP extraction studies in our laboratory is shown in Figure. 4.29.

Figure 4.29 The experimental RDC setup in the laboratory for ATP extraction studies

4.4.2 Separation of bioactive compounds in rotating disc contractor

The separation of bioactive compounds was studied at different dispersed phase velocities at a constant rotor speed of 120 rpm and a continuous phase velocity of 2.74 x 10^{-3} m/s. The efficiency of the column was evaluated at these operating conditions in terms of volumetric mass transfer coefficient, recovery, and separation efficiency of bioactive compounds in the presence of crude extract. The mass transfer of GL and IGL from salt rich continuous phase to 1-propanol rich dispersed phase was considered and evaluated in terms of mass transfer coefficient. But, the other compounds ACN, as well as HCA are retained in the continuous phase whose efficiency was evaluated in terms of recovery and separation efficiency in the salt rich continuous phase. The mass transfer coefficient of GL, IGL, recovery, and separation efficiency was observed to increase with the increase of dispersed phase velocity and rotor speed at constant continuous phase velocity (2.74 x 10^{-3} m/s). The results have been reported in Table 4.13. All the reported experiments for the RDC studies (separation and mass transfer experiments) were conducted in triplicates and average values were used for calculations and reported. The error in the analysis are below ± 2%.

Table 4.13 Separation of bioactive compounds in the RDC system.

V_C x 10^{-3} (m/s)	V_D x 10^{-3} (m/s)	Rotor speed (rpm)	GL			IGL			ACN		HCA	
			K_D^a (min^{-1})	SE^{top}	R^{top}	K_D^a min^{-1}	SE^{top}	R^{top}	SE^{top}	R^{bot}	SE^{top}	R^{bot}
2.74	1.00	120	0.137	90.04	57.30	0.092	79.98	50.89	13.96	95.54	7.17	97.15
2.74	1.58	120	0.231	90.94	66.69	0.159	80.43	58.98	18.59	89.51	8.19	94.99
2.74	2.16	120	0.344	92.41	92.41	0.304	89.23	89.23	23.69	77.20	9.90	90.86
2.74	2.74	120	0.436	91.79	91.79	0.368	86.18	86.18	21.24	72.02	13.95	83.96
2.74	3.24	120	Phase Inversion (PI)									

Where, Vc and Vd are velocities of continuous phase and dispersed phase and K_D^a, SE^{top}, R^{top}, R^{bot} are mass transfer coefficient of GL, IGL in dispersed phase, separation efficiency in dispersed phase for GL and IGL, recovery of GL and IGL in dispersed phase and recovery of ACNs, HCA in continuous phase respectively.

The separation efficiency, recovery, and mass transfer coefficient of GL and IGL gradually increases with increasing dispersed phase velocity. The increase in holdup showed the impact on mass transfer of GL, IGL compounds from the salt phase to the dispersed phase. The results hold a good agreement with the findings reported for different columns in the literature. The dispersed phase velocity shows the positive effect on dispersed phase holdup and volumetric mass transfer coefficient, as the interfacial area for mass transport is increasing. The similar observations were noticed in PRDC column (Porto et al. 2000) in the continuous extraction of BSA in PRDC using PEG/phosphate system, continuous extraction of α-toxin in PEG/phosphate system (Cavalcanti et al. 2008) and also in the continuous extraction of α – lactalbumin (Kalaivani and Regupathi 2016). Further, the other columns such as spray column in the extraction of horseradish peroxidase enzyme, York – scheibel column in the extraction for BSA, and amyloglucosidase (Jafarabad et al. 1992a) showed the similar results.

The increase in dispersed phase velocity has a positive effect on the separation efficiency and recovery of GL and IGL. The higher rotor speed with the increase in dispersed phase velocity creates high turbulence which aids the formation of smaller drops whose up-rising velocity decreases and retained for a longer duration in the column. The high interfacial area offered by the smaller drops and their improved residence time enhances the mass transfer and consequently enhances the activity recovery, purification factor, and separation efficiency. Porto et al. (2004) also reported that the activity recovery and purification factor of oxidoreductase increased to 23.6% and 34.3 respectively with the increase in dispersed phase velocity, which was following the present study. The maximum mass transfer coefficient (0.436 and 0.368 min^{-1}) and recovery (92.41 and 89.23%) was achieved at V_D 2.74 x 10^{-3} m/s, V_C 2.16 x 10^{-3} m/s, and at a rotor speed of 120 rpm. The higher separation efficiency and recovery observed in the present study were mainly due to the structural stability offered by the 1-propanol rich dispersed phase (Peters 1987) and the separation of ACN and HCA from the GL and IGL during the extraction. But maximum separation efficiencies of ACN and HCA in the bottom phase were observed at lower dispersed phase velocities. However, at the beneficial operating conditions for GL & IGL, only

the limited amount of ACN and HCA extracted to the dispersed phase with the extraction efficiency of about 21.24 % and 9.90 % for ACN and HCA, respectively, which implies that most of these remained in the bottom phase (Table 4.13). The GL and IGL compounds can be recovered with higher purity in the RDC.

CHAPTER 5

5. SUMMARY AND CONCLUSIONS

5.1 SUMMARY

- The valuable bioactive compounds present in the fruit of *garcinia indica* (GI) were explored for the simultaneous extraction and found that the ACN, HCA, GL, and IGL are present in a significant quantity.

- The solvent aqueous mixture containing 1-propanol (55 %) and ethanol (80%) can simultaneously extract all the bioactive compounds namely ACN, HCA, GL, and IGL from GI. The solvent to solid ratio studies revealed that the ratio of 1:10 able to provide the maximum yield of bioactive components in the crude extract.

- PEG-salt systems failed to partition the GL and IGL, however, the ACN and HCA were partitioned to the top and bottom phases of the ATPS, respectively.

- Ethanol-ammonium sulphate system showed better extraction efficiency of ACN (86.33 %), HCA (75.17%) in bottom phase, and GL (96.39 %), IGL (94.26 %) in top phase between the TLL values between 38.60 to 43.28 %.

- A maximum extraction efficiency of 94.20 % with K_{GL} = 17.797 for GL and 89.44 % with K_{IGL} = 9.047 for IGL into 1-propanol rich top phase and 90.51 % ACN with K_{ACN} = 0.444 and 96.31 % with (K_{HCA} = 0.162) in - magnesium sulphate rich bottom phase, respectively was observed in 1-propanol - magnesium sulphate ATPS having the tie lines the length from 20.378 to 43.796 %.

- The 1-propanol- ammonium sulphate showed higher extraction efficiency compared to other systems. The 20 % (w/w) 1-propanol resulted in a maximum extraction efficiency of 94.17 % (K_{ACN} = 0.205) of ACN and 98.01 % (K_{HCA} = 0.0675) of HCA in bottom phases. The maximum extraction efficiency achieved was 98.61 % (K_{GL} = 68.356) and 94.46 % (K_{IGL} = 23.792) for GL and IGL respectively at 30 % of 1-propanol. TLLs between 42.93 and 58.51 (%, w/w) found

suitable to simultaneously separate GL and IGL in the top phase and ACN and HCA in the bottom phase.

- To narrow down the specific phase composition to improve the efficiency this system is used for further optimisation using RSM studies.

- The desirability objective function based multi-response optimization revealed that the ATPS formed with 15.202 % w/w 1-propanol, 10.242 % w/w ammonium sulphate with the addition of 25g crude load is the suitable process condition for the maximum yield of 97.39 % GL (K=370.770) and 92.38 % IGL (K=120.581) in the top phase and ACNs 99.19% (K=0.080), HCA 99.83% (K=0.016) in the bottom phase.

- The purity of the bioactive compounds more than 99% was achieved in their respective phases of the second stage ATPE by implementing the enrichment step, however the partitioning limitation of the second step ATPE for the improvement of purity was realized from the reduction of an extraction efficiency of ACNs and HCA in the bottom phase when compared to the first step ATPE. The higher purity of the components and extraction efficiency of the ATPE is the specific credit of the process.

- The effect of dispersed phase velocity and rotor speed is found to be significant on the dispersed phase holdup than the continuous phase velocity in the RDC. The phase inversion was reached at lower rotor speed itself when compared to other liquid-liquid extraction systems due to their lower interfacial tension. The smaller window of operating conditions is identified for the alcohol-salt ATPS and hence easy to operate with lower energy consumption.

- For GL and IGL compounds, maximum volumetric mass transfer coefficient (0.344 and 0.304 min^{-1}) and separation efficiency and recovery (92.41 and 89.23%) were achieved at V_D 2.74 x 10^{-3} m/s, V_C 2.16 x 10^{-3} m/s and at a rotor speed of 120 rpm. At the same time recovery of ACN and HCA in the continuous outlet was decreased with dispersed phase flow rate, which was observed to be 77.20 and

90.86 at favourable conditions of GL and IGL. The GL and IGL compounds can be recovered with higher purity in the dispersed phase.

Table 5.1 The process conditions obtained at various stages of the ATPE process

System	Process Conditions/ variables (% w/w)	Extraction efficiency, EE (%)			
		ACN	HCA	GL	IGL
ATPE[1]					
PEG-Sodium sulphate	12.5 – 9.0, 1% crude load	97.53	74.96	---	---
PEG-trisodium citrate	12.5 – 10.0, 10% crude load	97.67	81.16	---	---
Ethanol-Ammonium sulphate	**25.0 - 19.0,** 10% crude load	86.33	75.17	96.39	94.26
1-Propanol-Magnesium sulphate	25.0 – 12.0, 10% crude load	90.69	96.37	94.20	89.44
1-Propanol-Ammonium sulphate	20.0 – 10.0, 10% crude load	94.17	98.01	95.16	92.03
Response surface methodology (RSM)					
1-Propanol-Ammonium sulphate	15.202 - 10.242, 25g crude load	99.19	99.83	97.39	92.38
Second Stage ATPE[2]					
1-Propanol-Magnesium sulphate	25.0 – 12.0, 10% crude load	**Recovery (%)**			
		77.55	89.72	95.38	91.66
		Purity (%)			
		99.08	99.08	60.38	60.38
1-Propanol-Ammonium sulphate	15.202 - 10.242, 25g crude load	**Recovery (%)**			
		83.27	96.26	99.17	97.96
		Purity (%)			
		99.99	99.99	99.45	99.45
Continuous ATPE in RDC					
1-Propanol-Ammonium sulphate	V_D =2.74 x 10^{-3} m/s, V_C =2.16 x 10^{-3} m/s Rotor speed = 120 rpm **Hold up = 0.6**	**Favourable Holdup and K_D[a]**			
		----	----	0.344	0.304
		Recovery %			
		77.20	90.86	92.41	89.23

5.2 CONCLUSIONS

The simultaneous ATPE based partitioning and enrichment of four different biomolecules from the natural crude extract is the first of its kind. The present investigation proclaims the utilization of underexploited fruit species kokum (*Garcinia indica*) for the extraction of four valuable bioactive compounds namely ACN, HCA, GL, and IGL present in it. The ATPE characteristics of ACN, HCA, GL, and IGL in the identified ATPS at various stages of the ATPE was presented in Table 5.1. The crude extract of four bioactive compounds with maximum concentration was achieved with aqueous propanol and ethanol as the solvents. The ATPS consisting of 1-propanol-ammonium sulphate proved to be the best ATPS for the differential partitioning of GL and IGL into 1-propanol rich top phase and ACNs and HCA into salt-rich bottom phase. The second stage ATPE could enrich these compounds to either of the phases with a yield of more than 95% with higher purity. The successful implementation of the ATPE in the continuous extractor (RDC) discloses the potentiality and suitability of the process for the continuous extraction and subsequent possibility of scale-up on the industrial scale. Thus, the present study contributes to identify an alternate bulk extraction process for multiple compounds from a natural source with relatively higher purity and extraction efficiency (Table 5.1). This may be further exploited for the recovery of high value multiple natural compounds in their native form from the single sources as well as complex biological mixtures.

5.3 LIMITATIONS OF PRESENT WORK

- Removal of phase components like 1-propanol and salts completely are important steps to be considered after ATPE.
- Removal of traces of proteins and carbohydrates present in bottom phases is to be considered.
- Complete fractionation of ACNs and HCA compounds need to be experimented for their specific applications.

5.4 SCOPE FOR FUTURE WORK

Commercial implementation of the process requires further research in the following area.

- Detailed experiments to be carried out to separate the partitioned bioactive compounds from both the phases by recycling the spent phase components.
- The detailed characterisation about compounds using LCMS, FTIR, NMR and biological characteristics like antioxidant properties of these compounds can be analysed and the lyophilised compounds may be studied for their effectiveness in various fields of application.
- More experiments need to be conducted at varied temperature and different TLLs for simultenaous separation in RDC and corelations can be used or developed for specific alcohol-salt system .
- Scale-up studies along with cost analysis in a pilot-scale unit to make the process viable for industries.

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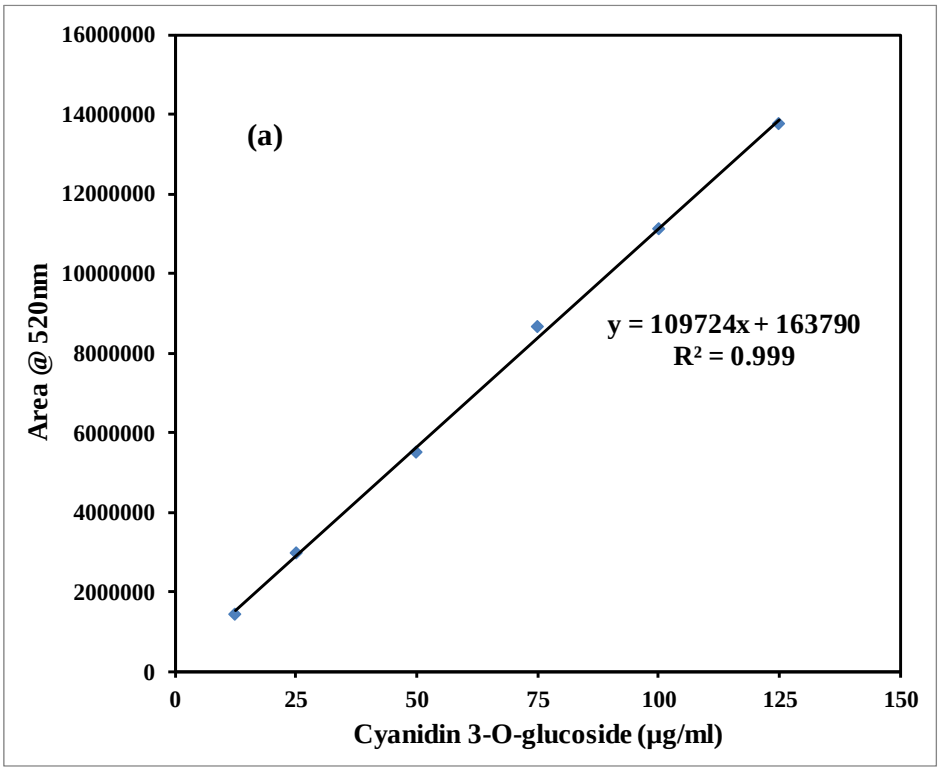
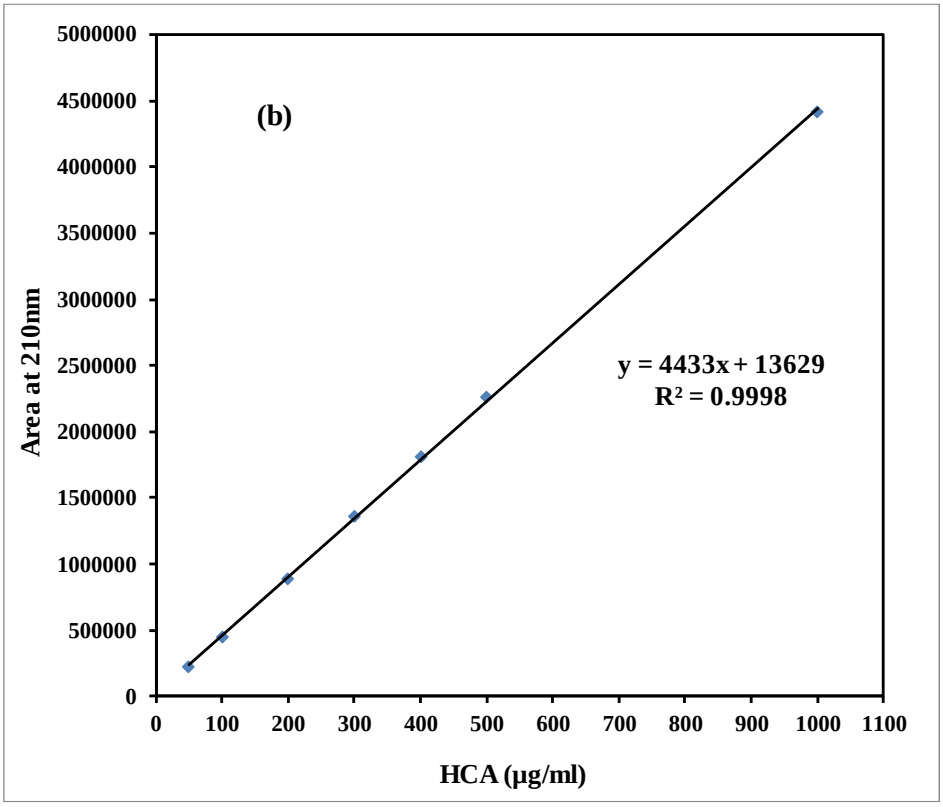
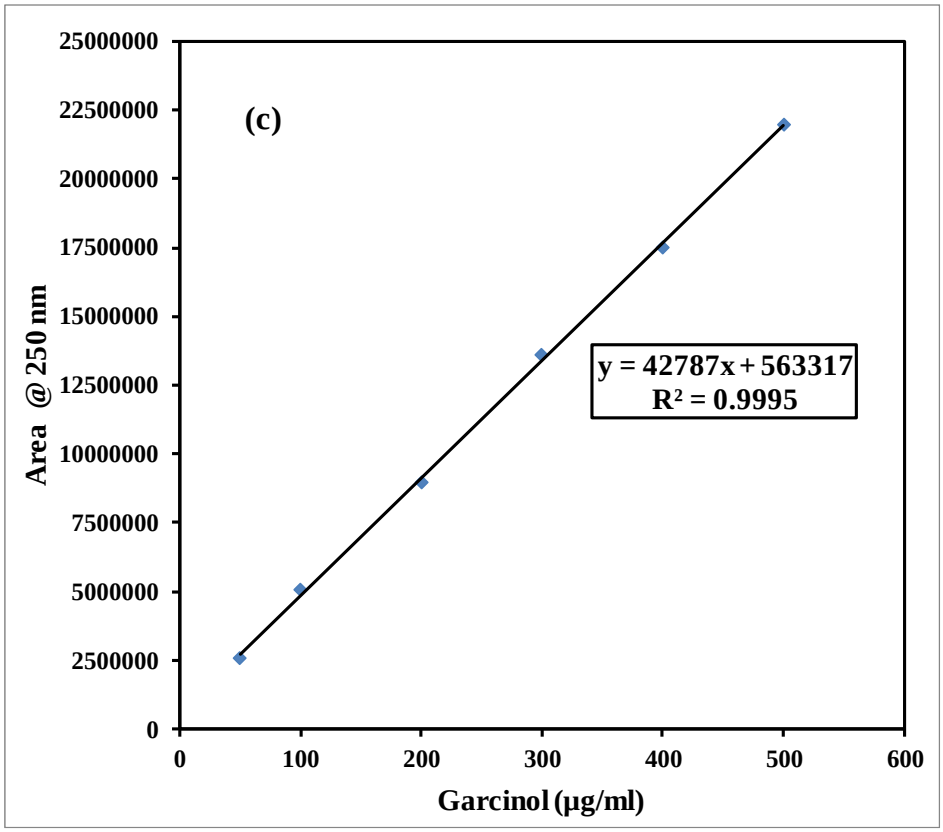
APPENDICES

APPENDIX I: Standard calibration plots of bioactive compounds







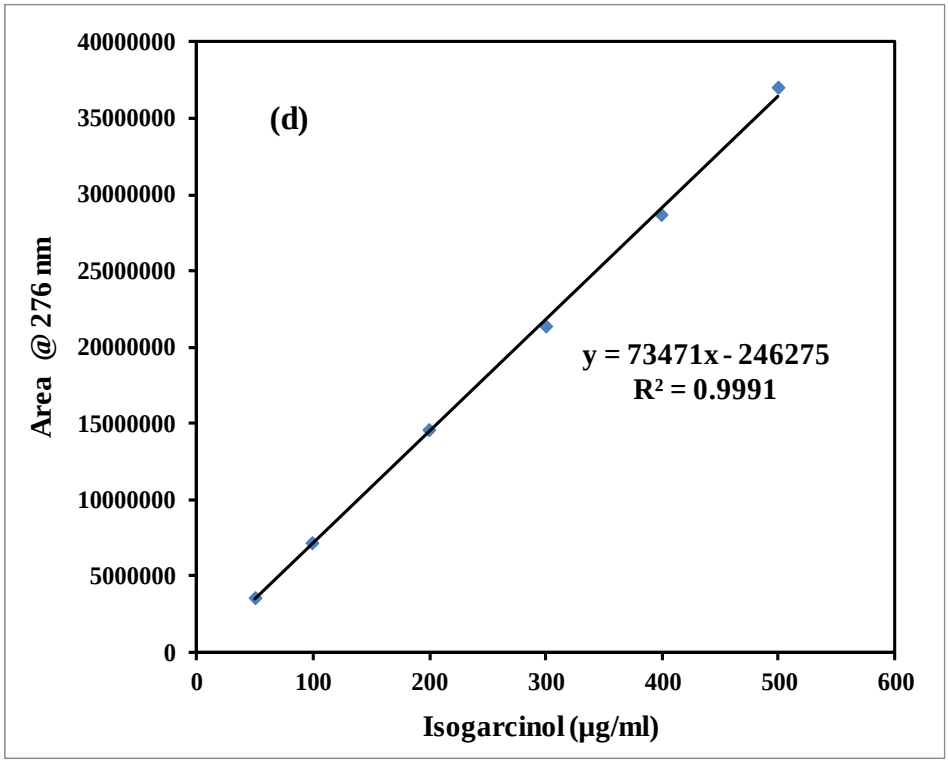


Figure A3.1 Calibration curve of (a) cyanidin 3-O-glucoside for anthocyanin estimation, (b) Garcinia acid (HCA) for HCA estimation and (c-d) for Garcinol and Isogarcinol estimation using HPLC methods.

APPENDIX II: Standard calibration plots of protein and carbohydrates

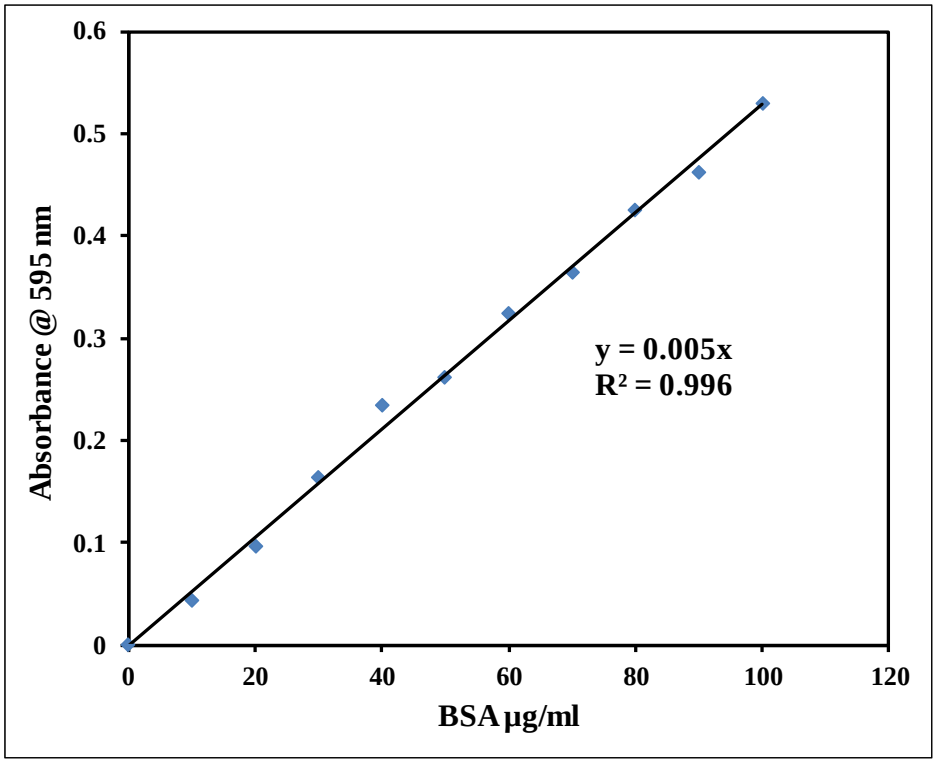


Figure A3.2 Calibration curve for protein estimation using Bradford method

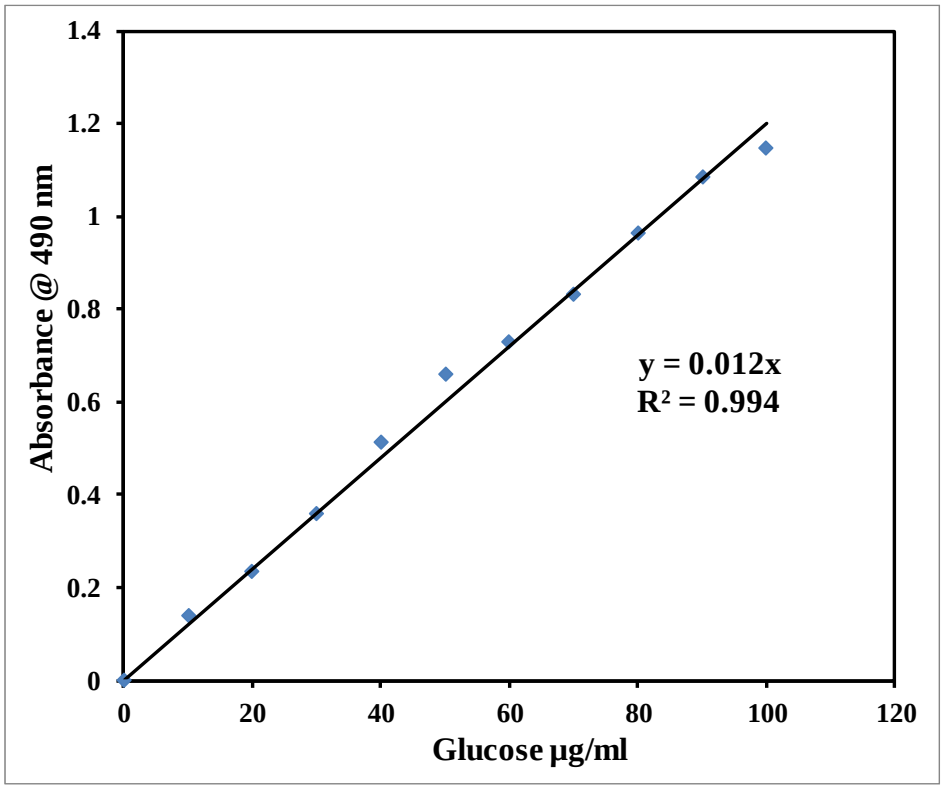


Figure A3.3 Calibration curve for carbohydrate estimation using Dubois method

APPENDIX III: Experimental binodal data

Table A3.4a Binodal data for Ethanol with Ammonium Sulphate salt system at 30 °C (W_1 = Ethanol (wt%) and W_2 = Ammonium Sulphate (wt%))

W_1	W_2	W_1	W_2	W_1	W_2	W_1	W_2
2.523	38.318	15.575	23.348	22.816	18.042	30.791	12.337
3.293	36.975	16.015	23.013	23.441	17.527	31.843	11.768
4.007	35.844	16.433	22.691	23.724	17.228	33.478	10.904
4.771	34.703	16.742	22.433	23.885	17.133	35.701	9.737
5.642	33.603	17.198	22.106	24.074	16.999	38.231	8.380
6.696	32.295	17.585	21.814	24.400	16.715	40.025	7.533
7.676	31.070	17.983	21.520	24.720	16.524	42.256	6.586
8.321	30.240	18.313	21.260	24.935	16.310	44.879	5.575
9.148	29.341	18.757	20.954	25.398	15.912	48.849	4.187
9.990	28.447	19.141	20.678	25.706	15.709	54.740	2.290
10.702	27.653	19.518	20.403	26.199	15.310	57.968	1.355
11.835	26.578	19.953	20.098	26.657	14.968		
12.376	26.034	20.397	19.793	27.031	14.745		
12.717	25.705	20.712	19.555	27.300	14.557		
13.040	25.438	21.094	19.289	27.557	14.376		
13.756	24.838	21.465	19.032	27.794	14.222		
14.094	24.543	21.846	18.771	28.064	14.019		
14.440	24.252	22.221	18.514	28.341	13.850		
14.828	23.941	22.581	18.248	28.520	13.724		
15.244	23.616	22.682	18.147	28.858	13.530		

Table A3.4b Binodal data for 1-Propanol with Magnesium Sulphate salt system at 30 ºC. (W_1 = 1-Propanol (wt%) and W_2 = Magnesium Sulphate (wt%))

W_1	W_2	W_1	W_2	W_1	W_2	W_1	W_2
5.700	47.299	15.395	15.685	24.707	9.381	38.345	4.089
5.745	44.086	15.708	15.352	24.842	9.352	39.297	3.786
5.928	42.380	16.365	14.759	25.186	9.104	40.012	3.489
6.123	39.992	17.114	14.095	25.847	8.788	41.969	2.951
6.424	37.135	17.133	13.991	26.181	8.774	43.939	2.756
6.513	35.593	17.344	13.780	26.789	8.498	45.637	2.474
6.745	34.667	18.235	13.135	27.529	8.233	47.258	2.144
7.012	32.946	18.740	12.856	27.697	8.099	51.372	1.402
7.321	31.335	19.147	12.623	28.572	7.763	52.890	1.223
7.579	30.112	19.534	12.348	29.029	7.595	53.563	1.113
7.810	29.057	19.955	11.904	29.555	7.350	54.751	1.071
8.067	27.567	20.631	11.576	30.279	7.031	55.564	0.735
8.313	26.789	20.883	11.522	30.548	6.827	56.765	0.581
8.423	26.123	21.180	11.227	31.522	6.318	57.372	0.388
9.224	23.576	21.634	11.004	31.786	6.220	59.012	0.232
10.062	21.988	22.049	10.830	32.177	6.127	60.433	0.193
11.184	20.071	22.169	10.743	33.614	5.623	61.871	0.161
12.016	18.901	22.179	10.480	33.936	5.499	62.871	0.112
12.691	18.277	22.883	10.266	34.890	5.257		
14.257	16.712	23.489	10.067	35.684	4.906		
14.557	16.412	23.496	10.006	36.180	4.854		
14.895	16.185	24.127	9.625	37.316	4.371		

Table A3.4c Binodal data for 1-propanol with Ammonium Sulphate salt system at 30 ºC. (W_1 = 1-Propanol (wt%) and W_2 = Ammonium Sulphate (wt%))

W_1	W_2	W_1	W_2	W_1	W_2	W_1	W_2
2.835	38.310	10.668	11.619	17.400	7.982	25.856	5.865
3.535	33.310	10.855	11.438	17.600	7.932	26.379	5.719
4.175	29.738	10.994	11.238	18.100	7.818	26.914	5.701
4.535	26.310	11.041	10.995	18.338	7.770	28.192	5.580
5.175	23.738	11.205	10.811	18.751	7.676	29.501	5.406
5.535	22.310	11.411	10.581	19.131	7.593	31.266	5.232
6.175	19.738	11.725	10.324	19.512	7.445	33.153	4.825
7.083	17.990	11.742	10.069	19.848	7.317	35.420	4.639
7.634	16.562	12.011	9.865	20.206	7.220	37.934	4.216
7.733	15.664	12.248	9.697	20.528	7.118	40.425	3.752
8.187	15.021	12.448	9.597	20.977	7.019	43.300	3.405
8.445	14.479	13.214	9.308	21.575	6.888	46.399	3.032
9.122	13.886	13.736	9.113	22.022	6.787	49.435	2.617
9.483	13.558	13.996	8.974	22.343	6.652	53.484	2.212
9.683	13.264	14.464	8.701	22.683	6.622	55.915	1.694
9.872	12.968	14.902	8.592	23.155	6.536	57.985	1.343
10.013	12.685	15.673	8.371	23.592	6.413		
10.117	12.337	15.958	8.280	24.113	6.306		
10.209	12.101	16.385	8.174	24.869	6.140		
10.371	11.880	17.017	8.021	25.424	6.037		

APPENDIX IV: Analysis of variance (ANOVA) results

Table A4.1 Analysis of Variance for the response surface quadratic models for partitioning coefficient as responses versus A- 1-propanol % (w/w), B- $(NH_4)_2SO_4$ % (w/w), C- Crude load (g).

Source	DF	KACN				KHCA				KGL				KIGL			
		SS	MS	F-Value	P-Value	SS	MS	F-Value	P-Value	SS	MS	F-Value	P-Value	SS	MS	F-Value	P-Value
Model	9	0.649447	0.072161	712.47	0.000	0.005279	0.000587	763.45	0.000	2489.69	276.632	83.73	0.000	1313.68	145.964	233.00	0.000
Linear	3	0.596739	0.198913	1963.94	0.000	0.004827	0.001609	2094.13	0.000	1360.45	453.484	137.25	0.000	704.85	234.950	375.04	0.000
A- 1-propanol % (w/w)	1	0.588350	0.588350	5808.98	0.000	0.002876	0.002876	3743.65	0.000	871.00	871.001	263.62	0.000	516.20	516.196	823.98	0.000
B- (NH4)2SO4 % (w/w)	1	0.002722	0.002722	26.87	0.000	0.001882	0.001882	2450.22	0.000	462.95	462.954	140.12	0.000	148.76	148.757	237.45	0.000
C- Crude load (g)	1	0.005667	0.005667	55.96	0.000	0.000068	0.000068	88.52	0.000	26.50	26.496	8.02	0.018	39.90	39.898	63.69	0.000
Square	3	0.044275	0.014758	145.71	0.000	0.000285	0.000095	123.87	0.000	727.95	242.650	73.44	0.000	365.76	121.919	194.61	0.000
A * A	1	0.040333	0.040333	398.22	0.000	0.000009	0.000009	11.33	0.007	292.86	292.862	88.64	0.000	257.07	257.065	410.34	0.000
B * B	1	0.003285	0.003285	32.43	0.000	0.000236	0.000236	307.11	0.000	568.47	568.472	172.06	0.000	180.35	180.346	287.88	0.000
C *C	1	0.010871	0.010871	107.33	0.000	0.000025	0.000025	32.93	0.000	19.66	19.655	5.95	0.035	10.72	10.717	17.11	0.002
2-Way Interaction	3	0.008432	0.002811	27.75	0.000	0.000167	0.000056	72.34	0.000	401.29	133.762	40.48	0.000	243.07	81.024	129.33	0.000
A * B	1	0.000061	0.000061	0.60	0.455	0.000107	0.000107	138.95	0.000	83.67	83.671	25.32	0.001	21.67	21.674	34.60	0.000
A * C	1	0.008232	0.008232	81.27	0.000	0.000032	0.000032	41.41	0.000	285.69	285.694	86.47	0.000	194.17	194.170	309.94	0.000
B * C	1	0.000140	0.000140	1.38	0.268	0.000028	0.000028	36.66	0.000	31.92	31.919	9.66	0.011	27.23	27.230	43.47	0.000
Error	10	0.001013	0.000101			0.000008	0.000001			33.04	3.304			6.26	0.626		

Lack-of-Fit	**5**	**0.000612**	**0.000122**	**1.52**	**0.328**	**0.000004**	**0.000001**	**1.12**	**0.450**	**20.97**	**4.193**	**1.74**	**0.280**	**3.07**	**0.614**	**0.96**	**0.516**
Pure Error	**5**	**0.000401**	**0.000080**			**0.000004**	**0.000001**			**12.07**	**2.415**			**3.19**	**0.639**		
Total	**19**	**0.650460**				**0.005287**				**2522.73**				**1319.94**			
S		**0.0100639**				**0.0008765**				**1.81769**				**0.791498**			
R-sq		**99.84%**				**99.85%**				**98.69%**				**99.53%**			
R-sq(adj)		**99.70%**				**99.72%**				**97.51%**				**99.10%**			
R-sq(pred)		**99.13 %**				**99.29%**				**92.39%**				**97.72%**			

A, B and C represent 1-Propanol, $(NH_4)_2SO_4$ % (w/w) and Crude load (g) respectively; SS, DF, and MS represent sum of squares, degree of freedom, mean square, coefficient of variation, respectively.

Table. A4.2

Analysis of Variance for the response surface quadratic models for extraction efficiency as responses versus A- 1-propanol % (w/w), B- $(NH_4)_2SO_4$ % (w/w), C- Crude load (g).

Source	DF	EEACN				EEHCA				EEGL				EEIGL			
		SS	MS	F-Value	P-Value	SS	MS	F-Value	P-Value	SS	MS	F-Value	P-Value	SS	MS	F-Value	P-Value
Model	9	2379.28	264.36	142.15	0.000	50.6507	5.6279	131.72	0.000	3345.71	371.75	50.27	0.000	6540.47	726.72	90.02	0.000
Linear	3	2096.07	698.69	375.69	0.000	46.2705	15.4235	360.98	0.000	2156.01	718.67	97.19	0.000	4747.05	1582.35	196.01	0.000
A- 1-propanol % (w/w)	1	2044.14	2044.14	1099.15	0.000	41.8948	41.8948	980.54	0.000	1967.51	1967.51	266.07	0.000	4643.46	4643.46	575.20	0.000
B- (NH4)2SO4 % (w/w)	1	44.04	44.04	23.68	0.001	4.0897	4.0897	95.72	0.000	51.61	51.61	6.98	0.025	90.54	90.54	11.22	0.007
C- Crude load (g)	1	7.88	7.88	4.24	0.067	0.2860	0.2860	6.69	0.027	136.89	136.89	18.51	0.002	13.05	13.05	1.62	0.232
Square	3	257.32	85.77	46.12	0.000	2.9198	0.9733	22.78	0.000	489.99	163.33	22.09	0.000	646.62	215.54	26.70	0.000
A * A	1	254.65	254.65	136.92	0.000	2.6585	2.6585	62.22	0.000	117.70	117.70	15.92	0.003	101.41	101.41	12.56	0.005
B * B	1	11.49	11.49	6.18	0.032	0.7182	0.7182	16.81	0.002	259.97	259.97	35.16	0.000	418.29	418.29	51.82	0.000
C *C	1	3.39	3.39	1.82	0.207	0.1626	0.1626	3.81	0.080	22.32	22.32	3.02	0.113	1.24	1.24	0.15	0.703
2-Way Interaction	3	25.89	8.63	4.64	0.028	1.4604	0.4868	11.39	0.001	699.71	233.24	31.54	0.000	1146.79	382.26	47.35	0.000
A * B	1	22.48	22.48	12.09	0.006	1.2561	1.2561	29.40	0.000	27.84	27.84	3.77	0.081	62.12	62.12	7.69	0.020
A * C	1	1.37	1.37	0.73	0.411	0.1129	0.1129	2.64	0.135	582.84	582.84	78.82	0.000	878.45	878.45	108.82	0.000
B * C	1	2.04	2.04	1.10	0.320	0.0913	0.0913	2.14	0.174	89.02	89.02	12.04	0.006	206.22	206.22	25.55	0.000
Error	10	18.60	1.86			0.4273	0.0427			73.95	7.39			80.73	8.07		
Lack-of-Fit	5	12.46	2.49	2.03	0.228	0.1481	0.0296	0.53	0.748	48.43	9.69	1.90	0.249	64.27	12.85	3.90	0.081

Pure Error	**5**	**6.14**	**1.23**			**0.2792**	**0.0558**			**25.52**	**5.10**			**16.46**	**3.29**		
Total	**19**	**2397.87**				**51.0779**				**3419.66**				**6621.20**			
S		**1.36372**				**0.206704**				**2.71934**				**2.84127**			
R-sq		**99.22%**				**99.16%**				**97.84%**				**98.78%**			
R-sq(adj)		**98.53%**				**98.41%**				**95.89%**				**97.68%**			
R-sq(pred)		**95.54%**				**97.02%**				**87.11%**				**91.52%**			

A, B and C represent 1-Propanol, $(NH_4)_2SO_4$ % (w/w) and Crude load (g) respectively; SS, DF, and MS represent sum of squares, degree of freedom, mean square, coefficient of variation, respectively.

APPENDIX Va: MATLAB Code/program used for calculation of equilibrium concentrations of phase components

```
%Program to evaluate weights for a given value of w1,w2 and lambda. Please %read the .txt file attached to know how to run the file.
clear all
x=input('Enter the value of weight of salt')
y=input('Enter the value of weight of polymer')
plot(x,y);hold on
w1=input('Enter the value of w1')  %command to enter the value of w1
w2=input('Enter the value of w2') %command to enter the value of w2
a1=input('Enter the value of a1') %command to enter the value of a
a2=input('Enter the value of a2') %command to enter the value of d
b1=input('Enter the value of b1') %command to enter the value of b
b2=input('Enter the value of b2') %command to enter the value of e
c=input('Enter the value of c') %command to enter the value of c
mt=input('Enter the value of mt') %command to enter the value of mt
mb=input('Enter the value of mb') %command to enter the value of mb
a=a1;b=b1;d=a2;e=b2;c=c;
lambda=mt./mb; %command to enter the value of lambda
for i=1:length(w1)
x0=[0.51 0.015 0.05 0.4]; % Please do not change this. This is the initial conditions for the non linear equations
options = optimoptions('fsolve','display', 'iter-detailed', 'TolFun',1e-16, 'TolX',1e-16,'Algorithm','trust-region-reflective')
[w]=fsolve(@(w)raj2(a,b,d,e,c,w1(i),w2(i),lambda(i),w),x0,options); %command to call the equations. The equations are written in seperate file named raj2.m
w1t(i)=w(1) %Displays the value of all weights.
w2t(i)=w(2)
w1b(i)=w(3)
w2b(i)=w(4)
end
plot(w2t,w1t);
plot(w2b,w1b);
```

APPENDIX Vb: MATLAB function call used for calculation of equilibrium concentrations of phase components

```
function [f] = raj2(a,b,d,e,c,w1,w2,lambda,w) % the file name is raj2. It should not be changed
f(1)=w(1)-(a.*exp(-(w(2)./b))+d.*exp(-(w(2)./e))+c) % The equaitons are all taken to LHS and written here a=a1;b=b1;d=a2;e=b2;c=c;
f(2)=w(3)-(a.*exp(-(w(4)./b))+d.*exp(-(w(4)./e))+c)
f(3)=lambda-(sqrt(((w(4)-w2).^2)+((w(3)-w1).^2))./sqrt(((w(2)-w2).^2)+((w(1)-w1).^2)));
f(4)=(w1-w(1))./(w2-w(2))-(w1-w(3))./(w2-w(4));
end
% w is a vector consisting of w(1),w(2),w(3) and w(4).
% w(1)=w1t,w(2)=w2t,w(3)=w1b,w(4)=w2b
```

PAPERS PUBLISHED AND CONFERENCES BASED ON THIS RESEARCH WORK

PEER-REVIEWED JOURNAL PUBLICATIONS

1. Nainegali, Basavaraj., I. Regupathi, and Prasanna Belur. D (2017). "Aqueous Two-phase Extraction of anthocyanin from fruits of *Garcinia indica*". ***International Journal of Earth Sciences and Engineering***, 10(3), 688-692. DOI:10.21276/ijee.2017.10.0330

2. Nainegali, B. S., Iyyaswami, R., & Belur, P. D. (2019). Simultaneous Extraction of four different Bioactive Compounds from *Garcinia indica* and their enrichment using Aqueous Two-Phase Systems. ***Food and Bioproducts Processing***, 114, 185-195. https://doi.org/10.1016/j.fbp.2019.01.002

3. Basavaraj S Nainegali, Regupathi Iyyaswami & Prasanna D. Belur (2020) Alcohol-based aqueous biphasic system applied to partition four different natural bioactive compounds from *Garcinia indica* Choisy. ***Separation Science and Technology***, 55(15), 1-17. https://doi.org/10.1080/01496395.2020.1802485

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2. **Basavaraj Nainegali**., Regupathi I., and Prasanna B. D, "Aqueous Two-phase Extraction of anthocyanin from fruits of *Garcinia indica*." **6th International Engineering Symposium - IES 2017** March 1-3, 2017, Kumamoto University, Japan

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Publications based on Ph.D. Research Work

Articles in SCI journals

1. **Nainegali, Basavaraj**., I. Regupathi, and Prasanna Belur. D (2017). "Aqueous Two-phase Extraction of anthocyanin from fruits of *Garcinia indica*". ***International Journal of Earth Sciences and Engineering***, 10(3), 688-692. DOI:10.21276/ijee.2017.10.0330
2. **Nainegali, B. S**., Iyyaswami, R., & Belur, P. D. (2019). Simultaneous Extraction of four different Bioactive Compounds from *Garcinia indica* and their enrichment using Aqueous Two-Phase Systems. ***Food and Bioproducts Processing***, 114, 185-195. https://doi.org/10.1016/j.fbp.2019.01.002

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Conferences/Seminars: International

5. **Basavaraj Nainegali**., Regupathi I., and Prasanna B. D, "Partitioning and preliminary purification of Bioactive Compounds from *Garcinia indica* using 1-propanol-magnesium sulphate Aqueous Two-Phase System" **4th International Conference on Bioscience and Biotechnology (BioTech-2019)** 21– 22 February 2019, Kuala Lumpur, Malaysia

6. **Basavaraj Nainegali**., Regupathi I., and Prasanna B. D, "Aqueous Two-phase Extraction of anthocyanin from fruits of *Garcinia indica*." **6th International Engineering Symposium - IES 2017** March 1-3, 2017, Kumamoto University, Japan

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Publications based on Previous Research Work

Articles in SCI journals

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Declaration

I hereby declare that all the statements made in this application are true and complete to the best of my knowledge and belief

Place: NITK
Date: 21.12.2020

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