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Novel Solvent Systems for the Extraction of Plant Metabolites

by

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DECLARATION

"I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief does not contain any material previously published or written by another person except where due reference is made in text."

Tristan Kilmartin

 on 19/12/2017

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SUMMARY

Plant extracts play an important role in human society with extracts finding uses in food, medicine and art to name a few. The preparation of tea and coffee along with the extraction of opioids from the opium poppy are just a few examples. There are a myriad of different technologies that have been developed over the years to achieve extraction of different chemical compounds from plant material. These utilise an extensive range of solvents, temperatures, contact methods, and pressures in order to selectively extract the components of interest. However, many of the solvents and processes are hazardous; either due to the chemical nature of the solvent or the extraction process. Further hazards can be posed by the environmental fate of the solvents, including mobilisation of toxic and or hazardous solvents and degradation products through air, soil, and water, and through the production of greenhouse gases. Industrially, there is often a trade-off between extraction efficiency and hazards in order to achieve an optimal extraction process for the target compounds, and in many cases hazardous, non-sustainable materials are used. Consequently, there is a need to produce 'green' extraction processes that utilise environmentally friendly, nonhazardous solvents, ideally obtained from renewable feedstocks.

This thesis describes the development of novel 'green' solvent systems for the extraction of metabolites from *C. officinalis* (Asteraceae). Systematic studies of glycerol-based composites in maceration and ultrasonic extraction processes led to the development of an acidified polysorbate/water solvent, which exhibited significant increases in peak areas and phenolic content when utilised in an ultrasonic extraction process.

Initial studies were conducted with aqueous acid and base, supercritical CO_2, and natural deep eutectic solvents and extracts were compared with those from a traditional hydroethanolic extraction process. Aqueous acidic and basic solutions, along with supercritical CO_2 were eliminated from the study on the basis of preliminary results. Preliminary extracts prepared using Natural Deep Eutectic Solvents (NaDES) made from two or more of sugars, acids, and glycerol exhibited a very similar extract profile to that of the traditional hydroethanolic process, with the glycerol based extracts having the most promise on the basis of their similar extraction performance and lower viscosity compared to the other NaDES tested.

Further investigations into the use of glycerol-based composites as extraction solvents, found that glycerol composites containing either sugar or acid demonstrated increased phytochemical content when compared to those prepared in neat glycerol. Strong positive correlations were observed between the extent of solvent chaotropicity and the extract phytochemical content (HPLC total peak area), phenolic

content (Folin-Ciocalteu assay), antioxidant activity ($KMnO_4$ chemiluminesence response), and radical scavenging activity (2,2-diphenylpicryl-1-hydrazyl assay). Results indicate that solvent mixtures with higher chaotropicity would be superior in their extraction efficiency for these types of plant extracts.

On the basis of these results, a novel solvent system was designed whereby the solvent chaotropicity was manipulated with the addition of polysorbates with differing chaotropicities. Aqueous and glycerolic polysorbate solutions were observed to result in significant increases in phytochemical content (HPLC total area) and phenolic content (Folin-Ciocalteau) when compared to neat aqueous and glycerolic extracts. Finally, the addition of organic acid to the aqueous polysorbate composites was trialled as a potential solvent system. These solvents demonstrated significant increases in phytochemical content when compared with traditional hydroethanolic solvents.

In summary, due to the often hazardous nature of current solvents and extraction processes, the development of alternate 'green' solvent systems is highly desirable. The research presented here represents a step in the development of novel solvent systems for the extraction of plant metabolites.

Dedicated to my family

Johanna and David

Sebastian and Jordan

Helene and John

Angeline and Michael

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LIST OF ACRONYMS

AAP	Aqueous Acidic Polysorbate
ABTS	2,2'-azino-bis(3-ethylbenzotiazoline-6-sulfonic acid)
CAD	Charged Aerosol Detection
CL	Chemiluminescence
CMC	Critical Micelle Concentration
DAD	Diode Array Detection
DI	Deionised
DPPH	2,2-diphenyl-1-picrylhydrazyl
FC	Folin-Ciocalteu
FIA	Flow Injection Analysis
FRAP	Ferric Reducing Antioxidant Potential
FTC	Ferric Thiocyanate
GAE	Gallic Acid Equivalent
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
HPLC	High Performance Liquid Chromatography
HPLC-CL	High Performance Liquid Chromatography - Chemiluminescence
HPLC-DAD	High Performance Liquid Chromatography – Diode Array Detection
HPLC-DAD-CL	High Performance Liquid Chromatography – Diode Array Detection - Chemiluminescence
HPLC-MS-MS	High Performance Liquid Chromatography – Mass Spectroscopy – Mass Spectroscopy
IL	Ionic Liquid
LC	Liquid Chromatography
LC-MS	Liquid Chromatography – Mass Spectroscopy
MS	Mass Spectroscopy
NMR	Nuclear Magnetic Resonance
NPC	Normal Phase Chromatography
ORAC	Oxygen Radical Absorbance Capacity
PS	Polysorbate
PS20	Polysorbate 20

PS80	Polysorbate 80
PTFE	Polytetrafluoroethylene
RPC	Reverse Phase Chromatography
RP-UHPLC	Reverse Phase – Ultra High Performance Liquid Chromatography
SF	Supercritical Fluid
SFE	Supercritical Fluid Extraction
SIA	Sequential Injection Analysis
SIA-CL	Sequential Injection Analysis - Chemiluminescence
UHPLC	Ultra High Performance Liquid Chromatography
UHPLC-DAD	Ultra High Performance Liquid Chromatography – Diode Array Detection
UV/Vis	Ultraviolet/Visible

1 INTRODUCTION

Humans have used plants and plant derived materials since ancient times as sources of food and textiles [1], construction materials [2], for aesthetic purposes [3], and for the treatment of illness and injury [1]. Plants were first used around 60-80,000 years ago when proto-humans adapted to convert long chain polyunsaturated fatty acids from plants into docosahexanoic and arachidonic acid, allowing them to utilise plants as a source of polyunsaturated fatty acids in place of fish and migrate from water sources [4]. Human preparation of plant extracts for use as food sources and/or in medicinal applications has occurred since ancient times using forms of extraction that utilised simple solvents such as water and ethanol in relatively crude extraction methods such as infusions to produce extracts with a wide range of different compounds. For example, Egyptians used willow bark and other salicylate-rich plants to prepare teas with pain-relieving properties [5]. Initially through trial and error, and later with increases in our understanding of chemistry and plant biochemistry, more complex and sophisticated solvents and extraction methods have been developed to selectively extract and isolate only the active compounds. In modern times, the preparation of many different drugs utilise these more sophisticated extraction technologies. Even common beverages such as coffee utilise many different extraction methods, with an estimated 2.25 billion cups of coffee being prepared and consumed per day [6].

Plants contain a wide variety of compounds beneficial to human health; however, they can also contain also a number of compounds that may be contraindicative to consumption. These compounds may impart an unpleasant taste, smell or texture (for example, the bitter taste of ginseng [7]), be toxic or harmful (for example, stinging nettles [8]), or simply be in an incorrect dosage that may preclude other hazards (for example, digoxin in foxglove [9]). By extracting the desired compounds from the plant material, it is possible to reduce the unwanted compounds and more accurately control the dosage of desired compounds in a form that is amenable to human use. Traditionally, the purpose of extraction has been to reduce or remove detrimental compounds present in plants, to isolate and/or concentrate the desired compounds, and to make the final product more palatable than the raw ingredients. This has been achieved using a number of methods (such as maceration and percolation) and extracting solvents (such as hot water or alcohol). Ancient Egyptians made use of willow and other salicylate-rich plants to prepare hot aqueous infusions (tea) with analgesic properties [5]. However, the active compound in the resulting willowbark tea (sodium salicylate) is bitter and astringent, and can cause throat pain as a result of prolonged exposure due the high acidity of the extract. Additionally, the low concentration of sodium salicylate in the tea required a relatively large quantity of tea to be consumed. In 1853, Charles Gerhardt acetylated this compound to form acetylsalicylic acid, commonly known as aspirin [10]. When using

aspirin compacted into a pill, the detrimental side-effect of the high acidity is removed, as is the bitter taste (as the pill is immediately swallowed), and a higher dosage is achievable compared with the tea (due to the more concentrated form).

1.1 PHYTOCHEMICAL EXTRACTION

To extract is to "obtain (a substance or resource) from something by a special method" [11]. Extraction of the desired components from plant materials is often achieved using non-polar or polar solvents [12] to isolate the soluble components from the insoluble components [13]. The resulting extracts are often complex mixtures of primary and secondary plant metabolites in either a liquid, semisolid or solid state, depending on the extraction solvent and process used. Plant extracts are also referred to as fluid extracts, decoctions, tinctures, infusions, powdered, or pilular (semisolid) extracts [14]. The extracts may be further processed through secondary extractions in order to remove undesirable components that may negatively impact the efficacy of the extract [13]. Extracting compounds from raw plant material can be hazardous; toxic or dangerous solvents may be used, and high pressures or temperatures may be involved in the extraction process [15]. Additionally, environmentally harmful waste products may be produced (directly or indirectly). High energy processes additionally impact negatively due to the larger quantities of greenhouse gases produced in the production of energy required to run the process. Alternative solvents and 'green' extraction methods should therefore be considered to reduce the environmental impact of phytochemical extraction.

The practice of extraction active components from plant material can be subdivided into two sequential processes: sample pre-treatment and extraction. A third step to isolate particular compounds is sometimes required. These three steps must be optimised depending on the desired product. Factors affecting the choice of extraction process include the nature and stability (chemical, thermal and chronological) of the desired compound, and the cost of raw material and solvent [13]. These factors can require additional costs in terms of time, equipment, or solvent and are often optimised to produce extracts with the best value for money rather than necessarily optimising for exhaustive extraction.

1.1.1 Sample Pretreatment

This step in the extraction process is the conversion of raw sample material into a form desirable for extraction or storage. This can be a multi-step procedure and can include freeze-drying, filtering, air-drying, centrifugation, ultrasonication, microwave treatment, heating or roasting, washing, chopping or grinding. There are advantages and disadvantages to different sample pretreatments. For example, grinding dried flower material before maceration increases the overall surface area, thereby allowing metabolites to more easily diffuse into the solvent. This results in decreased extraction time or increased extract concentration [16]. However, grinding the flower material too finely can lead to difficulties in later steps, for example, filtration to remove the insoluble cellulose waste. Additionally, finely ground samples risk higher exposure to oxygen, resulting in oxidation of phytochemicals. Sample pre-treatment can also include the addition of chemical additives, either to alter the compounds extracted in different extraction methods or as a method of altering the raw sample material (such as the addition of salt to lyse cells or the addition of water to rehydrate dried plant material). For example, *C. officinalis* has previously been pre-treated with ethanol before Supercritical Fluid Extraction (SFE) to increase the yield of phenolic and flavonoid compounds [17]. Since the researchpresented in this thesis primarily focusses on extraction processes and solvents, sample pre-treatment is not discussed in detail in this thesis.

1.1.2 Extraction

The phytochemical composition of the final extract is highly dependent on the nature of the solvent and the extraction method used [18]. Typical parameters that are modified for phytochemical extraction include pressure, time, temperature, solvent choice, and the contact process by which solvent interacts with the raw sample material. These parameters influence the underlying extraction process by modifying solvent-metabolite interactions and phytochemical diffusion processes, and can drastically alter the composition of the final extract. For example, Parenti *et al.* [19] found that aqueous coffee extracts prepared with a bar machine (92 ^{O}C, 9 bar, 25-30 second extraction time) and a capsule machine (92 ^{O}C, 12 bar, unknown extraction time) exhibited significantly different pH, room-temperature density, and refractive index, as well as significantly different quantities of total extracted solids.

There are a wide variety of different extraction techniques available for use which alter the parameters that influence the extraction process in different ways. These include traditional methods such as maceration, percolation, infusion, digestion, and distillation as well as modern methods such as counter-current, Sohxlet, ultrasonic, high pressure supercritical fluid, solid-phase microextraction, and matrix

solid-phase dispersion extractions [13]. Traditional methods typically utilise one or more of water, ethanol and oil as the extracting solvent, and usually result in quick, relatively safe to perform and cheap extractions that contain a reasonable amount of the desired compounds. However, such solvents and techniques are limited in selectivity and efficiency when compared with some modern methods. Modern extraction typically utilise superior heating methods, solvent/sample contact methods, or solvents. Some of the different extraction methods are discussed briefly below.

1.1.2.1 Extraction Methods

Maceration utilises whole or coarsely ground plant material placed in a stoppered vessel with the desired solvent (usually water). The mixture is left for a certain amount of time, typically days, with frequent agitation to let the soluble components within the plant material dissolve into the solvent. The extract is then collected and filtered or decanted and the solid waste material, known as the marc, is typically disposed of. Whilst time consuming, maceration is generally low cost, energy efficient and simple [20]. Modern solvents can replace the more traditional water/ethanol mixtures to increase extraction efficiency [21]. For example, the use of methanol in maceration has been shown to increase extraction of the total phenolic and flavonoid content in *C. officinalis* extracts [21].

Infusion extraction is typically a heated maceration in which the raw material and solvent contact for a shorter time period with the application of heat to rapidly solubilise only the most soluble metabolites [22]. The preparation of tea is a common example of infusion extraction.

Percolation is similar to maceration and is widely used among commercial applications to extract compounds. Here, the solid plant material is moistened with a specified amount of solvent and left for a period of time to allow absorption of the liquid. The material is then packed into a percolator which is then closed. Additional solvent is then added to the top of the percolator and allowed to macerate for a predetermined period of time, after which the bottom of the percolator is opened such that the solvent is allowed to drip out and be collected. Additional solvent is added and allowed to pass through the plant material until the desired amount of solvent is collected (approximately ¾ of the final volume desired). The plant material is then pressed to collect any remaining solvent. The final extract is made up to the desired volume with additional solvent and filtered or decanted [20, 22]. Pumping systems are sometimes used to cycle solvent from beneath the sample bed in order to achieve complete extraction.

Digestion extraction is another form of maceration where small amounts of heat are applied for extended periods of time (typically days) to increase phytochemical solubilisation [20].

Distillation uses heated solvent vapour to extract compounds from the raw material, which can be immersed in solvent, typically water (water distillation) which is then heated, placed above the boiling solvent (water and steam distillation), or placed in a separate vessel with subsequent introduction of solvent vapour from a different source (steam distillation) [20]. Distillation is typically used to extract the volatile essential components from plants [23]. The preparation of aromatic spirits such as gin or absinthe are examples of distillation extraction.

Counter-current extraction (Figure 1-1) utilises raw plant material that has been pulverised to fine slurry using toothed discs. In this form, the plant material moved through a cylindrical extractor, during which it comes in contact with the solvent. At the end of the extractor, the spent plant material (marc) is collected while extract is collected at the opposite end. This process can be conducted at room temperature, reducing the risk of temperature degradation of active compounds. The longer the extractor is, the further the raw plant slurry can move and therefore be in contact with solvent. Thus, complete extraction of active compounds can be achieved by careful optimisation of solvent flow and slurry movement [20].

Sohxlet extraction (Figure 1-2), also known as hot continuous extraction, utilises a condenser to recycle a fixed volume of solvent through a suspended bed of sample material over a long period of time This has the benefit of giving an almost complete extraction (as metabolites are continuously removed from the bulk of the solvent and concentrated in a smaller liquid solvent volume); however, to achieve full extraction, a long extraction time is required and, depending on the choice of solvent, a high temperature may be required, leading to the thermal degradation of extracted compounds [20].

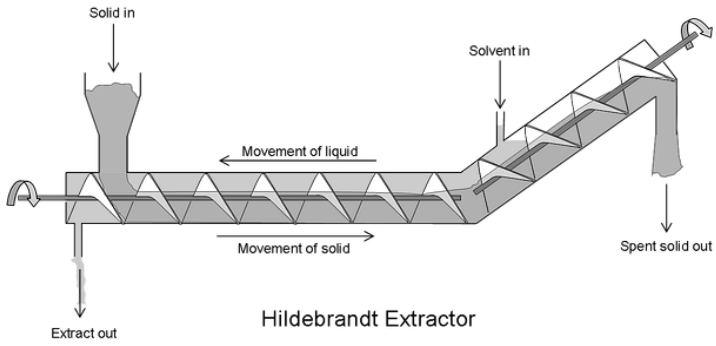


Figure 1-1: An image taken from Chakraborty [24] showing a Hildebrandt countercurrent extraction system.

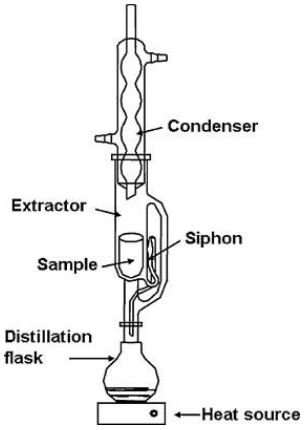


Figure 1-2: An image taken from Castro and Priego-Capote [25] showing a typical Soxhlet extraction system.

Ultrasonic assistance in extraction can be used either for sample pretreatment or as an extraction method. In sample pretreatment, ultrasonication can be used to rupture cell walls and oil glands, increasing surface area of the sample and solvent penetration into sample material [20]. If hydration of the sample prior to extraction is necessary, ultrasonication can also decrease hydration time. Ultrasonication during aqueous extraction allows the formation of microcavitations which have hydrophobic surfaces, increasing the solubility of non-polar species in highly polar media [26]. However, ultrasonic extraction can generate radical species [20], decreasing the overall antioxidant activity of the extract as the antioxidants act to remove the generated radicals [26]. Thus, optimisation of ultrasonic extraction time is necessary to maximise the extraction of phytochemicals whilst minimising the generation of radical species. Research has shown that ultrasonic extraction can give the extract with the highest antioxidant activity [27].

Microwave assistance in extractions can be used for sample pretreatment or extraction processes [13]. When used to aid extraction, microwaves offer a superior method of sample heating. Microwaves are typically used with polar solvents as the polar molecules change orientation with the oscillating electromagnetic field, resulting in heating. Nonpolar solvents exhibit poor heating due to the significantly lower interactions between the solvent and the electromagnetic field [28]. This extraction technique offers advantages over traditional heating methods as microwave ovens utilise significantly less power than traditional heating methods [28]. Microwave extraction is typically run with either open or closed vessels. Closed vessel systems have the added advantages of increased pressure and minimal solvent loss; however, safety risks can present due to the high pressures involved. These problems are mitigated in open vessel extraction systems; however solvent loss into the environment can present safety concerns.

Additionally, open vessel processes extract less compounds than closed vessel systems due to the lack of increased pressure.

High-pressure extraction systems have become increasingly popular for extraction of compounds from plants. The additional pressure present in the process can lead to deformation or rupture of the cellular membranes, increasing solvent penetration into the sample. Additionally, increased pressure increases the mass transfer mechanism, resulting in an overall increased yield of plant compounds compared to conventional atmospheric extractions [29]. Common solvents used in high-pressure extraction systems include water, ethanol (and mixtures thereof) and supercritical CO_2 [13]. The preparation of espresso from coffee grounds is a common example of high-pressure water extraction.

1.1.2.2 Extraction Solvents

Choice of extraction solvent is of critical concern in the extraction process, as solvent choice will often determine the composition of the final extract. Typical solvents used in traditional extraction processes are water and ethanol, as these have been readily available to humans for millennia [30]. Common modern solvents include water, methanol, ethanol, acetonitrile [31], petroleum ether [32], acetone [21], hexane [33], and isopropanol [34]. These can be used individually or as part of a solvent mixture, and as a result the choice is almost endless. Differences in solvent chemistry, polarity, and viscosity along with the pressures and temperatures used throughout the extraction process will change the resulting extract composition. For example, Iloki-Assangea *et al.* [35] found that hexanic, ethanolic, and aqueous extracts of *Bucida buceras L.* and *Phoradendron californicum* exhibited significantly different phytochemical profiles. Hexanic extracts contained carotenes, lactonic species, triterpenes and steroids, whereas ethanolic and aqueous extracts contained a more diverse range of phytochemicals including lactonic species, saponins, tannins, phenols, amines, amino acids, anthocyanins, flavonoids, triterpenes, and steroids. Additionally, the relative abundances of similar metabolites in different solvents varied between solvents, with hexanic extracts exhibiting higher concentrations of triterpenes and steroids than ethanolic extracts, which in turn exhibited higher abundances than aqueous extracts [35].

Though appealing for their efficiency, the use of organic solvents can have significant drawbacks. Organic solvents are often flammable and/or toxic, and can carry other health hazards such as carcinogenicity. They can also be an industrial hazard, for example flammable solvents may require special processes to ensure that an ignition event does not occur [36]. Table 1-1 shows different hazards associated with some common organic solvents. Organic extracts often require further processing in order to be made

suitable for human use, which can result in increased costs, as hazardous and toxic solvent residues must be thoroughly removed from the extract prior to use. In addition, many organic solvents are not obtained from sustainable resources and can negatively impact on the environment due to their toxicity. For example, toluene is produced through catalytic reforming of refined crude oil (an unsustainable resource). In Australia, organic waste streams such as those containing toluene are disposed of through incineration [37] which results in the release of greenhouse gases such as carbon dioxide into the atmosphere. Additionally, toluene poses acute short-term and chronic long-term toxicity to aquatic life, can damage plant cell membranes, and causes serious acute and chronic effects on the central nervous system and irritates the upper respiratory tract and eyes in humans [38]. Thus, there is often a trade-off between efficacies of given solvents in which a user must balance safer solvents that yield less product with more hazardous solvents that can offer greater yields.

Table 1-1: Typical solvents used in phytochemical extraction. Information taken from ChemWatch© Material Safety Data Sheets (MSDS) for respective solvents.

Solvent	*Flammable*	*Toxic*	*Irritant*	*Carcinogen*	*Other*
Methanol	✓	✓			
Ethanol	✓				
Isopropanol	✓	✓	✓		
Tetrahydrofuran	✓	✓	✓	✓	
Acetonitrile	✓	✓	✓		
Hexane	✓	✓	✓		Aspiration Hazard
Toluene	✓	✓	✓		Aspiration Hazard
Chloroform		✓	✓	✓	

Modern extraction methods often utilise high temperatures or pressures and use machinery such as pumping systems that consume electricity, thereby generating greenhouse gases. In addition to solvent changes, extraction processes can be made more sustainable through the substitution of energy-consumptive extraction processes with more energy-efficient systems. For example, Goktas *et al.* [39] used Soxhlet extraction to extract sterilising agents from *C. officinalis* and found that an elevated temperature and relatively long extraction (41 ^{O}C for 7 hours) was necessary to optimise the extraction process for aqueous extractions, resulting in a 90% metabolite extraction with the final extract demonstrating similar phytochemical composition to that "declared in the literature" [39]. This type of long, elevated-temperature extraction process consumes more energy than a room-temperature process such as percolation or maceration which can operate at ambient temperatures. However, a 90% phytochemical extraction at room temperature is relatively difficult to achieve. Consequently, trade-offs between energy consumption and extract efficiency are often required.

1.1.2.3 Alternative extraction solvents

Alternative solvents typically utilise 'unusual' chemical properties such as supercritical states or eutectic points in order produce solvents with similar extractive properties to common organic solvents. These solvents are often chosen because they are 'greener' or less hazardous than currently used organic solvents, but offer reasonable extractive properties. Alternate solvents include supercritical CO_2 [13], ionic liquids [40], deep eutectic solvents [41], natural deep eutectic solvents [42], and surfactant-based solvents [43]. These will be discussed below.

Supercritical Fluids (SF) are substances (typically gaseous or liquid) that exceed their critical temperature and pressure. By altering the temperature and pressure of the SF, the physical properties can be altered, resulting in a highly modifiable solvent that can be 'tuned' to extract specific compounds. Furthermore, SFs can be easily removed from the final extract by the simple process of exposing the extract to atmospheric conditions, resulting in the rapid phase change of the solvent from SF to gas. SFs can be used for the extraction of compounds and for chromatographic separation of liquids [44]. The properties of SFs can be further altered by doping; adding polar or non-polar solvents to the extraction to alter the polarity of the SF [17]. Carbon dioxide is a commonly used SF due to its low cost, ready availability and low environmental impact (compared with conventional solvents). A significant disadvantage of Supercritical Fluid Extraction (SFE) compared to other modern methods is the high initial costs; for example, advanced machinery is required to heat and pressurise CO_2 into a SF.

Ionic Liquids (IL) are generally composed of a bulky organic cation and an inorganic or organic anion, both of which are generally asymmetric. The most commonly used cations are pyridium and/or imidazolium species with one or more alkyl groups attached to the carbon or nitrogen atoms. The most commonly used anions are polyatomic inorganic fluorides such as PF_6^- or BF_4^-. ILs are molten at room temperature as they have additional ionic inter-molecular interactions not present in conventional solvents (such as dipole-dipole interactions, ion-dipole, Van-der-Waals interactions, and hydrogen bonding). These additional interactions also make ILs highly miscible with polar materials. The level of miscibility can be altered by the addition of alkyl chains on the cation, which increases miscibility with non-polar materials [45]. Additionally, the characteristics of ILs such as polarity, hydrophobicity, viscosity, and other chemical and physical properties that determine the application of the solvent can be altered by careful selection of the anion or cation, allowing for 'designer solvents,' increasing the range of potential applications. 1-butyl-3-methylimidazolium hexafluorophosphate $[C_4mim][PF_6]$ is a commonly used IL that is considered 'green' due to ease of preparation, hydrophobicity (allowing for liquid-liquid

extractions with water), low vapour pressure, non-flammability, re-useability [46], and superior extractive ability [47]. ILs are easily integrated into conventional extraction methods such as maceration extraction or microwave extraction and have previously been used for the liquid phase extraction of phytochemicals [46, 48], alkaloids [49], amino acids [50], essential oils [51], in the processing of fuel [52], for the dissolution of cellulose [53], in organic synthesis, electrochemistry, catalysis [54] and polymerisation processes [55, 56], as a chromatography additive [57], in ultrasonic [58-60], and microwave [61, 62] extraction from plant material.

Although imidazolium-based IL's are considered 'green' due to the aforementioned low vapour pressure, non-flammability and reusability, there are some disadvantages, particularly during IL synthesis. The environmental fate of ILs have had little study, as have the potential toxicity issues involved with prolonged IL use. Certain ILs have been studied and found to be prone to hydrolysis, leading to the formation of highly toxic byproducts such as HF and POF_3 [63]. To alleviate the issues presented by use of IL's, a secondary class of ILs was proposed in 2001 by Abbott *et al.* [64]. This class of solvents has been dubbed deep eutectic solvents and are discussed below.

Deep Eutectic Solvents (DES) are room-temperature liquids that predominantly feature quaternary ammonium salts (typically choline chloride) with a salt, organic acid or base. DES were proposed as being a 'green' alternative to the more toxic ILs that had previously been touted as 'green' due to their reusability and negligible vapour pressure but had been dismissed due to their toxicity [63]. These solvents present several advantages over the more traditional ILs such as ease of preparation, inexpensive materials, low toxicity, and generally use and produce more environmentally friendly products. DES have previously been used in the processing of lignin [65], and in microwave-assisted extraction of flavonoids from *Radix Scutellariae*, showing equivalent extractive capabilities to conventional water and ethanol solvents with nonhazardous materials and processes [66]. They have also been used in the extraction of terpenoids from C*hamaecyparis obtusa* leaves, showing rapid and simple extraction when compared to other techniques such as ultrasonic-assisted or reflux extraction [67].

Natural Deep Eutectic Solvents (NaDES) are a further subset of 'greener' DES have recently gained attention; NaDES have been prepared from naturally occurring plant metabolites such as amino acids, organic, acids and sugars [68] and form a room-temperature liquid when combined at certain molar ratios [69]. It has been hypothesised by Young *et al.* [55, 69] that certain naturally occurring products found in considerable quantities in most plants form NaDES and are responsible for the transport of compounds

that are poorly soluble in both water and lipid phases found in plant cells. NaDES have previously been shown to have comparable or higher affinities for phenolic compounds due to the high levels of hydrogen bonding interactions between the phenolic compounds and the NaDES [41]. Furthermore, the exact properties of a NaDES can be 'tuned' by the addition of a polar solvent, typically water, to optimise extraction of both polar and nonpolar molecules [70]. Typically, NaDES are prepared by dissolving the NaDES components in excess water. These aqueous solutions are then combined in varying molar ratios and the resulting mixture is either heated at low temperature or rotary evaporated until a constant weight is obtained [71]. NaDES have been prepared from a variety of different materials, including citric acid, choline chloride, malic acid, glucose, fructose, sucrose, and trehalose [69, 72]. Depending on the materials used, water can be retained in the NaDES as a necessary third component. It was found that the phenolic flavonoid Rutin was 50-100 times more soluble in various NADES than in water [69].

Surfactants are molecules that contain both a hydrophilic and hydrophobic chemical functionality, allowing them to interact with both polar and nonpolar molecules. These opposing functionalities give rise to aggregation and adsorption behaviours, in which the surfactant molecules self-arrange to minimise the unfavourable polar-nonpolar interactions [73]. In aqueous systems, non-ionic surfactants such as polysorbates form different structures depending on the relative concentrations of surfactant, water, and solubilised components (Figure 1-3). At low concentrations, surfactants exist as freely moving disordered molecules. At concentrations exceeding the Critical Micelle Concentration (CMC), surfactant molecules self-organise into spherical micelles [74] with the hydrophilic 'head' group forming the outer shell (Figure 1-4) and the hydrophobic 'tail' group forming the micelle core. The CMC for Polysorbate 20 and Polysorbate 80 in water have been reported previously as 0.007% w/v (55 μM) and 0.0017% w/v (13 μM) respectively [75]. As surfactant concentrations increase (typically above 10% w/w for non-ionic surfactants), micelle-micelle interactions become increasingly more common, and the spherical micelle structures convert to cylindrical rod-like structures in which the lipophilic surfactant tails form the central core of the cylinder. Further increasing the surfactant concentration results in the formation of a viscous isotropic phase, in which the surfactant and water forms a face- or body-centred cubic lattice. If the surfactant concentration is increased further, then a neat lamellar phase forms in which water is sandwiched between surfactant bilayers [74].Cubic lattice phases have previously been used for drug delivery. The phase behaviour of surfactants is strongly influenced by the relative concentrations of compounds in the solvent (shown in Figure 1-3), molecular geometry of the surfactant [76], temperature, and other solubilised compounds [77]. The formation of micelles in aqueous media allow for the formation of nonpolar 'domains' [78] that can more readily solubilise hydrophobic metabolites such as alkaloids and terpenes that would otherwise exhibit poor aqueous solubility. Surfactants such as those containing

polyoxyethylene functionalities, can be synthesised with larger or smaller hydrophilic and hydrophobic functionalities [79], allowing for increased selectivity over the extraction of different metabolites. Non-ionic surfactants have previously used in the aqueous two-phase extraction by heating *Salvia triloba* extract prepared with an aqueous surfactant mixture to extract oleanolic acid and ursolic acid [80]. Surfactants have also been used in the ultrasonic extraction of alkaloids from *Hyoscyamus muticus, Datura stramonium,* and *Ruta graveolens* [81], and in high-pressure aqueous extraction of anthroquinones from *Morinda citrifolia* [43].

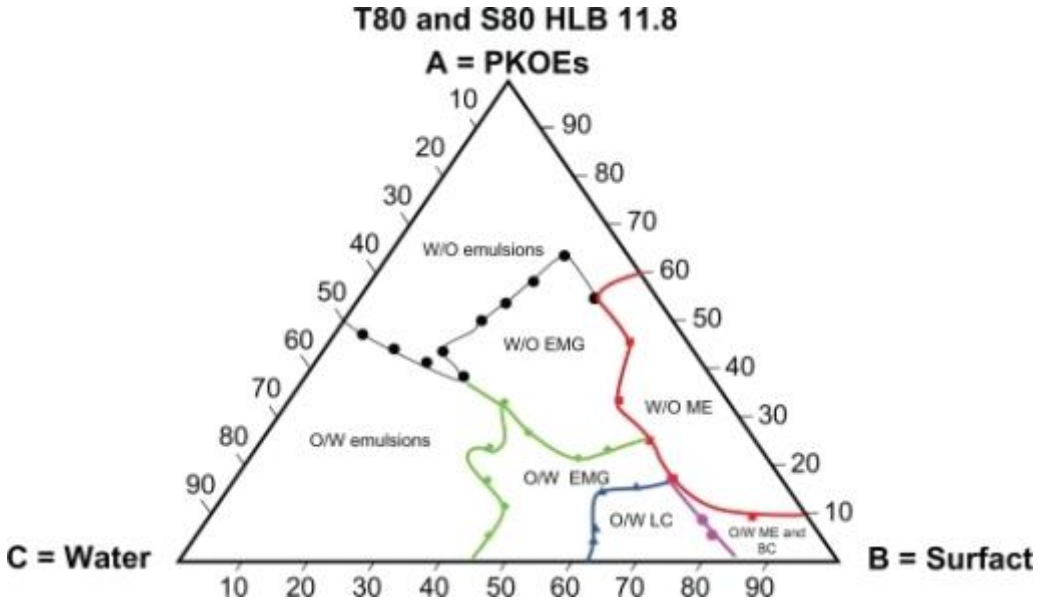


Figure 1-3: Schematic phase diagram taken from Mahdi et al. *[82] detailing the micellar structure formed by polysorbate 80/water/oil mixtures at varying concentrations.*

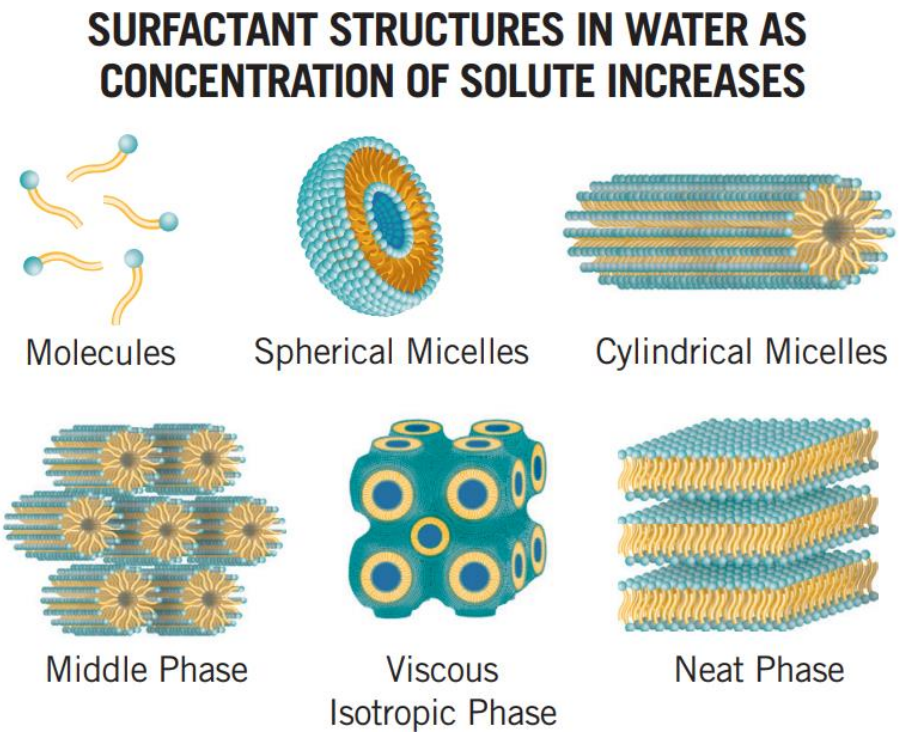


Figure 1-4: Image taken from Particle Sciences® [83] showing different surfactant structures in water at increasing surfactant concentrations, with concentrations below the CMC displaying random molecular motion, and higher concentrations displaying spherical, cylindrical, middle, viscous isotropic, and then neat phase micellar structures.

1.1.3 Isolation

Isolation is an optional tertiary step in in the extraction process that involves isolating the desired components in order to either remove undesirable compounds or to concentrate the desired compounds for a more potent dosage. Traditional methods of isolation may involve decantation or filtration to remove impurities and waste material, or heating the extract to a specific temperature to denature specific undesirable components [84]. Modern isolation methods may involve distillation [23], liquid-liquid extraction, chromatographic processes such as silica column chromatography, solid phase extraction, or matrix solid phase dispersion to isolate particular compounds or classes of compounds for elucidation, further analysis [33], or consumption. Since this research primarily focusses on extraction processes and solvents, isolation is not discussed in detail in this thesis except as a means of chemical analysis.

1.2 'GREEN' APPROACHES TO PLANT EXTRACTION

Although modern extraction methods can be highly efficient, particularly compared with traditional extraction techniques, they often utilise more hazardous solvents and more energy-consumptive processes. The use of these solvents and processes can result in significant environmental effects through disposal of the solvent (in landfill or through incineration) and subsequent movement (through solubilisation with water or volatilisation into the atmosphere, adsorption to mobile soils and clays, or through consumption by organisms). Furthermore, additional compounds are formed as the solvents degrade or react with other chemicals present in the environment, resulting in a large 'cocktail' of different compounds with a wide variety of physicochemical properties that may further damage the environment [85]. There are additional long-term concerns with the use of energy-consumptive processes, as the electricity needed for such methods is typically produced with fossil fuels such as coal, which then result in increased contributions to climate change. Thus, there is a need for novel 'green' chemical approaches in order to reduce or eliminate chemical hazards, energy consumption, and the use of non-renewable solvents. The use of benign solvents and renewable feedstocks along with energy efficient processes is of particular interest to industrial manufacterers of plant extracts. These are three of the core principles of green chemistry as defined by Anastas and Warner (Table 1-2) [86]. By replacing organic solvents with renewable, nonhazardous solvents, phytochemical extracts can be prepared with decreased risk and costs [68].

Table 1-2: Anastas and Warner's 12 Principles of Green Chemistry [86].

1	Prevent Waste
2	Atom Economy
3	Less Hazardous Synthesis
4	Design Benign Chemicals
5	Benign Solvents & Auxiliaries
6	Design for Energy Efficiency
7	Use of Renewable Feedstocks
8	Reduce Derivatives
9	Catalysis (vs. Stoichiometric)
10	Design for Degradation
11	Real-Time Analysis for Pollution Prevention
12	Inherently Benign Chemistry for Accident Prevention

In an ideal situation, a 'green' solvent would selectively extract desirable metabolites with an excellent yield and efficiency and with negligible hazards; however, this is often not the case. More often, large volumes of hazardous solvents from non-renewable feedstocks are used to extract metabolites that could not otherwise be extracted through environmentally friendly means. This generates large volumes of

hazardous chemical wastes which must be appropriately treated and disposed of. Consequently, there is a trade-off between extract yield and the 'greenness' of the solvent. Alternative extraction approaches that are low-cost, and that use less hazardous solvents obtained (ideally) from sustainable feedstocks are worthy of research towards transforming large scale processing into greener, more environmentally friendly, and sustainable industries. This may be achieved by modifying current 'green' solvents such as water in order to alter the extractive properties to better extract the desired compound. Some plant extracts (for example, *C. officinalis* extracts) are used in 'crude' forms for traditional medicine and cosmetic applications in which they can be immediately added to the desired preparation without further processing. For *C. officinalis*, this may be in such preparations as mouthwashes or skin creams. When extracts are used for such applications, 'green' solvent choices are imperative as the solvents used must be nonhazardous if they are to become part of the final preparation used by the consumer.

1.3 CALENDULA

1.3.1 General information

Calendula officinalis is an annual or perennial shrub belonging to the *Asteraceae* family, along with daisies, ragweed and chrysanthemums. The root word 'Calendula' originates from the Latin word '*Calends*,' meaning 'first day of the month,' thought to be in reference to Calendula flowering every month [87]. Originally known as 'Gold's', in Old English, *C. officinalis* became associated with the virgin Mary and later Queen Mary and became known as 'Mary's Gold,' or 'Marigold' [88]. *C. officinalis* composed of between 15-20 different genera [89], and is typically used as a food source, an ornamental plant, as medicine, a cosmetic aid, a dye for textiles, in the preparation of medicines, and as food. *C. officinalis* is believed to have been widely cultivated from the Mediterranean region to Iran. It has been used since the 12th century in traditional medicine in Europe [90] and is currently cultivated commercially in Europe and the Americas. *C. officinalis* is a small, hardy bush which can grow up to 2 ft. in diameter [91], is hermaphroditic (the flowers can be male or female and are both found on the same plant) and does not bear fruit. The shrub is tolerant to temperatures reaching -3 ^{O}C and grows well in sunny locations with soil of acidic or alkaline nature but prefers moist soil. *C. officinalis* requires little or no shade [92].

1.3.2 Uses

C. officinalis extracts have historically been used in both traditional and homoeopathic preparations as an antipyretic (used to reduce or prevent fevers), to treat conjunctivitis (inflammation of the eye) [93], pharyngitis (sore throat), aphthous stomatitis (benign mouth ulcers), gingivostomatitis (mouth inflammation), diaper rash, haemorrhoids, minor burns, herpes, measles, smallpox, jaundice, constipation, psoriasis, leprosy, and varicose veins, as an emmenagogue (promoting menstrual flow), to heal wounds and reduce scar tissue, for blood purification, and as a stimulant, antispasmodic, cholagogic (inducing bile flow), diaphoretic (inducing perspiration) and as an antiseptic. However, the efficacy of *C. officinalis* for a number of these conditions (particularly in homoeopathic preparations, in which active compounds have been diluted far beyond any efficacious concentration) has been disputed.

Traditional preparations of *C. officinalis* extracts include as tinctures, within lotions and ointments [94], washes, teas, creams [93], infusions, and as a fluid extract from fresh plant material. These are used orally, topically, and through ingestion. In modern medicine, *C. officinalis* extracts are mainly found as an ointment component to treat dermatological conditions such as ulcers eczema, burns and haemorrhoids,

and to treat conjunctivitis. *C. officinalis* extracts have also shown broad-spectrum antibacterial [95] and antifungal [31] activity. *C. officinalis* is also used as a food colourant to replace saffron in a dish [96]. Additionally, *C. officinalis* petals can be added to cakes, jams or teas to impart a golden colour.

1.3.3 Compounds within Calendula officinalis

The compounds present in *C. officinalis* are varied and are composed of terpenes, flavonoids, quinones, amino acids, alcohols, phenols, and amino acids, as well as volatile oil components [21]. Different compounds have widely different chemical characteristics and play different roles in *C. officinalis*. Many of these compounds are believed to be physiologically beneficial to humans, such as Rutin (antioxidant, anti-inflammatory, anti-diabetic, anti-adipogenic, and neuroprotective activities [97]) or Thymol (antioxidant, radical scavenging, anti-inflammatory, analgesic, antispasmodic, antibacterial, antifungal, antiseptic, and antitumor activities [98]).

Phenolics. Phenolic compounds are the largest group of secondary plant metabolites and are responsible for the pigmentation of fruit. Phenolics have multiple plant functions, the most important of which are herbivore and pathogen defence, and as antioxidants. Phenolics are comprised of multiple subclasses, including flavonoids, phenolic acids, quinones, xanthones, coumarins, and tannins [99]. Some phenolic compounds previously identified in *C. officinalis* extracts are detailed in Table 1-3.

Table 1-3: Some phenolic compounds previously identified in C. officinalis *plants [100, 101]. Extraction methods and solvents have been listed where reported.*

Compound	*Maceration*	*Steam Distillation*	*Percolation*	*SFE*
Gallic Acid	✓ (methanol/water)			
(E)-Anethole		✓		
Methyl salicylate		✓		
Octyl phenol				✓
Chlorogenic acid	✓ (ethanol/water)			
Caffeic acid	✓ (ethanol/water)			
Ferulic acid	✓ (ethanol/water)			
Coumaric acid	✓ (ethanol/water)			
Rosmarinic acid	✓ (ethanol/water)			

Flavonoids. Structurally, flavonoids are polyphenolic compounds that contain more than one benzene ring within their structure and are derived from parent compounds known as flavans [102]. Flavonoids often exhibit antioxidant and free radical scavenging activity, and act as pigments in flowers and fruits [103]. Flavonoids can have anti-inflammatory or antiviral properties [104]. Flavonoid nomenclature does not have a singular suffix. Flavonoids previously identified in *C. officinalis* extracts are detailed in Table 1-4.

Table 1-4: Flavonoids previously identified in C. officinalis *plants [91, 100, 101, 105, 106]. Extraction methods and solvents have been listed where reported.*

Compound	*Maceration*	*Steam Distillation*	*Percolation*	*SFE*
(E)-α-ionone		✓		
quercetin	✓			
isorhamnetin	✓			
isoquercetin				
isorhamnetin-3-O-glucoside	✓ (chloroform, ethanol)			
narcissin	✓ (methanol)			✓
rutinoside	✓(chloroform, ethanol)			
calendoflavobioside				
rutin	✓ (ethanol/water, methanol/water)			
isoquercitrin	✓			
neohesperidoside	✓(chloroform, ethanol)			
isorhamnetin-3-O-neohesperidoside	✓(chloroform, ethanol)			
2G-rhamnosylrutinoside	✓(chloroform, ethanol)			
quercetin-3-O-neohesperidoside	✓(chloroform, ethanol)			
quercetin-2G-rhamnosylrutinoside	✓(chloroform, ethanol)			
quercetin glucoside	✓(chloroform, ethanol, methanol)			
quercetin-3-O-rutinoside	✓			
Scopoletin-7-O-glucoside	✓ (methanol/water)			

Saponins. Saponins are glycosides that are made up of two main groups; steroid and triterpene saponins. Typically soluble in water and alcohols, saponins are usually insoluble in non-polar solvents. Saponins are known to exhibit hypolipidemic and anticancer activities, are highly poisonous, and can cause haemolysis [103]. Saponins previously identified in *C. officinalis* extracts are detailed in Table 1-5.

Table 1-5: Saponins previously identified in C. officinalis *plants [107-110]. Extraction methods and solvents have been listed where reported.*

Compound	*Maceration*	*Steam Distillation*	*Percolation*	*SFE*
Oleanic acid	✓ (hot methanol)			
Ursolic acid	✓ (hot methanol)			
Calendasaponin A	✓ (hot methanol reflux)			
Calendasaponin B	✓ (hot methanol reflux)			
Calendasaponin C	✓ (hot methanol reflux)			
Calendasaponin D	✓ (hot methanol reflux)			
Officinoside A	✓ (hot methanol reflux)			
Officinoside B	✓ (hot methanol reflux)			

Terpenes. Terpenes, also known as terpenoids or isoprenoids, are the most widespread and chemically diverse group of phytochemicals, containing over 25-30,000 different compounds with both antagonistic and beneficial interactions among organisms [111]. Structurally, terpenes are unsaturated hydrocarbons usually found in resins, oleoresins, and essential oils and are classified according to the number isoprene ($CH_2=C(CH_3)CH=CH_2$) units present as either mono-, di-, tri-, tetra-, sesqui- or polyterpenoids (Table 1-6) [103]. Terpenes are present in plants, animals and microorganisms and defend against predators, pathogens and damage by oxidation or free radical activity. For example, drimanes, a bicyclic sesquiterpene subclass, have powerful antibacterial and antifungal effects and are toxic to aquatic life, insects, and nematodes, and as such are used to deter predation [112]. Plants typically produce complex mixtures of terpenes rather than one particular variety. Why this occurs is not fully understood; however, it has been hypothesised that the production of complex mixtures may be a method of impeding the ability of predators to develop resistance [111]. Terpenes previously identified in *C. officinalis* extracts are detailed in Table 1-7.

Table 1-6: Nomenclature of terpenoids classified according to the number of isoprene subunits

Terpenoid nomenclature	*Isoprene units present*
Monoterpene	2
Sesquiterpene	3
Diterpene	4
Triterpene	6
Tetraterpene	8
Polyterpene	>8

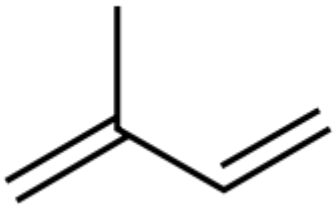


Figure 1-5: Structure of a basic isoprene subunit

Table 1-7: Terpenes previously identified in C. officinalis *plants [91, 106, 110, 113-115]. Extraction methods and solvents have been listed where reported.*

Compound	*Maceration*	*Steam Distillation*	*Percolation*	*SFE*
cedrol				✓
guaiol				✓
1-epi-cubenol		✓		
carotol		✓		
globulol		✓		
viridiflorol		✓		✓
elemol		✓		
epi-cubebol		✓		✓
valencene		✓		✓
cis-β-guaiene		✓		
geranyl acetone		✓		
longifolene		✓		
terpinene-4-ol		✓		
Menthone		✓		
γ-terpinene		✓		
tricyclene		✓		
β-eudesmol		✓		✓
cubenol		✓		✓
Ledol		✓		✓
Thymol		✓		✓
sigmasterol	✓ (Methanol/diethyl ether/water, petroleum)			
taraxasterol	✓ (petroleum, diethyl ether)			✓
faradiol-3-O-palmitate	✓ (dichloromethane)			✓
faradiol-3-O-myristate	✓ (dichloromethane)			✓
faradiol-3-O-laurate	✓ (dichloromethane)			
arnidiol-3-O-palmitate	✓ (dichloromethane)			
arnidiol-3-O-myristate	✓ (dichloromethane)			
arnidiol-3-O-laurate	✓ (dichloromethane)			
Calenduladiol-3-O-palmitate	✓ (dichloromethane)			

Compound	*Maceration*	*Steam Distillation*	*Percolation*	*SFE*
Calenduladiol-3-O-myristate	✓ (dichloromethane)			
Calenduladiol-3-O-laurate	✓ (dichloromethane)			
cis-sabinene hydrate		✓		
Linalool		✓		
cis-limonene oxide		✓		
cis-chrysanthemol		✓		
terpinene-4-ol		✓		✓
α-terpineol		✓		✓
trans-carveol		✓		✓
Neral		✓		✓
Carvone		✓		✓
Geranic acid		✓		✓
geranyl acetate		✓		✓
α-cedrene		✓		✓
heliantriol C	✓		✓	
heliantriol F	✓		✓	
Ursatriol	✓		✓	
longispinogenine	✓		✓	
lupenetriol	✓		✓	
Decanoic acid	✓ (Methanol, diethyl ether, water)			
undecanoic acid	✓ (Methanol, diethyl ether, water)			
lauric acid	✓ (Methanol, diethyl ether, water)			
tridecanoic acid	✓ (Methanol, diethyl ether, water)			
myristic acid	✓ (Methanol, diethyl ether, water)			
pentadecanoic acid	✓ (Methanol, diethyl ether, water)			
palmitic acid	✓ (Methanol, diethyl ether, water)			
margaric acid	✓ (Methanol, diethyl ether, water)			
stearic acid	✓ (Methanol, diethyl ether, water)			
oleic acid	✓ (Methanol, diethyl ether, water)			
linoleic acid	✓ (Methanol, diethyl ether, water)			
linolenic acid	✓ (Methanol, diethyl ether, water)			
eicosanoic acid	✓ (Methanol, diethyl ether, water)			

Compound	*Maceration*	*Steam Distillation*	*Percolation*	*SFE*
eicosatrienoic acid	✓ (Methanol, diethyl ether, water)			
arachidic acid	✓ (Methanol, diethyl ether, water)			
behenic acid	✓ (Methanol, diethyl ether, water)			
α-amyrin	✓ (diethyl ether, petroleum)			
β-amyrin	✓ (diethyl ether, petroleum)			
Calenduladiol	✓ (petroleum)			
lauric acid		✓		✓
isoamyl laurate		✓		✓
mystiric acid				✓
methyl myristate		✓		✓
ethyl myristate		✓		
palmitic acid		✓		
ethyl palmitate		✓		✓
butyl palmitate				✓
stearic acid				
methyl stearate		✓		✓
oleic acid				
linoleic acid				
methyl linoleate		✓		✓
linolenic acid				
methyl linolenate		✓		✓
hexanoic acid		✓		✓
methyl pentadecanoate				
methyl margarate		✓		✓
9,12-octadecadienal				✓
methyl eicosanoate				✓
methyl docosanoate				✓
stearyl alcohol				✓

Sterols and Stanols are lipids that can exist as free metabolites, glycosides, and as esters [116]. In humans, plant sterols are believed to lower low density lipoprotein cholesterol when ingested [117]. The general structure of sterols and stanols are shown in Figure 1-6 and Figure 1-7 respectively. Sterols contain an unsaturated double bond, whereas stanols do not (indicated in green). Sterols and stanols previously identified in *C. officinalis* are listed in Table 1-8.

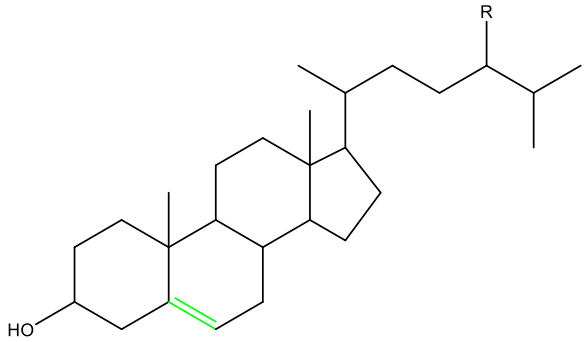


Figure 1-6: General structure of sterols where R is typically an organic moiety [118].

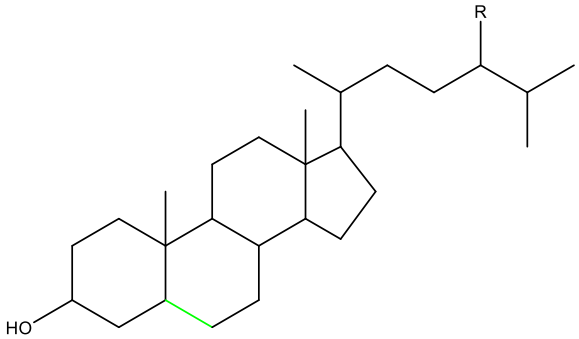


Figure 1-7: General structure of stanols where R is typically an organic moiety [119].

Table 1-8: Sterols and stanols previously identified in C. officinalis [120, 121]*. Extraction methods and solvents have been listed where reported.*

Compound	*Maceration*	*Steam Distillation*	*Percolation*	*SFE*
clerosterol	✓ (Methanol, diethyl ether, water)			
methylenecholesterol	✓ (Methanol, diethyl ether, water)			
isofucosterol	✓ (petroleum)			
cholest-7-en-3β-ol	✓ (Methanol, diethyl ether, water)			
cholesterol	✓ (Methanol, diethyl ether, water)			
campesterol	✓ (Methanol, diethyl ether, water)			
sitosterol	✓ (Methanol, diethyl ether, water, petroleum)			
cholestanol	✓ (Methanol, diethyl ether, water)			
campestanol	✓ (Methanol, diethyl ether, water)			
sigmasterol	✓ (Methanol, diethyl ether, water)			

Carotenoids are a subclass of terpenoids. There are approximately 500 different naturally occurring carotenoids which serve two important roles in plants. Firstly, carotenoids aid photosynthesis by transferring the absorbed energy to the chlorophylls. Secondly, by absorbing extra light, the carotenoid compounds protect plants that can be overexposed to light [122], which can result in damage to the plant. Carotenoids are thought to reduce the risk of disease, particularly eye disease and certain types of cancer due to their antioxidant activities [123]. Carotenoids previously identified in *C. officinalis* extracts are detailed in Table 1-9.

Table 1-9: Carotenoids previously identified in C. officinalis *plants [91, 124]. Extraction methods and solvents have been listed where reported.*

Compound	*Maceration*	*Steam Distillation*	*Percolation*	*SFE*
Luteoxanthin				
Flavoxanthin	✓ (methanol, ethyl acetate, petroleum)			
Lutein	✓ (methanol)			
Antheraxanthin	✓ (methanol, ethyl acetate, petroleum)			
Rubixanthin	✓ (methanol, ethyl acetate, petroleum)			
α-Carotene	✓ (methanol, ethyl acetate, petroleum)			
β-Carotene	✓ (methanol, ethyl acetate, petroleum)			
Lycopene				
γ-Carotene	✓ (methanol, ethyl acetate, petroleum)			
9Z-neoxanthin	✓ (methanol)			
violaxanthin	✓ (methanol)			
luteoxanthin	✓ (methanol, ethyl acetate, petroleum)			
auroxanthin				
9Z-violaxanthin	✓ (methanol)			
13Z-violaxanthin	✓ (methanol)			
mutatoxanthin	✓ (methanol)			
neoxanthin	✓ (methanol, ethyl acetate, petroleum)			
9Z-anthroxanthin				
9/9'Z-lutein	✓ (methanol)			
13/13'Z-lutein	✓ (methanol)			
α-cryptoxanthin	✓ (methanol)			
β-cryptoxanthin	✓ (methanol)			
Lycopene	✓ (methanol, ethyl acetate, petroleum)			
β-Ionone		✓		✓

Quinones. Quinones are present in plants typically as benzoquinones, naphthoquinones, anthroquinones, or polyquinones [125] and are highly redox active compounds [126] that may play a role in photosynthesis and (plant) cellular respiration, functioning as electron transport cofactors [127]. *In vivo*, Quinones create a variety of hazardous effects that include carcinogenesis, immunotoxicity, and acute cytotoxicity [126]. Quinones previously identified in *C. officinalis* extracts are detailed in Table 1-10.

Table 1-10: Quinones previously identified in C. officinalis *plants [91]. Extraction methods and solvents have been listed where reported.*

Compound	*Maceration*	*Steam Distillation*	*Percolation*	*SFE*
phylloquinone		✓		✓
α-tocopherol		✓		✓
ubiquinone		✓		✓
plastoquinone		✓		✓

Coumarins. Coumarins are produced in response to pathogenic attack and environmental stresses in plants [128]. The four coumarin subtypes are simple coumarins, dimeric coumarins, furanocoumarins, and pyranocoumarins [128]. Coumarins are believed to exhibit a large variety of different properties, including anti-inflammatory, antimicrobial, antifungal, antiviral, antioxidant, analgesic [128], anticancer, and antidiabetic properties [129]. Coumarins previously identified in *C. officinalis* extracts are detailed in Table 1-11.

Table 1-11: Coumarins previously identified in C. officinalis *extracts [91]. Extraction methods and solvents have been listed where reported.*

Compound	*Maceration*	*Steam Distillation*	*Percolation*	*SFE*
scopoletin	✓			
umbelliferone				
esculetin	✓			

Volatile (essential) oils. Essential oils are the products of various plant and animal species that are odorous and volatile. An essential oil can contain over 200 different chemical compounds [103]. It has been hypothesised that the volatile constituents selectively attract and repel certain species, which both aids in pollination or seed spreading, or protects the plant from predators. Additionally, it has been hypothesised that certain compounds protect the plant from bacteria and other microscopic parasites. Volatile oil components previously identified in *C. officinalis* are detailed in Table 1-12.

Table 1-12: Volatile essential oil components previously identified in C. officinalis *extracts [91, 114, 130]. Extraction methods and solvents have been listed where reported.*

Compound	*Maceration*	*Steam Distillation*	*Percolation*	*SFE*
α-thujene		✓		
α-pinene				✓
sabinene				✓
β-pinene		✓		✓
Myrcene		✓		
limonene		✓		✓
tridecane		✓		✓
D-Limonene		✓		
1,8-cineol		✓		
p-cymene		✓		
trans-β-ocimene		✓		
γ-terpenene				✓
δ-3-carene		✓		
nonanal		✓		✓
terpene-4-ol				
4-methyl-3-cylohexene-1-ol		✓		
α-phellandrene				
α-terpeneol		✓		✓
geraniol				✓
carvacrol		✓		✓
bornyl acetate		✓		✓
sabinyl acetate		✓		
α-cubebene		✓		✓
β-copaene		✓		✓
α-copaene		✓		✓
α-bourbonene		✓		
β-bourbonene		✓		✓
β-cubebene		✓		
α-gurjunene		✓		✓
β-gurjunene		✓		✓
aromadendrene		✓		✓
β-caryophyllene		✓		
α-ylangene				✓
α-humulene		✓		
epi-bicyclo-sesquiphellandrene		✓		
germacrene D				
α-amorphene		✓		
Estragole		✓		✓
alloaromadendrene		✓		✓

Compound	*Maceration*	*Steam Distillation*	*Percolation*	*SFE*
β-saliene		✓		✓
calarene		✓		✓
Germacrene D		✓		✓
α-muurolene		✓		✓
γ-muurolene		✓		
γ-cadinene		✓		✓
δ-cadinene		✓		
cadina-1,4-diene		✓		
α-cadinene		✓		✓
β-cadinene	✓			
nerolidol	✓	✓		✓
palustron		✓		✓
β-endobourbonene		✓		✓
Oplopanone				✓
δ-cadinol				✓
α-cadinol				✓
T-Cadinol				✓
T-muurolol				✓
5-diene cis-Muurola-4-(14)				✓
6,7-Dimethyl 1,2,3,5,8,8α-hexahydronaphthalene				✓
cis-Muurola-3,5-diene				✓
trans-Muurola-3,5-diene				✓
α-caryophyllene				✓
trans-cadina-1(6),4-diene				✓
trans-cadina-1,4-diene				✓
α-cadinene				✓
caryophyllene oxide				
1-hexadecene				
2-methylpropanoic acid				
1,10-di-epi-cubenol				
1-epi-cubenol				
Acetyl Eugenol				✓

Amino Acids. Amino acids play an essential role in the regulation of plant stress due to external conditions and are precursors for hormones that control plant growth [131]. Amino acids previously identified in *C. officinalis* extracts are detailed in Table 1-13.

Table 1-13: Amino acids previously identified in C. officinalis *plants [91]. Extraction methods and solvents have been listed where reported.*

Compound	*Maceration*	*Steam Distillation*	*Percolation*	*SFE*
alanine				
arginine				
aspartic acid				
aspargine				
valine				
histidine				
glutamic acid				
leucine				
lysine				
proline				
serine				
tyrosine				
threonine				
methionine				
phenylalanine				
alloaromadendrene oxide				✓

1.4 ANALYSIS OF PLANT EXTRACTS

The pharmacological activity of plants are generally attributed to the secondary metabolites present such as flavonoids, volatile oil components, carotenoids, terpenes, saponins, and other polyphenolic compounds. In particular, flavonoids and phenolic compounds are of interest due to their properties as antioxidants, free-radical scavengers and peroxide-inhibitors [93]. Some common methods of characterisation for these properties include 2,2-diphenyl-1-picrylhydrazyl (DPPH) (free-radical scavenging), Folin-Ciocalteau (phenolic content) [132], and chemiluminescence [133], and can be combined with HPLC-MS for compound elucidation and quantification [31, 101, 134]. HPLC has also been used previously in conjunction with DPPH assays to determine the phytochemical composition of *C. officinalis* leaves [135] or in conjunction with chemiluminescence to determine the antioxidant activity of *C. officinalis* flowers [133].

1.4.1 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is a common chromatographic technique that separate mixtures of different compounds based on the differential affinity between an analyte and the mobile liquid phase and solid stationary phase. HPLC is a modular and versatile technique that can utilise different stationary and mobile phases in order to achieve separation. HPLC is typically used to analyse liquid samples and has previously been used in the detection of carotenoids [124], flavonoids [136], and other compounds [21, 100] in *C. officinalis* extracts.

HPLC systems are typically composed of a pumping system (typically either binary or quaternary), an injection system, and an analytical column coupled to one or more detection systems. Guard columns and in-line filters are often used to reduce column contamination in order to minimise instrument maintenance. This is shown in Figure 1-8.

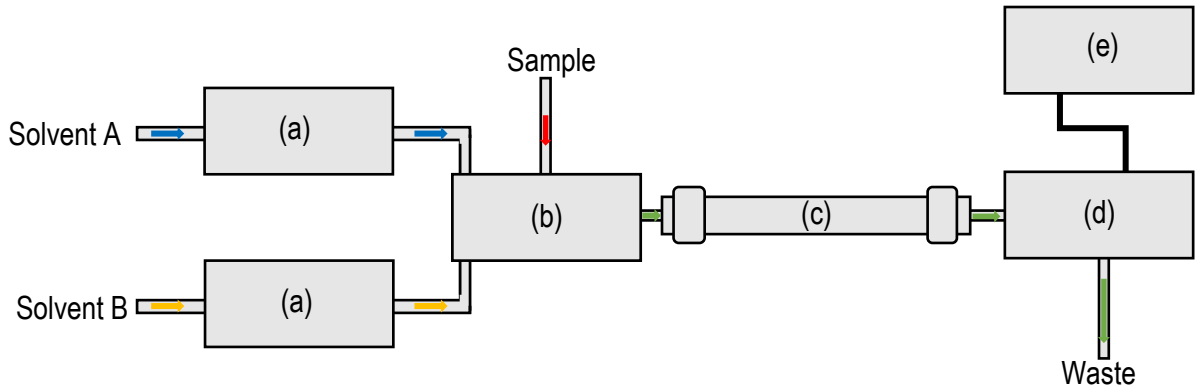
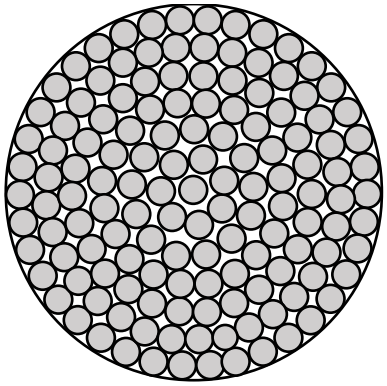


Figure 1-8: Diagram of a binary HPLC/UHPLC system with a) pump, b) injector, c) analytical column, d) detector, e) computer.

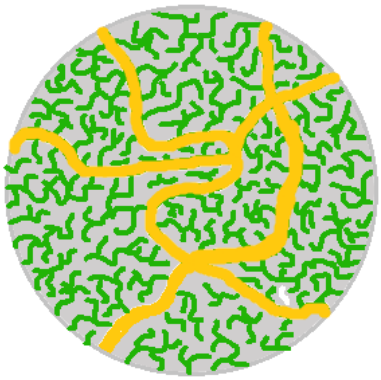
During analysis, a mixture of analytes is injected onto the analytical column that is held at constant temperature. A liquid mobile phase under high pressure is then used to force the analyte mixture through the analytical column which is uniformly and densely packed with particles of uniform diameter. Particle sizes can vary, with smaller particles requiring higher pressures in order to force the analyte through the column. Pressures for HPLC typically range from 2000-4000 psi [137]. HPLC systems that operate under higher pressures (typically 6,000-19,000 psi) are often called Ultra-High Performance Liquid Chromatography (UHPLC) systems [137, 138]. UHPLC systems typically require less sample material and mobile phase than HPLC whilst offering comparable or improved separation due to a smaller, more densely packed column which increases the number of interactions that occur between the analyte and the stationary phase. In both HPLC and UHPLC systems, analytes are separated based on their relative attraction to stationary phase present in the analytical column, and to the mobile phase. In Normal Phase Chromatography (NPC), a polar stationary phase is used. Polar compounds are more strongly attracted to the stationary phase and are retained longer than nonpolar compounds that more strongly adsorb to the less polar mobile phase. Conversely, in Reverse Phase Chromatography (RPC), a nonpolar stationary phase is used which results in nonpolar compounds adsorbing to the stationary phase, resulting in a longer retention than more polar compounds which are more strongly attracted to the polar mobile phase. Nonpolar solvents used in NPC include hexane or tetrahydrofuran. Polar solvents used in RPC include water and organic solvent/water mixtures (such as methanol/water, isopropanol/water, and acetonitrile/water) [138, 139]. Soluble additives can be added to mobile phases in order to modify the interactions between analytes and the mobile and stationary phases; for example, the addition of formic acid to a polar mobile phase will increase protonation of acidic functionalities present in the analyte, resulting in an increased affinity for the nonpolar stationary phase and assisting in the separation of mixtures. RPC is more commonly used than NPC due to increased resolution of analytes [138].

HPLC mobile phases can be run in either isocratic or gradient modes. Isocratic mobile phases do not change composition throughout the analysis, and are typically used when analytes have very similar polarity and therefore retention time. When different analytes have substantially different retention times, however, isocratic mobile phases can exhibit poor separation of low-retention compounds, peak broadening of strongly retained compounds, and increased run times in order to elute strongly retained compounds. Additionally if the affinity of the analyte for the stationary phase is significantly higher than the affinity for the mobile phase, the analyte may not elute at all, leading to column contamination and affecting future analyses. Gradient HPLC mobile phases are typically used to overcome some of these issues. Gradient HPLC usually involved the on-line mixing of multiple mobile phases in order to change the polarity of the mobile phase over time, thus changing the relative affinity of the analyte to the stationary and mobile phases. In RPC, the mobile phase will typically decrease in polarity. This has the resulting effect of eluting multiple analytes of different polarities over a significantly shorter time period. Binary gradient mobile phases are common, but some HPLC systems are able to use ternary or quaternary gradients to effect a significant change in the mobile phase polarity.

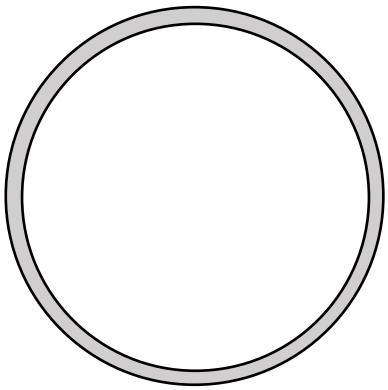
Stationary phases in HPLC are extremely varied and may contain different materials, structures, and chemical functionalities. Silica is a common material used and typically comes as either monolithic or packed columns of varying length. Monolithic columns are typically composed of crosslinked polymers or porous silica materials [140]. Packed columns are typically composed of silica particles densely packed into the column. A range of different particle structures can be used depending on the analytes in question and are shown in Figure 1-9. These include microporous particles, perfusion particles nonporous particles (which typically have a film present), or microporous particles with a solid nonporous core. Particles can also vary in size depending on the desired column efficiency, with smaller particles demonstrating increased separation but also requiring higher pressures (and therefore more advanced instrumentation) [141]. Pores can also vary in size depending on the desired retention, selectivity, and mass transfer of the analyte [140]. Porous particles are preferred for most analyses due to their higher surface area. Particles are typically silica-based which have polar surfaces due to silanol functionalities present. Whilst plain silica particles are typically used for normal phase chromatography (with a polar stationary phase and nonpolar mobile phase) in which polar compounds are retained, nonpolar chemical functionalities (typically long carbon chains or aromatic moieties) can be covalently attached to the exterior surface of the silica particle in order to increase the selectivity to nonpolar analytes. In this case, a polar mobile phase is typically used [138].



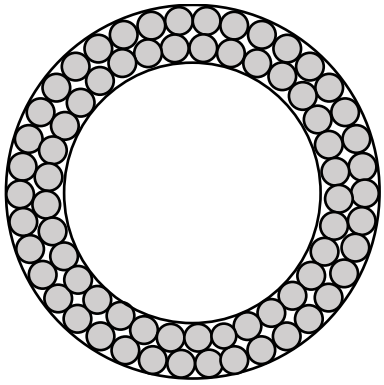
Microporous particle



Perfusion Particle – Orange and Green indicate thorough and diffusive pores respectively.



Nonporous Particle – Grey indicates a liquid or ion exchange film



Core-shell Particle

Figure 1-9: Particles types used in packed columns for HPLC [138].

HPLC can be coupled to a number of different detection systems including Diode Array Detection (DAD) [142], Charged Aerosol Detection (CAD) [139], chemiluminescence (CL) [143], and Mass Spectroscopy (MS). The detection system can be changed depending on the analyte and the desired information, and multiple detection systems can be coupled together (such as HPLC-DAD-CL [144] or HPLC-MS-MS [145]). DAD detects analytes based on the UV/Vis absorbance of the analytes. CAD detects nonvolatile analyte aerosols that have been desolvated and electrostatically charged, and MS detects ionised compounds based on their mass-to-charge ratio.

1.4.2 Sequential Injection Analysis Chemiluminescence

Sequential Injection Analysis Chemiluminescence (SIA-CL) is an analytical technique that uses acidified potassium permanganate injected sequentially with liquid analytes and reagents to determine the total antioxidant capacity of an analyte through the measurement of chemiluminescence emission.

Chemiluminescence (CL) is "the emission of light during a chemical reaction which does not produce significant quantities of heat [11]." CL can be used as a rapid and sensitive chemical analysis technique and has previously been used to determine the antioxidant levels in fruit juices [146]. In general, chemiluminescence occurs when two different compounds (typically the analyte and an oxidant) react, sometimes in the presence of a catalyst, and an excited state is formed by one or more components of the reaction. This excited component then undergoes a radiative energy loss, emitting light (shown in Figure 1-10). Typical chemiluminescence systems used in analytical chemistry include luminol [147], tris(2,2'-bipiridyl)ruthenium(III) [148] and acidified potassium permanganate [146]. Permanganate chemiluminescence has previously been used to determine the antioxidant activity of *C. officinalis* extracts [133, 149].

$$A + B \rightarrow C^* \rightarrow C + \gamma$$

Figure 1-10: General chemiluminescence reaction where A and B are reagents, C and C are the product and excited product respectively, and γ is the emitted photon*

CL can be enhanced by the inclusion of additional reagents to increase the emission of light and thereby increase the sensitivity of the chemiluminescence assay. In permanganate CL, this is typically done with enhancers such as polyphosphates, formaldehyde, or surfactants which act to prevent disproportionation of Mn(II) and reduce the non-radiative relaxation pathways of the Mn(II)* excited species [150]. The use of sodium hexametaphosphate is of particular interest here as 50-fold increases in CL have been observed when used in acidified potassium permanganate CL systems [150].

In SIA-CL, buffer and permanganate solutions are prepared. Analyte is sandwiched between aliquots of buffer in the holding coil in order to prevent chemiluminescence reactions occurring with the permanganate carrier solution outside of the reaction coil (Figure 1-11). Sample and reagent lines were flushed with respective solutions prior to analysis in order to minimise carryover. Once the analyte has been stored in the holding coil, the analyte and buffer aliquots are flushed into a mixing coil with permanganate carrier solution where the CL reaction occurs and emissions are detected with a photomultiplier tube in a lightproof housing.

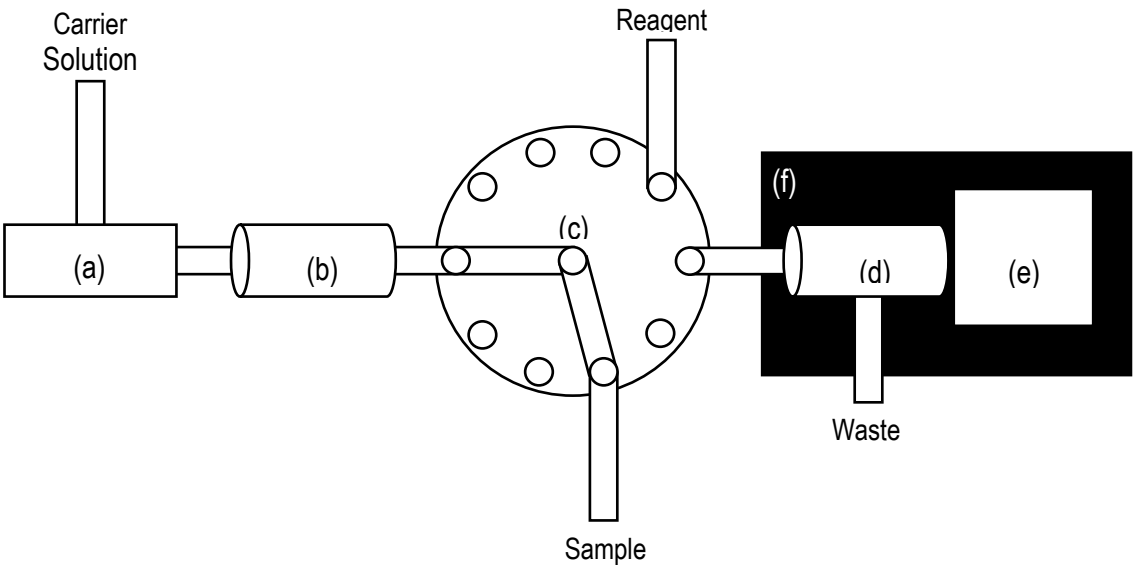


Figure 1-11: Schematic of SIA-CL manifold comprising of (a) pump, (b) holding coil, (c) selection valve, (d) reaction coil, (e) photomultiplier tube, and (f) lightproof housing.

1.4.3 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay is a colourimetric wet chemistry technique that utilises alcoholic DPPH solutions titrated with a liquid analyte to determine the radical scavenging activity of the analyte. DPPH is a stable free radical with a single delocalised electron (shown in Figure 1-12 attached to the nitrogen 'bridge') that gives rise to a deep purple colour with a localised absorbance maximum at 517nm. When a DPPH radical is reacted with a hydrogen-donating species (typically antioxidants), DPPH is reduced and becomes colourless. DPPH is typically solubilised in alcohols (usually methanol or ethanol) prior to titration with the analyte. This mixture is allowed to react for a significant amount of time (typically up to an hour) at ambient temperatures before analysis. This offers some advantages in sensitivity over similar antioxidant-measuring assays such as chemiluminescence as the DPPH redox reaction is irreversible and, given sufficient time, will react even with weak antioxidants. Conversely, CL is extremely rapid and suitable for quick analyses; however, the limited reagent contact and analysis times may not allow for reaction with weaker antioxidants. DPPH may also be utilised for the analysis of lipophilic antioxidants (such as tocopherols and carotenoids[151]) as the alcoholic solvents can solubilise lipophilic compounds to a greater extent than water-based assays. DPPH assays have been widely used to determine the radical scavenging activity of *C. officinalis* extracts [21, 100, 152-154].

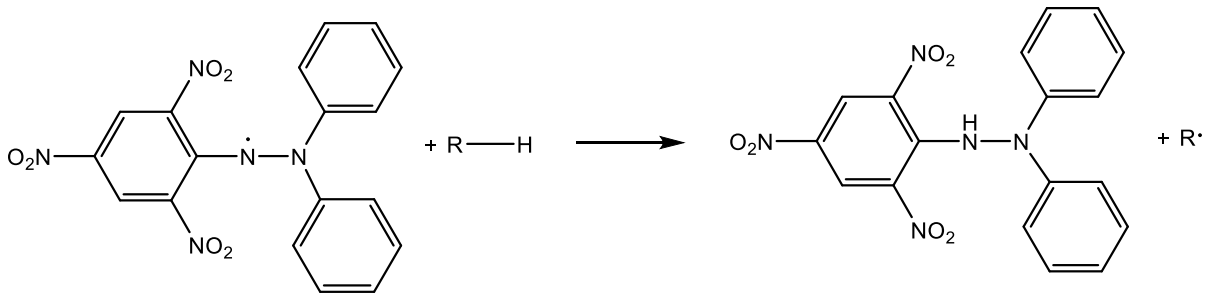


Figure 1-12: Reaction of DPPH molecule with a hydrogen-donor.

1.4.4 Folin-Ciocalteu Total Phenolic Content Assay

Folin-Ciocalteau's (FC) reagent is an acidic mixture of tungstate and molybdate (VI) complexes and other reagents such as lithium salts. When titrated with a phenolic analyte under basic conditions, a single electron transfer occurs between the deprotonated phenolic functionality and the Mo(VI) complex, reducing the Mo(VI) to Mo(V) and resulting in the formation of a phosphotungstic-phosphomolybdic complex with a deep blue chromophore that exhibits a local absorbance maximum at 765 nm [155]. The structure and underlying chemistry of this complex is not fully understood [156]. Although originally developed for the measurement of compounds that contain phenolic moieties, FC reagent will also react with metals, carbonyls, and radicals [157]. Folin-Ciocalteu assays have previously been used for the determination of the total phenolic content of *C. officinalis* extracts [21, 152].

1.4.5 Statistical Testing

T-tests are statistical tests used to determine if two samples are significantly different. The null hypothesis for this test in that there is no difference between two samples. Rejection or acceptance is determined by comparison between a t-score determined using the means and variances of the two populations and a t-threshold determined from the degrees of freedom and confidence (typically 95%) between the samples [158]. A secondary statistical test is typically carried out prior to T-testing with a view to determining the variance of samples prior to T-testing. If the sample sets display equal variance, then Student's T-test is used. If unequal sample set variance is observed, then Welch's T-test is used. Independent T-testing is used when determining if two independent sample sets are significantly different.

Certain assumptions are made in order to use Student's T-test. First, the scale of measurement is identical for both data sets. Second, the relationship between the two data sets must be linear and continuous (or ordinal). Third, the sample set is normally distributed and representative of the population

being tested. Additionally, Student's T-test is best used for large data sets ($n>5$); however, T-testing is still applicable for smaller data sets ($n\leq5$) if a large difference is observed [159].

1.5 CONCLUSION AND PROJECT PREMISE

In view of the complications presented by the use of hazardous solvents and energy-consumptive processes in the extraction of metabolites from plants, there is a need for alternative green solvent systems that operate at room temperature and extract equivalent or greater levels of plant metabolites when compared with current solvent systems are desirable.

Thus, the overarching aim of this project was to develop a suite of non-hazardous, environmentally friendly and sustainable solvent mixtures and test their extractive ability with a model plant material, namely the flowerheads from *Calendula officinalis*. A secondary aim was to achieve an understanding of physical and chemical factors influencing the extraction efficiency and to use this to inform the design of new solvent mixtures.

The sub aims were as follows:

1. Chapter 4 investigated the potential of alternative solvents including aqueous acids and bases, supercritical fluids, and natural deep eutectic solvents to produce crude extracts of equivalent phytochemical composition, antioxidant activity, and radical scavenging activity to current hydroethanolic solvents used in industrial applications.

2. Chapter 5 investigated the effect of altering the chaotropicity and dielectric constant of glycerolic solvents upon extract composition, antioxidant activity, and radical scavenging activity with a view to increasing phytochemical extraction when compared to neat glycerolic extractions.

3. Chapter 6 investigated the potential of highly chaotropic surfactants in glycerolic and aqueous extractions to produce crude extracts of equivalent or greater phytochemical composition, antioxidant activity, and radical scavenging activity to neat glycerolic or aqueous extractions.

4. Chapter 7 investigated the potential of acidified aqueous polysorbate extractions to produce crude extracts of equivalent or greater phytochemical composition, antioxidant activity, and radical scavenging activity to current aqueous ethanol solvents used in industrial applications

2 GENERAL METHODS

2.1 GENERAL REAGENTS

DPPH (≤100%) and potassium permanganate (>99%) were purchased from Sigma-Aldrich (St Louis, MO, USA). Sodium hexametaphosphate was purchased from British Drug House Laboratories (Poole, UK). Sulfuric acid (95-97%) was purchased from Merck (Kilsyth, VIC, AUS). Methanol (99.96%), and Folin-Ciocalteau reagent (2 N) were purchased from Chem-Supply (Gillman, SA, AUS). Sterile filters (0.22 µm, PTFE), and disposable UV-Vis cuvettes (3.5 mL, polycarbonate) were purchased from Sarstedt (Nümbrecht, DE). UHPLC solvent filters (0.22 µm, nylon) were purchased from AdeLab Scientific (Thebarton, SA, AUS). Ultrapure water (Optima® LC-MS), formic acid (Optima® LC-MS), and methanol (Optima® LC-MS) were purchased from Fisher Scientific (Waltham, MA, USA). Sodium Carbonate (99.5%) was purchased from Ajax Chemical (Bulimba, QLD, AUS). Deionised water was purified to 18 MΩ using a Thermo Fisher Scientific (Waltham, MA, USA) Barnstead™ E-Pure™ water system.

2.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

Three LC systems were used in this research. An Agilent Technologies 1100 Series HPLC system (Mulgrave, VIC, AUS) equipped with a two-line Flow Injection Analysis (FIA) manifold was utilised for the determination of antioxidant phytochemicals from *C. officinalis* detailed in Chapter 3. A Perkin Elmer Flexar-10 UHPLC system (Waltham, MA, USA) equipped with diode array detection (DAD) was utilised for the analysis of extracts detailed in Chapter 4. A Thermo Vanquish UHPLC system (Waltham, MA, USA) equipped with DAD was utilised for the analysis of extracts prepared in Chapters 3, 5, 6, and 7.

Hewlett Packard 1100 LC Series. A Hewlett Packard 1100 LC Series HPLC system (Agilent Technologies, Mulgrave, VIC, AUS) equipped with a Flow Injection Analysis (FIA) manifold was utilised as detailed by Anastos *et al.* [160]. As detailed in the manuscript, "*The mixed reagent system used a Hewlett Packard 1100 series liquid chromatograph (Agilent Technologies) equipped with a Hewlett Packard analogue digital interface box (Agilent Technologies) for analogue input from the chemiluminescence detector, based on that of Lenehan* et al*.... …Control of the HPLC pump, UV detection at 269 nm, and data acquisition from the chemiluminescence detector were achieved using Hewlett Packard Chemstation Software (Agilent Technologies). A flow rate of 0.5 mL/min was employed with a run time of 5 min. The column eluate and chemiluminescence reagent were merged at a T-piece in front of a spiral flow cell comprising 0.5 mm i.d. PTFE mounted flush against a photomultiplier tube (THORN-EMI 9924BS, ETP Ltd., Salsbury, Australia) which was operated at 900 V, using a stable power*

supply (THORN-EMI Model PM28BN) via a voltage divider supply (Thorn EMI Model C611, ETP Ltd.) which monitored the resultant emission. The flow cell and photomultiplier tube were enclosed in a light tight housing. Delivery of the postcolumn reagent was achieved using a Gilson Minipuls 3 peristaltic pump (John Morris, Chatswood, Australia) with PVC pump tubing (1.85 mm i.d., A.I. Scientific, Clontarf, Australia) to propel both reagent streams at a flow rate of 2.5 mL/min." [160].

Filtered (0.22 µm, PTFE) extracts were injected (10 µL) onto a Zorbax Eclipse C18 reverse phase column (4.6 x 150 mm, 5 µm) and eluted using the gradient flow detailed in Table 2-1. Mobile phase A was 0.1% formic acid in ultrapure water. Mobile phase B was ultrapure methanol. $KMnO_4$ solution was prepared according to the method outlined in Chapter 2.3. Mobile phases were filtered prior to use. UV-Vis data was collected at 254 nm. After each analysis, the UHPLC system was allowed to equilibrate for 10 minutes at initial gradient conditions (step 0).

Table 2-1: Hewlett Packard 1100 LC Series run conditions

Step Number	*Step Type*	*Step Time (min)*	*%A*	*%B*
0	Equilibration	10	100	0
1	Run	50	0	100
2	Run	5	100	0
3	Run	5	100	0

Perkin Elmer Flexar FX-10. A Perkin Elmer Flexar-10 UHPLC system (Waltham, MA, USA) equipped with DAD was utilised. Filtered (0.22 µm, PTFE) extracts were injected (5 µL) onto a Perkin Elmer phenyl-hexyl reverse phase column (2.1 x 100 mm, 2.7 µm) and eluted using the gradient flow detailed in Table 2-2. Mobile phase A was 0.1% v/v formic acid in ultrapure water. Mobile phase B was ultrapure methanol. Mobile phases were filtered prior to use. UV-Vis data was collected from 200-400 nm. Detection was by UV-Vis absorbance at 254 nm. After each analysis, the UHPLC system was allowed to equilibrate for 10 minutes at initial gradient conditions (step 0).

Table 2-2: Perkin Elmer Flexar FX-10 UHPLC run conditions

Step Number	*Step Type*	*Step Time (min)*	*%A*	*%B*
0	Equilibration	10	95	5
1	Run	0.1	95	5
2	Run	4.9	90	10
3	Run	15	20	80
4	Run	1	0	100
5	Run	4	0	100

Thermo Vanquish. A Thermo Vanquish UHPLC system (Waltham, MA, USA) equipped with DAD was utilised. Filtered (0.22 µm, PTFE) extracts were injected (5 µL) onto a Perkin Elmer phenyl-hexyl reverse phase column (2.1 x 100mm, 2.7 µm) and eluted using the gradient flow detailed in Table 2-3 . Mobile phase A was 0.1% formic acid in ultrapure water. Mobile phase B was ultrapure methanol. UV-Vis data was collected from 200-400 nm. Detection was by UV-Vis absorbance at 254 nm. After each analysis, the UHPLC system was allowed to equilibrate for 10 minutes at initial gradient conditions (steps 0 & 4).

Table 2-3: Thermo Vanquish UHPLC run conditions

Step Number	***Step Type***	***Step Time (min)***	***%A***	***%B***
0	Equilibration	5	95	5
1	Run	15	0	100
2	Run	5	0	100
3	Run	2	95	5
4	Run	5	95	5

2.3 SEQUENTIAL INJECTION ANALYSIS CHEMILUMINESCENCE.

The SIA manifold setup and reagent chemistry used was developed within Flinders University and is reported by Hughes *et al*. [133]. As stated in the manuscript, *"The SIA manifold consisted of a bi-directional MilliGAT™ pump (Global FIA, Fox Island, WA, USA) and a Valco 10-port multi-position valve (Global FIA, Fox Island, WA, USA) operating synchronously. Reagents and samples were connected to the multi-position valve by polytetrafluoroethylene (PTFE) tubing (ID 0.5 mm) (Global FIA, Fox Island, WA, USA) and aspirated sequentially by the pump into a holding coil (400 µL, PTFE tubing). The pump direction was switched, sending reagents and samples into a clear PTFE reaction coil (ID 0.76 mm) (Pro Tech Group, Coolum, Australia) placed flush against the window of a photomultiplier tube (PMT) (Electron Tubes Limited, type 9828SB, Uxbridge, UK). The PMT was operated at 800 V by a modular power supply (Electron Tubes Limited, PS1800/12F, Uxbridge, UK). The PMT, modular power supply and the reaction coil were all contained inside a custom-built lightproof housing. Data acquisition from the PMT was achieved using a LabJack U12 (Lakewood, CO, USA) data acquisition module using a differential 12-bit analogue input. The SIA-CL instrument was controlled by software written in-house using National Instruments LabVIEW® version 8.2 (Austin, TX, USA)."* [133]

Sodium hexametaphosphate was dissolved in deionised water to 1% w/v and acidified using sulfuric acid (conc.) to pH 2.25. Potassium permanganate ($5x10^{-4}$ M) was dissolved in sodium hexametaphosphate

(1% w/v, pH 2.25). Sample (5 µL) was sandwiched between aliquots (100 µL) of sodium hexametaphosphate solution in order to prevent chemiluminescence reactions occurring with the permanganate carrier solution outside of the reaction coil. Sample and reagent lines were flushed with respective solutions prior to analysis in order to minimise carryover. Flow rates are detailed in Table 2-4. Assays were performed in quintuplicate.

Table 2-4: Flow rates used for determination of total antioxidant activity of C. officinalis *extracts*

Step	*Solution*	*Volume (µL)*	*Flow Rate (µL.s^{-1})*	*Flow Direction*
1	Sodium hexametaphosphate	100	10	Reverse
2	Sample	5	1	Reverse
3	Sodium hexametaphosphate	100	10	Reverse
4	Potassium permanganate	700	100	Forward

2.4 2,2-DIPHENYL-1-PICRYLHYDRAZYL RADICAL SCAVENGING ASSAY.

A Thermo Scientific Evolution Array UV-Vis spectrometer (Waltham, MA, USA) was utilised for the analysis of all extracts. The DPPH assay used in this experiment was adapted from Politeo *et al.* [161]. DPPH stock solution (6x10^{-5} M) was prepared by dissolving DPPH in methanol. Sample (200 µL) was added to a polycarbonate UV/Vis cuvette. DPPH solution (2 mL) was added and analysed using UV/Vis at 517 nm after 60 minutes. Inhibition of the DPPH radical was calculated with Equation 2-1.

$$\%\text{Inhibition} = \left(\frac{\text{Abs}_{\text{Blank}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Blank}}}\right) \times 100$$

Equation 2-1: Equation for calculating % inhibition of DPPH radical where Abs_{Blank} and Abs_{Sample} are the absorbances of the matrix blank and sample respectively.

2.5 FOLIN-CIOCALTEU'S TOTAL PHENOLIC ASSAY.

A Thermo Scientific Evolution Array UV-Vis spectrometer (Waltham, MA, USA) was utilised for the analysis of all extracts. The Folin-Ciocalteau assay used was adapted from Ercetin *et al.* [162]. Sodium carbonate solution (10% w/v) was prepared by dissolving sodium carbonate in deionised water. An aliquot of extract (200 µL) was added to a disposable UV cuvette. Folin-Ciocalteau reagent (200 µL) was then added and diluted with deionised water (1 mL). An aliquot of Na_2CO_3 solution (600 µL) was then added with a subsequent aliquot of deionised water (500 µL). The resulting mixture was left for 60 minutes prior to analysis. Analyses were performed in triplicate.

2.6 STUDENT'S T-TEST AND UNSCRAMBLER.

Sample variance was determined with F-testing. Independent Student's T-testing ($p<0.05$) was used to determine statistical significance of extracts where multiple samples were produced with equal variance between sample sets. Independent Welch's T-testing ($p<0.05$) was used to determine statistical significance of extracts where multiple samples were produced with unequal variance between sample sets. Pearson's correlation values were determined using The Unscrambler™ software (Magnolia, TX, USA) and classified according to a guide by Evans [163].

3 ADAPTATION OF INDUSTRY METHOD

3.1 CHAPTER SYNOPSIS

This chapter presents method development for adapting a standard industrial percolation method for the preparation of extracts using dried *C. officinalis* flower heads to a laboratory-scale extraction process. Dried *C. officinalis* flowers were used as a model plant material due to their high content of antioxidant and polyphenolic phytochemicals. The chemiluminescence response of individual compounds was determined with HPLC-CL. Total phytochemical content and individual phytochemical concentrations were determined by UHPLC-DAD analysis. Total antioxidant activity, radical scavenging activity, and total phenolic content were determined by $KMnO_4$ chemiluminescence, DPPH, and Folin-Ciocalteu assays respectively. Hydroethanolic extracts were prepared and it was found that a number of extracted phytochemicals that demonstrated UV/Vis absorbance at 254 nm exhibited antioxidant activity. Dried *C. officinalis* flower heads were ground using a commercial blender in order to maintain sample homogeneity during laboratory-scale extractions, and it was found that the process of grinding did not significantly affect the phytochemical content, radical scavenging activity, chemiluminescence response (as a measure of antioxidant activity), or phenolic content of hydroethanolic extracts when compared with extracts prepared with whole flowers. An ultrasonic extraction method for the extraction of phytochemicals with high-viscosity solvents was developed, and it was found that the ultrasonication process did not significantly affect the phytochemical content, radical scavenging activity, chemiluminescence response, or phenolic content when compared with hydroethanolic extracts prepared with a maceration extraction process.

3.2 INTRODUCTION

C. officinalis extracts are highly desirable in traditional medicine and in cosmetic applications due to their high level of antioxidant activity. In particular, many of the flavonoid phytochemicals present in *C. officinalis* exhibit antioxidative effects. For example, the flavonoids rutin, isorhamnetin, and thymol found in *C. officinalis* demonstrate antioxidant activity [164-166]. Determination of the antioxidant-active metabolites in *C. officinalis* is therefore desirable in order to tailor extraction approaches with a view to maximising extraction of these phytochemicals.

Typically, *C. officinalis* extracts are prepared on an industrial scale by percolating solvent through a packed bed of dried whole flower heads. A typical industrial percolation extraction can use pumping

equipment to percolate large solvent volumes (typically litres) through a large quantity of sample material (typically kilograms) over a prolonged period of time (typically hours or days) until the solvent has reached a phytochemical concentration equilibrium with the flower material. In contrast, a laboratory scale extraction uses substantially smaller quantities of solvent (typically millilitres) and plant material (typically milligrams). This reduction in the quantities of solvent and flower material poses problems with extract homogeneity due to the size of whole *C. officinalis* flower heads. Grinding dried plant material was posed as a means of maintaining sample homogeneity in laboratory scale extraction processes. The process of grinding sample material offers both advantages and disadvantages; the higher surface area and smaller particle size can offer increased speed of extraction due to the increased surface area and decreased mean diffusion path length for phytochemicals to enter the bulk solvents. However, the process of grinding can generate of heat in the sample material, which may result in the thermal degradation of some thermally sensitive compounds. Additionally, the increase in surface area which can assist in phytochemical extraction also increases the surface area at which phytochemical degradation can occur through oxidative and photodegradative mechanisms when exposed to air and light.

In Chapters 5, 6, and 7, high-viscosity solvents are utilised for extraction. The Stokes-Einstein equation (Equation 3-1) demonstrates that diffusivity that results from the Brownian motion of a particle through a solvent is directly proportional to temperature and inversely proportional to its viscosity [167]. High viscosity therefore limits diffusion, resulting in the non-viability of techniques such as maceration, infusion, or percolation that rely on diffusion as the primary means of phytochemical extraction. Ultrasonic extraction has previously been shown to increase the diffusion of phytochemicals through rupturing of cell membranes and improved mass transfer resulting in minimised saturation of local solvent [168]. Cavitation effects that occur within an ultrasonically treated fluid cause localised high pressures and temperatures [169] which, over time, will result in a moderate increase in bulk solvent temperature and thereby result in reduced solvent viscosity [167]. However, the local high temperatures that result from cavitation induce thermal decomposition of water in aqueous systems to produce hydrogen and hydroxyl radicals [170]. Degradation of phytochemicals has also been observed in non-aqueous systems [171]. This can result in degradation of antioxidant and radical scavenging phytochemicals in aqueous *C. officinalis* extracts. Thus, there is a trade-off between the increased phytochemical extraction observed in ultrasonic phytochemical extraction and the degradation of phytochemicals through cavitation-induced radical formation.

$$d = \frac{kT}{3\pi\eta D}$$

Equation 3-1: Stokes-Einstein equation where d =hydrodynamic diameter (m), k = Boltzmann constant ($kg.m^2.s^{-2}.K^{-1}$), T = temperature (K), η = solvent viscosity ($kg.m^{-1}.s^{-1}$), D = diffusion coefficient ($m^2.s^{-1}$)

This chapter presents method development for adapting a standard industrial percolation method for the preparation of extracts using dried *C. officinalis* flower heads to a laboratory-scale extraction process. The antioxidant activity of different phytochemicals extracted from *C. officinalis* was investigated in order to identify desirable phytochemicals for extraction in subsequent chapters. The effect of reducing the particle size of the plant material in order to maintain sample homogeneity during laboratory-scale extractions was investigated, and it was hypothesised that the reduction in particle size may increase the extraction of metabolites from *C. officinalis* through reduction of the mean path length. An ultrasonic extraction method was tested for use with high viscosity solvents, and it was hypothesised that ultrasonic extraction processes would be a suitable replacement for maceration extraction processes.

3.3 EXPERIMENTAL

Calendula officinalis samples. Dried *C. officinalis* flower heads were used as supplied. Flowers were grown in the Adelaide Hills, South Australia on a certified biodynamic farm (Jurlique International, certified under the National Association for Sustainable Agriculture, Australia), hand-picked and air-dried in sheds. Dried *C. officinalis* was stored in sealed plastic containers in darkness at ambient conditions prior to use.

Chemicals and Reagents. Ethanol (AR grade) was purchased from Chem-Supply (Gillman, SA, AUS). Centrifuge vials (50 mL, polypropylene) were purchased from Sarstedt (Nümbrecht, DE). Filter paper (cellulose) was purchased from Advantec (Dublin, SA, USA). Deionised water was purified to 18 MΩ using a Thermo Fisher Scientific (Waltham, MA, USA) Barnstead™ E-Pure™ water system.

Preparation of *C. officinalis* extracts for HPLC-CL analysis. Hydroethanolic solvent was prepared by titrating ethanol with deionised water to 30% v/v. Extracts were prepared by macerating *C. officinalis* flower material (1 g) with hydroethanolic solvent (10 g) for 120 minutes before filtration. Samples were filtered prior to HPLC-CL analysis. Process blanks were prepared and analysed concurrently using identical techniques.

Comparison of whole and ground flower material. Hydroethanolic solvent was prepared by titrating ethanol with deionised water to a final concentration of 30% v/v ethanol/water. Unground extracts were prepared by macerating *C. officinalis* flower material (1 g) with hydroethanolic solvent (10 g) for 120 minutes before filtration. Ground extracts were prepared by macerating *C. officinalis* material (0.15 g) with 30% v/v ethanol/water (3 g) for 120 minutes before filtration. Extracts were prepared in triplicate. Process blanks were prepared and analysed concurrently using identical techniques.

Comparison of ultrasonic and maceration extraction methods. Hydroethanolic solvent was prepared by titrating ethanol with deionised water to a final concentration of 30% v/v ethanol/water. Extracts were prepared by combining *C. officinalis* material (0.15 g) with hydroethanolic solvent (3 g). Ultrasonic extracts were prepared by ultrasonication (Elmasonic S30, 120 min). Macerated extracts were prepared by maceration (65 ^{O}C, 120 mins) with stirring to mimic the higher temperature observed in ultrasonic extraction. Resulting extracts were filtered and stored in darkness under ambient conditions until analysed. Extracts were prepared in triplicate. Process blanks were prepared and analysed concurrently using identical techniques.

UHPLC Analysis. HPLC-CL analyses were conducted on a Hewlett Packard 1100 LC Series using the method detailed in Chapter 2.2. UHPLC analyses were conducted on a Thermo Vanquish UHPLC using the method detailed in Chapter 2.2.

Chemiluminescence response. SIA-CL assays were conducted using the method detailed in Chapter 2.3. A reference standard of methanolic quercetin (25 μM) was run concurrently with extracts prepare with ultrasonic and maceration extraction processes.

Radical Scavenging Activity. DPPH assays were conducted using the method detailed in Chapter 2.4.

Phenolic Content. Folin-Ciocalteu assays were conducted using the method detailed in Chapter 2.5.

Statistical Testing. F-testing and T-testing to determine statistical significance was conducted using the method outlined in Chapter 2.6.

3.4 Results & Discussion

3.4.1 Identification of peaks

Individual chemiluminescence detection. HPLC-CL analyses were conducted with a view to determining phytochemicals in hydroethanolic *C. officinalis* extracts that exhibited antioxidant activity. Figure 3-1 shows the phytochemical composition of a hydroethanolic *C. officinalis* extract with subsequent CL responses for individual compounds. It can be seen that there are significantly more CL peaks present compared with the number of UV active peaks; this indicates that there are a number of antioxidant compounds extracted that do not exhibit UV absorbance. Peaks at 1.7, 13.5, 21.8, 22.8, 24.2, 24.7, 28.4, and 34.5 minutes demonstrated CL activity and good HPLC resolution. These were labelled Peaks A-H respectively.

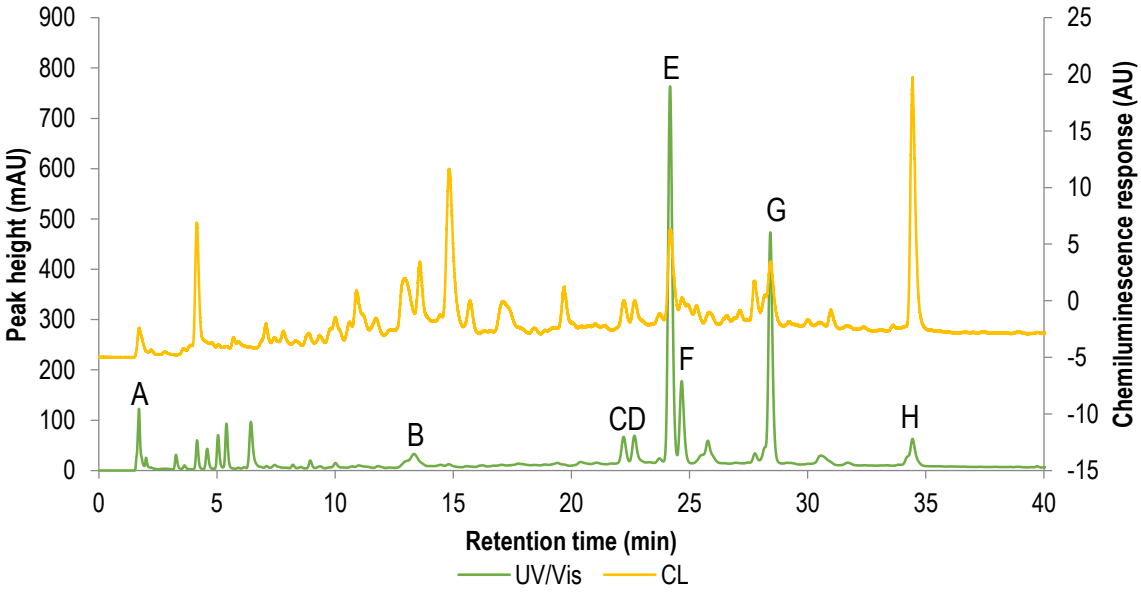


Figure 3-1: HPLC-CL chromatogram of a hydroethanolic C. officinalis *extract.*

3.4.2 Comparison between whole and ground flowers.

Individual Peak Analysis. UHPLC analyses were conducted with a view to determining the individual peak heights of phytochemicals present in *C. officinalis* extracts prepared with whole and ground flowers. Peaks at 0.8, 8.5, 11.05, 11.2, 11.5, 11.9, 12.8, and 13.3 minutes were selected for further analysis due to clear peak resolution and antioxidant activity determined in Chapter 3.4.1 and labelled A-H respectively. Figure 3-2 shows the heights of peaks A-H in *C. officinalis* extracts prepared with whole and ground flowers. It can be seen that although some variation in phytochemical content can be observed, there was no significant difference in peak height between extracts prepared with whole or ground flower material. This result indicates that the process of reducing flower material does not induce thermal degradation, and does not result in increased extraction due to shorter diffusion pathways, thereby producing an extract of equivalent phytochemical composition to an extract prepared with whole flowers.

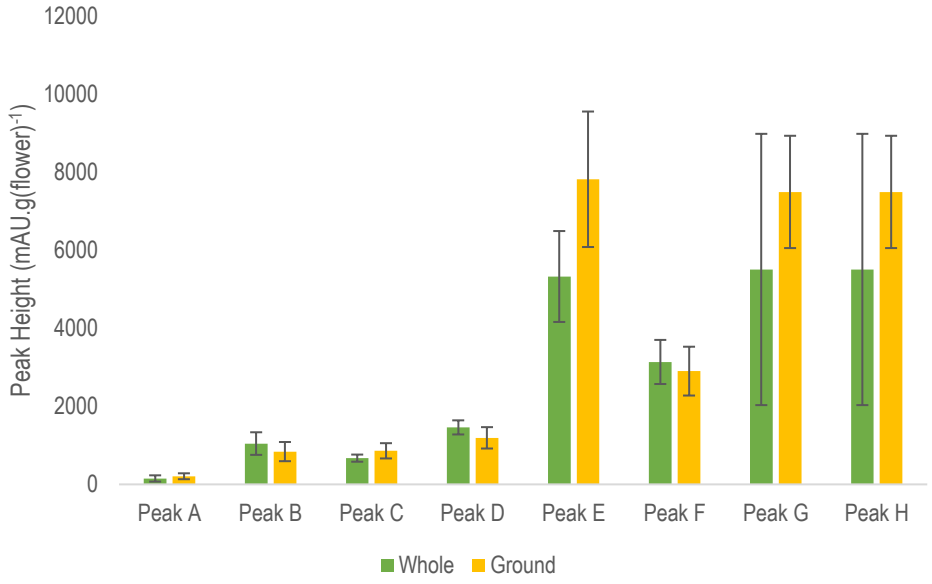


Figure 3-2: Heights of peaks A-H in hydroethanolic C. officinalis *extracts prepared with whole and ground flowers. Data is presented as means ± standard deviations of replicates (n=3). Asterisks indicate statistically significant differences (p<0.05) in extracts prepared with ground flower extracts compared with whole flower extracts.*

The total peak area of UHPLC chromatograms of hydroethanolic *C. officinalis* extracts prepared with whole and ground flowers was calculated. Figure 3-3 shows the total peak area of hydroethanolic *C. officinalis* extracts prepared with whole and ground flowers. It can be seen that although extracts prepared with ground flowers exhibit an apparent increase in total peak area when compared with extracts

prepared with whole flowers, this was not determined to be significant ($p<0.05$). Results from individual peak analyses and comparison of total peak areas indicate that that the process of grinding the flower material does not result in phytochemical degradation, and may result in a slight increase in phytochemical extraction due to the increased surface area and decreased mean diffusion path length in the plant material.

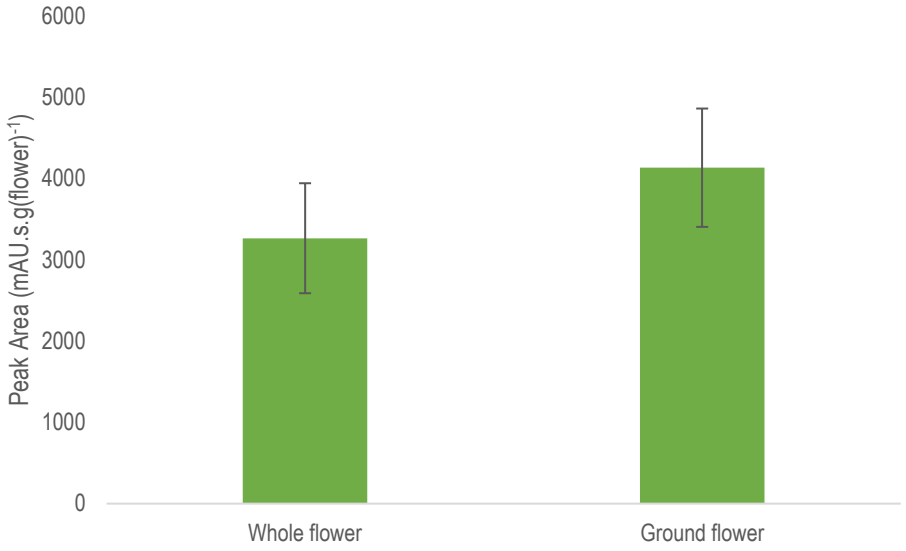


Figure 3-3: Total peak areas of UHPLC chromatograms of hydroethanolic C. officinalis *extracts prepared with whole and ground flowers. Data is presented as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) of ground flower extracts compared with whole flower extracts.*

Chemiluminescence response. SIA-CL assays were conducted with a view to determining the antioxidant activity of hydroethanolic *C. officinalis* extracts prepared with whole and ground flowers. Figure 3-4 shows the chemiluminescence response of hydroethanolic *C. officinalis* extracts prepared with whole and ground flowers. It can be seen that although extracts prepared with ground flowers exhibit an apparent increase in chemiluminescence when compared with extracts prepared from whole flowers, this was not found to be significant ($p<0.05$). This indicates that the process of grinding *C. officinalis* flowers does not negatively impact on the antioxidant activity of the extract.

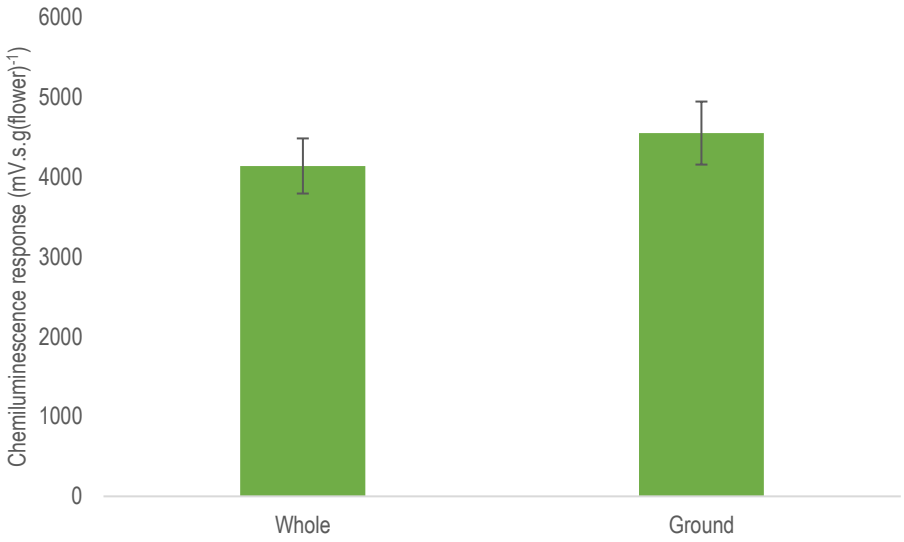


Figure 3-4: Chemiluminescence response of hydroethanolic C. officinalis *extracts prepared with whole and ground flowers. Data is presented as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) of ground flower extracts compared with whole flower extracts.*

Radical Scavenging Activity. DPPH analyses were conducted with a view to determining the radical scavenging activity of hydroethanolic *C. officinalis* extracts prepared with whole and ground flowers. Figure 3-5 shows the ability of hydroethanolic *C. officinalis* extracts to scavenge stable DPPH radicals. It can be seen that extracts prepared with ground flowers exhibit an apparent increase in radical scavenging activity when compared with extracts prepared with whole flowers. However, this increase was not found to be significant ($p<0.05$). This result indicates that the process of grinding *C. officinalis* flowers does not negatively impact on the radical scavenging ability of the extract.

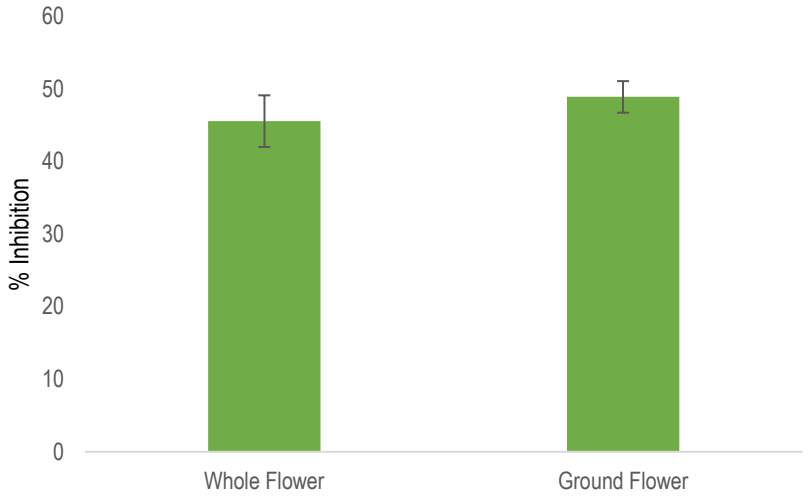


Figure 3-5: Radical scavenging activity of hydroethanolic C. officinalis *extracts prepared with whole and ground flowers. Data is presented as means ± standard deviations of replicates (n=9). Asterisks indicate statistical significance (p<0.05) of ground flower extracts compared with whole flower extracts.*

Phenolic Content. Folin-Ciocalteu assays were conducted with a view to determining the total phenolic content of hydroethanolic *C. officinalis* extracts prepared from whole and ground flower material. Figure 3-6 shows the phenolic content of *C. officinalis* extracts prepared with whole and ground flower material. It can be seen that extracts prepared with ground flower material exhibit significantly higher phenolic content when compared with extracts prepared with whole flowers under similar extraction conditions (1.7646 ± 0.13599 mg(GAE).g(flower)$^{-1}$ and 2.3240 ± 0.035215 mg(GAE).g(flower)$^{-1}$ for whole and ground flower material respectively) . This indicates that the use of ground flower material in the preparation of hydroethanolic extracts does not result in a decrease in phenol content; indeed, the increased surface area and decreased mean diffusion path result in increased phenolic extraction.

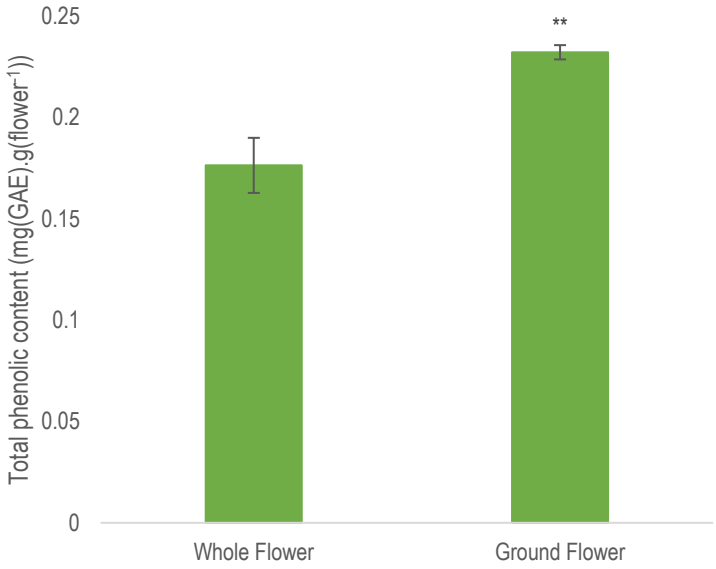


Figure 3-6: Phenolic content of hydroethanolic C. officinalis *extracts prepared with whole and ground flowers. Data is presented as means ± standard deviations of replicates (n=3). Asterisks indicate statistically significant (p<0.05) differences in extracts prepared with ground flower compared with whole flower extracts.*

3.4.3 Comparison between ultrasonic and maceration extraction methods.

Individual Peak Analysis. UHPLC analyses were conducted with a view to determining the heights of selected peaks in hydroethanolic *C. officinalis* extracts prepared using maceration and ultrasonication extraction methods. It can be seen from Figure 3-7 that extracts prepared with ultrasonic and maceration extraction methods exhibit identical phytochemical compositions, indicating that the use of ultrasonic extraction methods do not result in metabolite degradation. Peaks were selected at 0.8, 8.5, 11.1, 11.2, 11.5, 11.7, 12, 12.75, and 13.3 minutes due to clear peak resolution and antioxidant activity determined in Chapter 3.4.1 and labelled A-H respectively (Figure 3-7). Figure 3-8 shows the heights of peaks A-H in hydroethanolic *C. officinalis* extracts prepared with maceration and ultrasonication extraction methods. This result suggests that extracts prepared with an ultrasonic extraction method produces an extract with equivalent phytochemical concentrations to an extract prepared using a maceration extraction method. The slight apparent decrease in metabolite concentration in ultrasonic extracts may be due to the extended extraction time causing phytochemical degradation; Qiao *et al.* [172] showed that quercetin found in citrus degrades during ultrasonication. It is not unreasonable to suggest that degradation of quercetin-based flavonoids may be occurring during ultrasonic extraction.

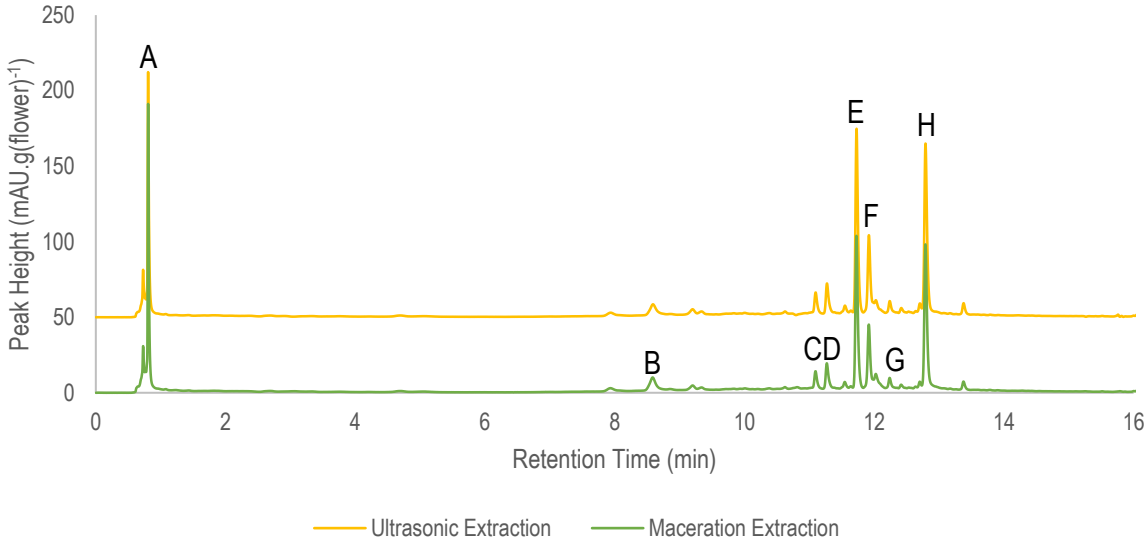


Figure 3-7: UHPLC chromatogram of C. officinalis *extracts prepared with ultrasonic and maceration extraction methods. Sequential chromatograms are offset vertically by 50 mAU.g(flower)$^{-1}$.*

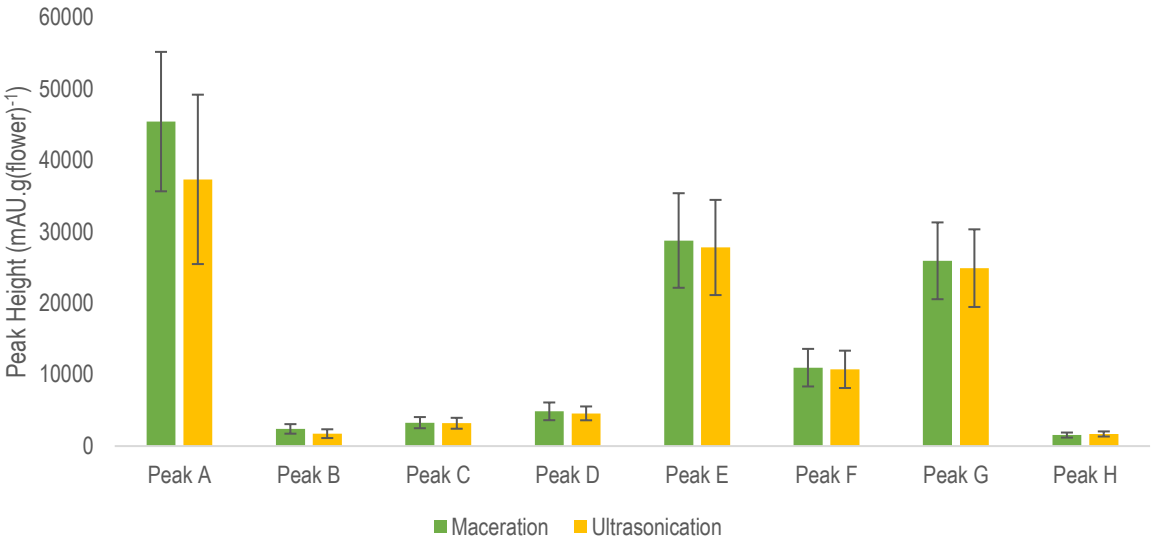


Figure 3-8: Heights of peaks A-H in hydroethanolic C. officinalis *extracts prepared with maceration and ultrasonication extraction methods. Data is presented as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) of ground flower extracts compared with whole flower extracts.*

The total peak areas of UHPLC chromatograms of hydroethanolic *C. officinalis* extracts prepared with ultrasonic and maceration extraction methods were calculated. Figure 3-9 shows the total peak area of hydroethanolic *C. officinalis* extracts prepared with whole and ground flowers. It can be seen that extracts prepared with ground flowers exhibit an apparent increase in total peak area when compared with extracts prepared with whole flowers; however, this was not determined to be significant ($p<0.05$). This result indicates that the process of grinding the flower material does not result in phytochemical degradation.

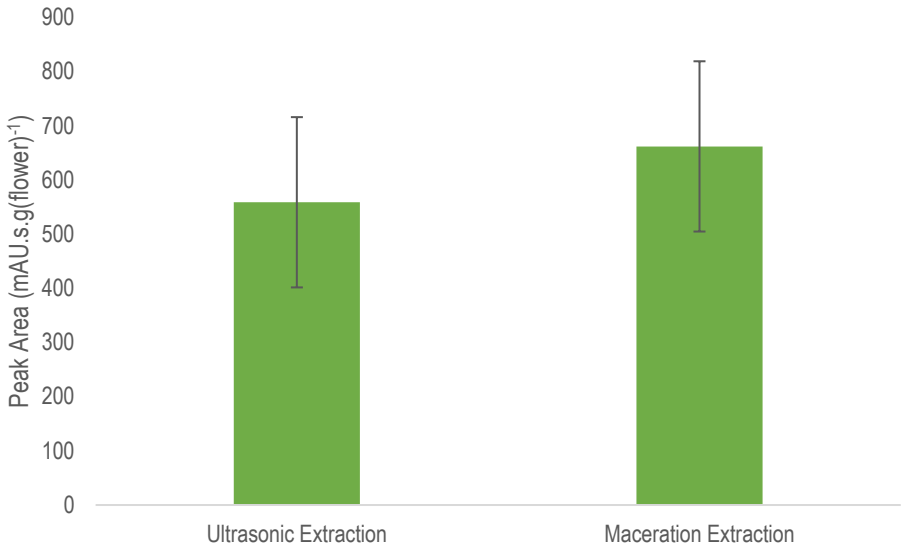


Figure 3-9: Total peak areas of C. officinalis *extracts prepared with ultrasonic and maceration extraction processes. Data is presented as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) of ground flower extracts compared with whole flower extracts.*

Chemiluminescence response. SIA-CL assays were conducted with a view to determining the antioxidant activity of hydroethanolic *C. officinalis* extracts prepared using ultrasonic and maceration extraction methods. Figure 3-10 shows the chemiluminescence response of hydroethanolic *C. officinalis* extracts prepared using ultrasonic and maceration extraction methods. It can be seen that although extracts prepared with maceration extraction exhibit an apparent increase in chemiluminescence when compared with extracts prepared using maceration extraction methods; however, this was not found to be significant ($p<0.05$). This indicates that utilisation of ultrasonic extraction processes do not result in a loss of antioxidant activity in hydroethanolic *C. officinalis* extracts.

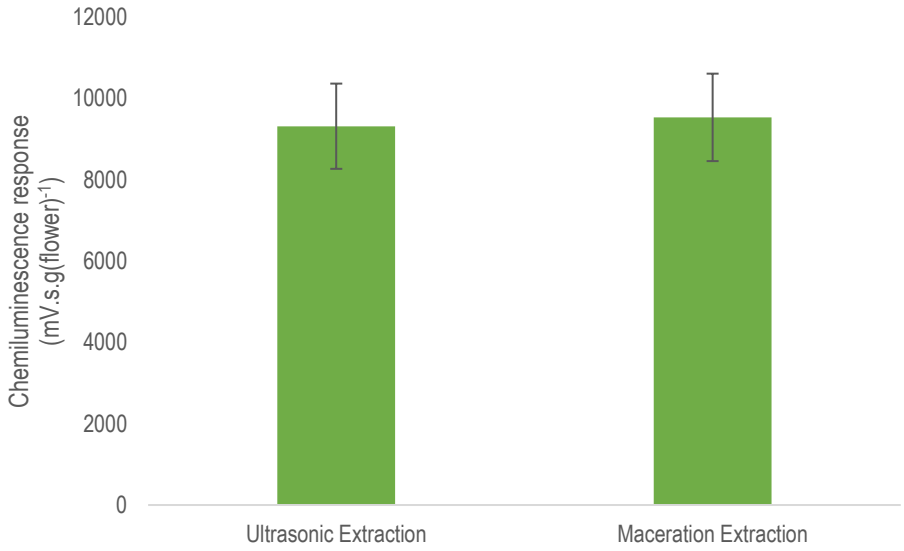


Figure 3-10: Chemiluminescence response of hydroethanolic C. officinalis *extracts prepared with ultrasonic and maceration extraction processes. Data is presented as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance ($p<0.05$) of ultrasonic extraction methods when compared with maceration extraction methods.*

Radical Scavenging Activity. DPPH assays were conducted with a view to determining the radical scavenging activity of hydroethanolic *C. officinalis* extracts prepared with maceration and ultrasonication extraction methods. Figure 3-11 shows the ability of hydroethanolic *C. officinalis* extracts to scavenge stable DPPH radicals. It can be seen that the radical scavenging activity of an extract prepared by maceration is slightly higher than an extract prepared by ultrasonication (0.63025 ± 0.16033 mg(quercetin).g(flower)$^{-1}$ and 0.72042 ± 0.13593 mg(quercetin).g(flower)$^{-1}$) respectively); however, this result is not significant. It was expected that ultrasonication would result in decreased radical scavenging activity as phytochemicals responsible for the radical scavenging activity react with the free •H and •OH radicals that are produced during cavitation [170].

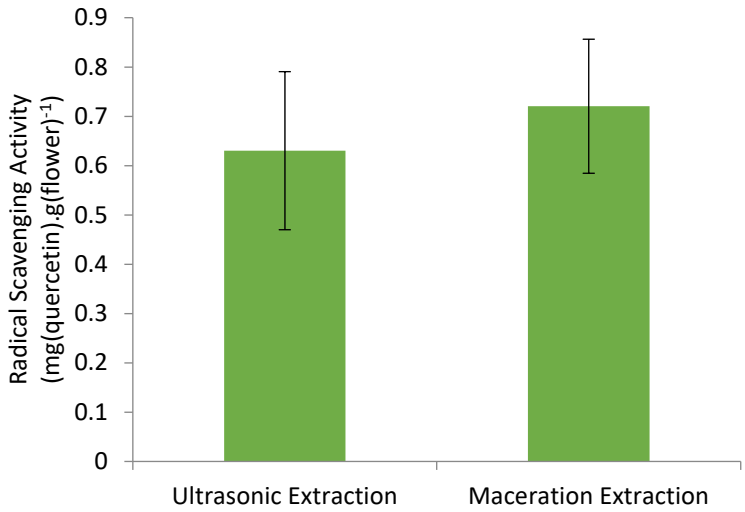


Figure 3-11: Radical scavenging activity of C. officinalis *extracts prepared with ultrasonic and maceration extractions. Data is presented as means ± standard deviations of replicates (n=9). Asterisks indicate statistical difference (p<0.05) of ultrasonic extracts compared with macerated extracts.*

Phenolic content. Folin-Ciocalteu assays were conducted with a view to determining the total phenolic content of hydroethanolic *C. officinalis* extracts prepared with maceration and ultrasonication extraction methods. Figure 3-12 shows the total phenolic content of *C. officinalis* extracts prepared with maceration and ultrasonication extraction methods. It can be seen that maceration extraction methods appear to extract a higher number of phenolic compounds than ultrasonic extraction (1.7298 ± 0.39857 mg.g(flower)$^{-1}$ and 2.0798 ± 0.41698 mg.g(flower)$^{-1}$ respectively); however, this difference was not significant. This result indicates that ultrasonic and maceration extraction methods extract equivalent levels of phenolic compounds.

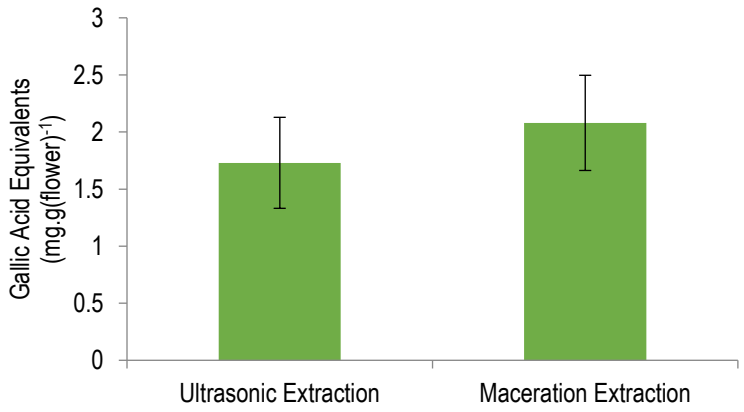


Figure 3-12: Total phenolic content of C. officinalis *extracts prepared with ultrasonic or maceration extraction. Data is presented as means ± standard deviations of replicates (n=9). Asterisks indicate statistically significant differences (p<0.05) between extracts prepared with maceration and ultrasonication extraction methods.*

3.5 SUMMARY

Experiments were conducted with a view to determining if the process of grinding dried *C. officinalis* flowers causes a change in phytochemical composition, antioxidant activity, radical scavenging activity, and phenolic content when prepared with maceration and ultrasonication extraction methods. Apparent differences in phytochemical content were observed; however, these were not found to be significant. Similarly, hydroethanolic *C. officinalis* extracts prepared with ground flowers gave an apparent increase in radical scavenging activity, antioxidant activity, and total phenolic content when compared with extracts prepared with whole flowers; however, these increases were not significant. This indicates that the process of grinding *C. officinalis* flowers has little impact on the final extract. It was found that maceration extracts exhibited an apparent increase in phytochemical content, antioxidant activity, radical scavenging activity, and phenolic content; however, this was not found to be significant. It was hypothesised that this apparent increase was due to ultrasonic degradation of the metabolites due to the relatively long ultrasonic extraction time.

It was determined that extracts prepared with ground flowers and using ultrasonic extraction methods gave equivalent extracts to those prepared with whole flowers and using maceration extractions with an aqueous ethanol solvent.

4 EXTRACTION SOLVENTS

4.1 CHAPTER SYNOPSIS

This chapter explores potential replacement solvents for more hazardous organic solvents (such as ethanol) for the extraction of phytochemicals from *C. officinalis*. Dried *C. officinalis* flowers were used as a model plant material due to their high content of antioxidant and polyphenolic phytochemicals. Total phytochemical content and individual phytochemical concentrations were determined by UHPLC-DAD analysis. Total antioxidant activity, radical scavenging activity, and total phenolic content were determined by $KMnO_4$ chemiluminescence, DPPH, and Folin-Ciocalteu assays respectively. Aqueous acid and base were investigated due to their ability to alter the aqueous solubility of molecules through protonation/deprotonation. Supercritical fluids were investigated potential replacements due to the 'green' nature of the solvents used and the purity of the Supercritical Fluid (SF) residue after desolvation. Natural Deep Eutectic Solvent (NaDES) extracts were investigated as potential replacements due to their large hydrogen bonding capability, 'green' characteristics, and low cost. Aqueous acids and bases demonstrated similar phytochemical composition but reduced chemiluminescence response (as a measure of antioxidant activity), DPPH radical scavenging activity, and phenolic content and were eliminated as potential alternative solvents. Similarly, SF residues demonstrated negligible water-soluble phytochemicals and were eliminated as potential alternative solvents. NaDES prepared with glycerol exhibited similar phytochemical composition, and equivalent chemiluminescence response and radical scavenging activity and showed potential as alternative solvents for phytochemical extraction.

4.2 INTRODUCTION

Currently, a large variety of organic solvents are employed for the purpose of phytochemical extraction. The use of organic solvents carries multiple safety hazards for both operators and end users, including flammability and toxicity, which then can then increase the potential for contamination in products for human use. Additionally, mitigation of these hazards can incur increased costs during product manufacture. There is demand for 'green' solvents that reduce the hazards (and therefore costs) associated with using organic solvents for phytochemical extraction. Anastas' 5th and 7th principles (safer solvents and auxiliaries, and use of renewable feedstocks respectively) [86] can be applied to phytochemical extraction to develop a 'greener' extraction system. One such way of making 'greener' extraction solvents is to modify currently used green solvents (such as water) in order to increase the solubility of desirable compounds. The addition of acids (or bases) is well known to influence the aqueous solubility of organic compounds containing acidic and basic functionalities [173]. For example, the addition of acid to a solution containing organic compounds with basic functionalities can increase aqueous solubility by protonating the base as shown in Figure 4-1. Here, the resulting cation becomes more water soluble due to ion-dipole interactions between the protonated base and the solvent molecule. Conversely, increasing the solution pH by adding base can reduce the solubility of organic base by decreasing protonated basic functionalities and thereby reducing ion-dipole interactions. The addition of base to a solution containing organic compounds with acidic functionalities will result in deprotonation of the acid (Figure 4-2), increasing ion-dipole interactions and as a result, increasing aqueous solubility. Thus, the use of aqueous acid or base as a solvent has potential to alter the solubility of molecules with acidic of basic functionalities. Flavonoids are a class of polyphenols that contain acidic phenol functionalities, the solubility of which may be enhanced by the addition of base. Conversely, alkaloids, which contain basic amine functionalities, may become more soluble upon the addition of acid.

Figure 4-1: Protonation of amine functionality by hydrogen cation

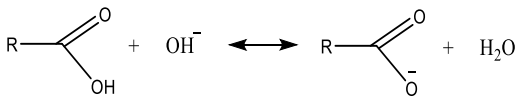


Figure 4-2: Deprotonation of carboxylic acid functionality by hydroxide anion

Whilst the use of acids and bases may seem appealing, there are some limitations to this approach. The solubilities of phytochemicals that do not contain acidic or basic functionalities will be minimally affected. At low pH values, the addition of acid can result in hydrolysis. Acid hydrolysis (Figure 4-3) is the cleavage of chemical bonds (including glycoside linkages) through nucleophilic substitution, and results in the

production of two molecules; flavonoid glycosides, for example, can undergo acid hydrolysis to produce a flavonoid aglycone and a sugar glycone [174]. Rutin (quercetin-3-O-rutinoside) undergoes acid hydrolysis to produce quercetin (aglycone) and rutinose (glycone), shown in Figure 4-3. Acid hydrolysis can therefore be used to assist in the quantitative analysis of a flavonoid by removing glycoside functionalities that can interfere with spectrophotometric analyses that utilise retention times such as UHPLC as the hydroxyl moieties on the glycoside functionality will alter the interactions between the molecule and the polar phase.

At high pH values, the addition of base results in alkaline hydrolysis; that is, nucleophilic substitution of a chemical bond in which a hydroxide anion acts as a nucleophile. The alkaline hydrolysis of triglycerides and long chain fatty acids results in saponification. Alkaline hydrolysis can be used to remove organic acid ester functionalities from flavonoid molecules [174, 175].

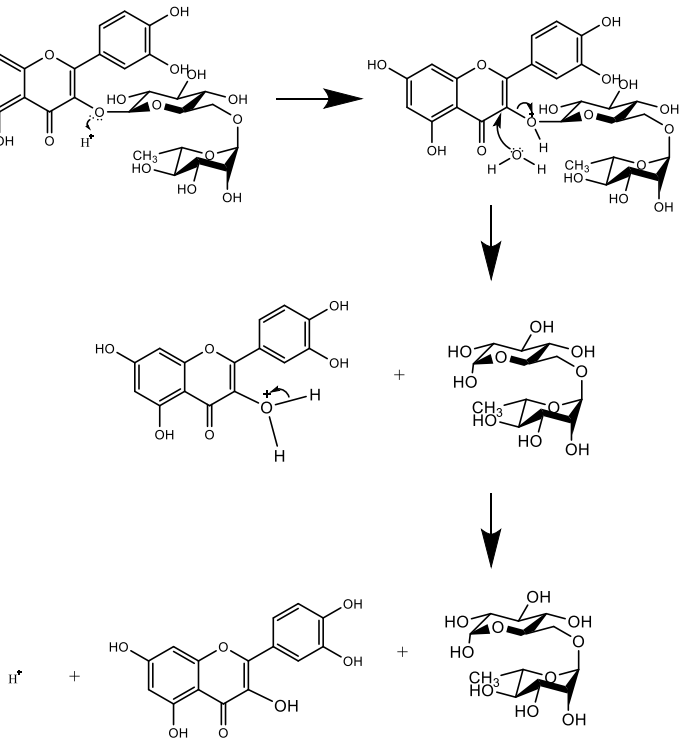


Figure 4-3: Mechanism of acid-catalysed hydrolysis of rutin (quercetin-3-O-rutinoside) to quercetin and rutinose.

Supercritical Fluids (SF) are an alternative solvent class that have recently gained attention as 'green' alternatives to current organic solvents. SFs result when substances exceed their critical temperature and pressure, forming a supercritical state. By altering the exact temperature and pressure of the supercritical fluid, the physical properties can be altered, resulting in a highly modifiable solvent that can be 'tuned' to extract specific compounds. Furthermore, supercritical fluids can be easily removed from

the final extract by the simple process of exposing the extract to atmospheric conditions, resulting in the rapid phase change of the solvent from supercritical fluid to gas. SFs are considered 'green' due to the nonhazardous and renewable nature of the gases used. Carbon dioxide, for example, is nonhazardous at atmospheric conditions (assuming adequate ventilation to prevent suffocation), and is produced in a myriad of organic systems. Additionally, supercritical CO_2 rapidly transitions to the gaseous phase when the temperature and pressure return to atmospheric conditions and does not leave any residues. SFs exhibit a range of properties that make them attractive for phytochemical extraction including high diffusion rates, low viscosities, and densities, dielectric constants, dipole moments, and partition coefficients that can be 'tuned' by slightly altering temperature and pressure [176]. Additionally, cosolvents such as ethanol or water can be added to the SF in order to further modify the properties of the SF [17, 177]. Supercritical CO_2 has previously been used in the extraction of monoterpenes, sesquiterpenes, sesquiterpene alcohols [178], and alkanes [114], but exhibits poor extraction of antioxidant [179] and phenolic compounds [27]. Supercritical CO_2 has shown promise in the selective extraction of compounds from plants and has been used in processes such as the decaffeination of coffee beans or preparation of hops concentrate [44].

Deep Eutectic Solvents (DES) are room-temperature eutectic mixtures that have been prepared from quaternary ammonium salts (typically choline chloride) with a salt, organic acid or base (such as those listed in Table 4-1) [180]) in which the components are a hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) that, when combined, form a liquid with a melting point below that of any individual component. DES were proposed as alternatives to the more toxic imidazolium-based Ionic Liquids (IL) that had previously been touted as 'green' due to their reusability and negligible vapour pressure, but were dismissed due to their toxicity and biotoxicity [63]. DES present several advantages over more traditional ILs; ease of preparation, inexpensive materials, low toxicity, and in general use and produce more environmentally friendly products. DES have previously been used in the processing of lignin [65], in microwave-assisted extraction of flavonoids from *Radix Scutellariae* (showing equivalent extractive capabilities to conventional water and ethanol extractions, with nonhazardous materials and processes [66]), and in the extraction of terpenoids from C*hamaecyparis obtusa* leaves, showing rapid and simple extraction when compared to other techniques such as ultrasonic-assisted or reflux extraction [181]. Further uses include the removal of glycerol from biodiesel [182], the solubilisation of metal oxides [183] and the preparation of cellulose derivatives [184].

Table 4-1: Some components previously used in the preparation of DES [64].

Hydrogen Bond Donors	*Hydrogen Bond Acceptors*
Urea, Acetamide, 1-methyl urea, 1,3-dimethyl urea, 1,1-dimethyl urea, thiourea, benzamide, glycerol, ethylene glycol, malonic acid, benzoic acid, adipic acid, oxalic acid, succinic acid, citric acid	Choline Chloride, N-ethyl-2-hydroxy-N,N-dimethylethanaminium chloride, 2-(chlorocarbonyloxy)-N,N,N-trimethylethanaminium chloride, N-benzyl-2-hydroxy-N,N-dimethylethanaminium chloride

Natural Deep Eutectic Solvents (NaDES) are DES that have been prepared from naturally occurring materials and exhibit the desirable 'green' properties such as low toxicity, vapour pressure, flammability, and environmental impact, whilst maintaining high levels of hydrogen bonding [68]. Like DES, NaDES are composed of an organic hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) that, when combined, form a liquid with a melting point below that of any individual component. Water is sometimes retained in NaDES in order to achieve this liquid state [69]. Components used in the preparation of a NaDES are typically naturally occurring plant metabolites such as organic acids and sugars (such as those listed in Table 4-2) that often can act as both HBDs and HBAs. A typical method of NaDES preparation dissolves individual components in excess water. These aqueous solutions are then combined and the resulting mixture is either heated or rotary evaporated until a constant weight is achieved [71]. Depending on the materials used, water can be retained in the DES as a vital third component. Choi *et al.* [69] prepared NaDES using this method and found that a certain quantity of water was strongly retained within the NADES, suggesting that water forms a vital component in these NADES. NaDES are hypothesised to be the means by which phytochemicals with low aqueous solubility are solubilised in organisms [185]. NaDES have previously been used in the extraction of isoflavones [186], flavonoids [66, 187], and anthocyanins [188] from plant-based sources. Additionally, it was found that rutin, a compound only slightly soluble in the water or lipid phases within plant cells, was 50-100 times more soluble in the various NADES than in water [69].

Table 4-2: Typical components used in the preparation of NaDES [187, 188].

Acids	*Sugars*	*Other*
Citric, Tartaric, Malic, ascorbic, oxalic, L-Proline, L-Alanine, Glycine, L-Histidine, L-Threonine, L-Lysine, L-Arginine	Sucrose, Glucose, Fructose, Galactose, Lactose, Xylose	Glycerol, choline chloride, urea

This chapter presents an investigation into alternative 'green' solvents that were used in order to prepare *C. officinalis* extracts with similar chemical composition to that of a traditional hydroethanolic extract without the use of hazardous substances such as ethanol. Given the potential of acid and base

extractions to alter the relative aqueous solubility of molecules with acidic and basic functionalities, it was hypothesised that the manipulation of aqueous pH would allow for increased flavonoid extraction to mimic a traditional hydroethanolic solvent. As supercritical fluid extraction has previously demonstrated efficacy in the extraction of phytochemicals from plant material, it was hypothesised that SFs would allow preparation of a chemically similar *C. officinalis* extract to hydroethanolic solvents whilst reducing the hazardous nature of the solvent when compared with organic extraction solvents. NaDES have previously been shown to contain high levels of hydrogen bonding and have been used to solubilise phytochemicals with relatively poor aqueous solubility, and it can be hypothesised that the use of NaDES as extraction solvents would provide an extract that has similar chemical composition whilst completely eliminating the hazardous nature of the solvent itself.

4.3 EXPERIMENTAL

Calendula officinalis samples. Dried *C. officinalis* flower heads were used as supplied. Flowers were grown in the Adelaide Hills, South Australia on a certified biodynamic farm (Jurlique International, certified under the National Association for Sustainable Agriculture, Australia), hand-picked and air-dried in sheds. Dried *C. officinalis* was ground using a commercial grinder (Sunbeam Coffee Grinder, purchased commercially) and stored in sealed plastic containers in darkness at ambient conditions prior to use.

Chemicals and Reagents. Sodium hydroxide (>99%) and Ethanol (99.9%) was purchased from Chem-Supply (Gillman, SA, AUS). Tartaric and citric acids (Food grade, McKenzie's Foods), sucrose (Food grade, CSR Sugar Australia), fructose (Food grade, Fruisana) and glucose (Food grade, Glucodin) were purchased from local supermarkets. Sterile filters (0.22 µm, nylon), centrifuge vials (10 mL, polypropylene) and storage vials (3 mL, polypropylene were purchased from Sarstedt (Nümbrecht, DE). Plastic syringes (1 & 5 mL, polypropylene) were purchased from Livingstone (Rosebery, NSW, AUS). Phenoxyethanol (as Euxyl 9010) was supplied by Jurlique International (Mount Barker, SA, AUS). Hydrochloric acid (37%) was purchased from British Drug House Laboratories (Poole, UK). Deionised water was purified to 18 MΩ using a Thermo Fisher Scientific (Waltham, MA, USA) Barnstead™ E-Pure™ water system.

Solvent Preparation

Aqueous acid and base. Acidic aqueous solvents in the pH range 0-6 were prepared by titrating deionised water with hydrochloric acid (conc.) to the desired pH. Basic aqueous solvents in the pH range 8-14 were prepared by dissolving solid sodium hydroxide in water to achieve the desired pH.

NaDES. The method used to prepare the NaDES was adapted from Dai *et al*. [189] Naturally occurring compounds (Table 4-3) were combined in 1:1 molar ratios and dissolved in small quantities of water until a clear homogenous solution was achieved. The resulting solutions were then heated (70 ^{O}C, 72 hours) until the resulting solution was of consistent weight (water weights available in Appendix 3).

Table 4-3: Combinations used for preparing preliminary NaDES.

NaDES #	*Fructose*	*Glucose*	*Sucrose*	*Glycerol*	*Citric Acid*	*Water*
1	✓		✓			✓
2		✓	✓			✓
3			✓	✓		✓
4			✓		✓	✓
5	✓	✓				✓
6	✓			✓		✓
7	✓				✓	✓
8		✓		✓		✓
9		✓			✓	✓
10				✓	✓	✓
11	✓	✓		✓		✓
12	✓	✓			✓	✓

Extract Preparation.

Reference Extracts. Hydroethanolic solvent was prepared by titrating ethanol with deionised water to a final concentration of 30% v/v ethanol/water. Reference extracts for aqueous acid and base and SCF extractions were prepared in triplicate by combining ground *C. officinalis* flower heads (0.15 g) with hydroethanolic solvent (3 g) with subsequent stirring for 120 minutes. Resulting extracts were gravity filtered (110 mm, Advantec) and Euxyl PE9010 was added (to 0.5% w/w) in order to inhibit bacterial growth. Extracts were stored in darkness at (4 ^{O}C) prior to analysis. Process blanks were prepared and analysed concurrently using identical techniques.

Reference extracts for NaDES extractions were prepared in triplicate by combining ground *C. officinalis* flower heads (0.25 g) with hydroethanolic solvent (5 g) with heating (70 ^{O}C) for 120 minutes in order to mimic the NaDES extraction method. Resulting extracts were centrifuged and filtered through a syringe packed with clean cotton wool to remove remaining flower material. Extracts were stored in darkness under ambient conditions until analysed. Process blanks were prepared and analysed concurrently using identical techniques. Extracts and blanks were diluted with hydroethanolic solvent.

Aqueous acid and base. Extracts were prepared by combining ground *C. officinalis* flower heads (10 g) with aqueous acid or base (100 g) and subsequently heated (40 ^{O}C) stirring for 150 minutes. Resulting extracts were filtered (Advantec) and Euxyl PE9010 was added (to 0.5% w/w) in order to inhibit bacterial growth. Extracts were stored in darkness at 4 ^{O}C prior to analysis. Process blanks were prepared

and analysed concurrently to the extracts using identical techniques. Samples were adjusted to pH 7 with aqueous HCl or NaOH to analysis.

SCF extraction. SCF extracts were prepared using an Applied Separations Spe-ed SFE-2 7071 multi-vessel simultaneous oven-based extraction system (Allentown, PA, USA) equipped with a 100 mL stainless steel extraction vessel. Ground *C. officinalis* flower heads (3 g) were sandwiched between layers of glass wool and sand in the stainless steel extraction vessel (Figure 4-4). Supercritical CO_2 (100 ^{O}C, 600 bar) was then pumped through the column. Solvent evaporation occurred upon extract collection at atmospheric pressure and the remaining *C. officinalis* extract residue was collected in an amber glass vial. Triplicate extracts were combined and stored in darkness at 4 ^{O}C.

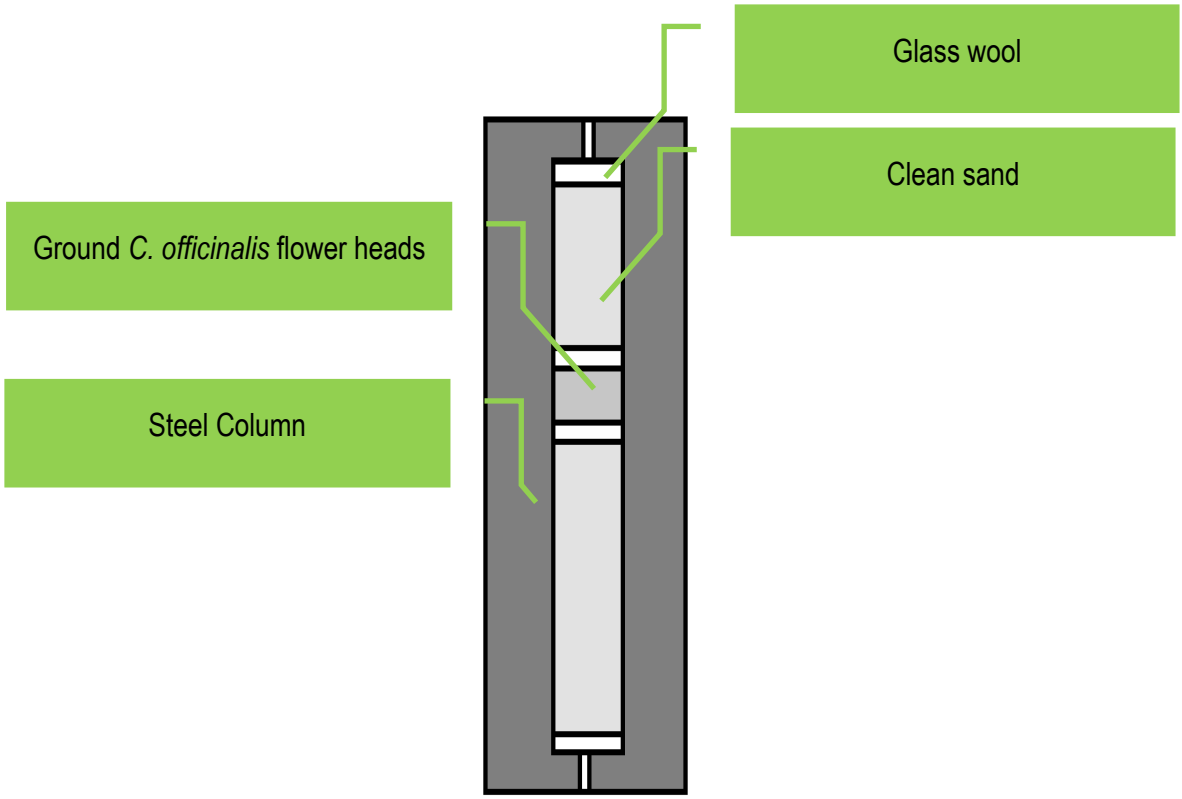


Figure 4-4: Layout of SCF column used for supercritical CO_2 extraction of C. officinalis.

Secondary SCF extract liquid/liquid extraction. Liquid/liquid extracts were prepared by combining SCF residues (0.1 g) with aqueous hydrochloric acid (pH 0), sodium hydroxide (pH 14), deionised water, and acetonitrile solvents in separate sealed glass vials with stirring for 60 minutes. Resulting extracts were then centrifuged (Clements Orbital 325, 3000 rpm, 30 min) and the supernatant was collected. Solid material observed in the basic extraction was collected and dried (60 ^{O}C, 60 minutes). Extracts were stored in darkness under ambient conditions until analysis.

NaDES extraction. Extracts were prepared by combining ground *C. officinalis* flower heads (1 g) with NaDES #4-7 and #10-12 (10 g) shown in Table 4-3 with heating (70 ^{O}C) for 120 minutes. NaDES #1-3 and 8-9 were excluded as the NaDES precipitated at room temperature. Resulting extracts were centrifuged (Clements Orbital 325, 3000 rpm, 30 min), and filtered through clean cotton wool to remove remaining flower material. Samples were stored in darkness under ambient conditions until analysed. Process blanks were prepared and analysed concurrently using identical techniques. Samples and blanks were diluted tenfold (w/w) with hydroethanolic solvent (30% v/v ethanol/water) prior to analysis.

Chemiluminescence response. SIA-CL assays were conducted using the method detailed in Chapter 2.3.

Radical Scavenging Activity. DPPH assays were conducted using the method detailed in Chapter 2.4.

Phenolic Content. Folin-Ciocalteu assays were conducted using the method detailed in Chapter 2.5. Reference standards of aqueous gallic acid (10, 20, 30, 40, and 50 mg/mL) were run concurrently.

Statistical Testing. F-testing and T-testing to determine statistical significance was conducted using the method outlined in Chapter 2.6.

4.4 RESULTS & DISCUSSION

4.4.1 Acid/base extraction.

Initial experiments focused on the use of acidic and basic aqueous solutions, with a view to altering the aqueous solubility of extract components on the basis of acid-base equilibrium reactions. During extractions using low pH solvents (pH 0-1), both the flower tips and the resulting extract were observed to change colour from the typical yellow to a bright pink-red. This may be due to the presence of anthocyanins that exhibit different colours at different pH [190]. Anthocyanins including cyanidine-3-o-rutinoside, petunidin-3-O-glucopyranoside, pelargonidin-3-O-glucopyranoside, peonidin-3-O-glucopyranoside, malvidin-3-O-glucopyranoside, delphinidin-3-O-glucopyranoside, pelargonidin-3,5-di-O-glucopyranoside, and cyanidine-3,5-di-O-glucopyranoside have previously been detected in *C. officinalis* by Ollenikov *et al.* [191]. Extracts prepared with pH 0-1 and pH 13-14 solvents changed to a brown colour upon neutralisation; however, these extracts were still distinguishable from extracts prepared with pH 2-12 solvents due to slight variations in colour. Additionally, extracts prepared with pH 0-1 solvents were observed to be slightly gelatinous when compared with extracts prepared with pH 2-12 solvents. This was hypothesised to be the result of increased pectin extraction as low pH (typically 1-2.5) is the preferred acidity for the extraction of pectin [192]. Pectin has been previously identified in *C. officinalis* petals by Slavov *et al.* [193]. Extracts prepared with highly alkaline (pH 13-14) solvents were observed to contain small quantities of dark material; there were hypothesised to be the results of plant tissue digestion.

UHPLC Analysis. UHPLC analyses were conducted with a view to determining the phytochemical composition of *C. officinalis* extracts prepared with aqueous acid and base. Figure 4-6 shows the phytochemical composition of *C. officinalis* extracts prepared with a hydroethanolic solvent, and aqueous pH 0, 7, and 14 solvents. Full UHPLC chromatograms for pH 0-14 aqueous extracts are available in Appendix 3. It can be seen that *C. officinalis* extracts prepared with a hydroethanolic solvent contains similar phytochemical constituents to extracts prepared with pH 0-12 water. The large peak present at 12 minutes was determined to be the phenoxyethanol preservative by comparison with a standard. Extremely acidic (pH 0-1) and basic (pH 13-14) aqueous extracts exhibit fewer peaks than aqueous extracts prepared with less concentrated acids and bases. These metabolites were hypothesised to have degraded under the extreme conditions used.

Peaks at 0.85, 8.69, 12.5, 12.75, 13.1, 13.37, 13.65, 14.7, and 15.37 minutes were selected (Figure 4-5) for further analysis due to clear peak resolution and antioxidant activity determined in Chapter 3.4.1 and labelled A-I respectively. Figure 4-7, Figure 4-8, Figure 4-9, and Figure 4-10 show the hieghts of Peaks A, B, G, and H respectively. It can be seen that peak heights of unretained compounds (Figure 4-7) show that extracts prepared with moderately acidic solvents (pH 2-14) exhibit increased peak height when compared with a hydroethanolic extract prepared under similar conditions. This was anticipated as aqueous solvents, particularly those containing acid and base, would be more polar than a hydroethanolic solution (dielectric constants for water and the hydroethanolic solvent are 80.1 and 63.7 respectively [194]) and as such would extract higher concentrations of polar metabolites. Extracts prepared with highly concentrated acid (pH 0-1) exhibited decreased peak A heights; this was hypothesised to be the result of degradation due to the acid concentration, as shown in Figure 4-6. Extracts prepared with concentrated base (pH 14) exhibited a large increase in peak height compared with hydroethanolic solvents and other aqueous acid and base solvents; this was hypothesised to be the elution of hydroxylated plant metabolites as UHPLC chromatograms (Figure 4-6) showed decreased phytochemical peak heights in concentrated aqueous base, and the addition of hydroxyl moieties would reduce the retention time of compounds in RP-UHPLC. Retained compounds (peaks B, G, and H) exhibited decreased peak heights when compared with a hydroethanolic extract prepared under similar conditions. Whilst this result was in line with expectations regarding the polarities of water and hydroethanolic solvents, it also indicates that increasing the aqueous solubility of nonpolar metabolites through deprotonation of acidic functionalities with aqueous base is not a viable means of phytochemical extraction.

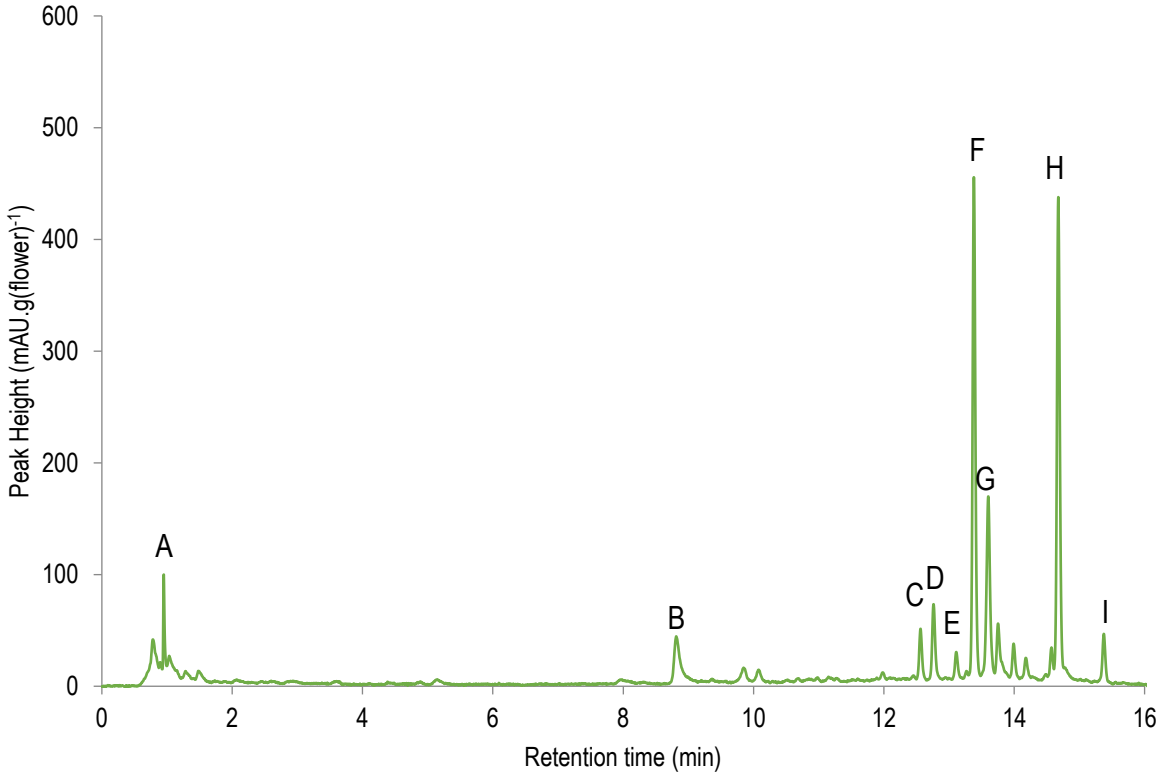


Figure 4-5: UHPLC analysis of a C. officinalis *extract prepared with hydroethanolic solvent. Labelled peaks were chosen for further investigation due to clear resolution and antioxidant activity as per Chapter 3.4.1.*

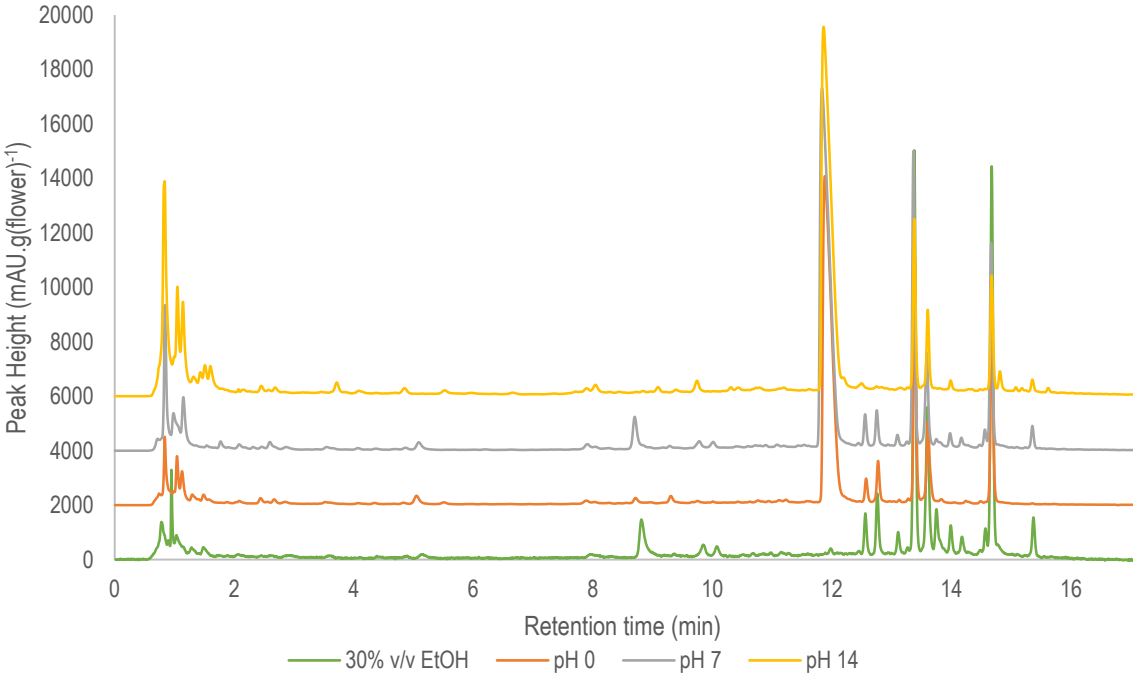


Figure 4-6: UHPLC chromatograms of C. officinalis *extracts prepared with hydroethanolic and pH 0, 7, and 14 solvents. Sequential chromatograms are offset vertically by 2000 mAU.g(flower)$^{-1}$.*

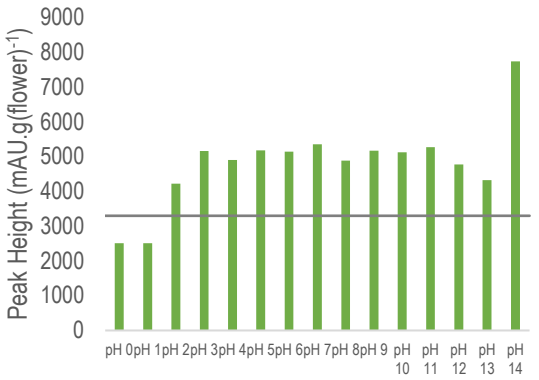


Figure 4-7: Height of Peak A in C. officinalis *extracts prepared with aqueous acid, base, and hydroethanolic solvents. The line represents the height of Peak A in a hydroethanolic extract.*

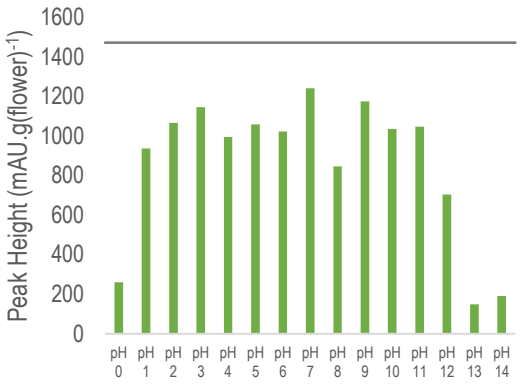


Figure 4-8: Height of Peak B in C. officinalis *extracts prepared with aqueous acid, base, and hydroethanolic solvents. The line represents the height of Peak B in a hydroethanolic extract.*

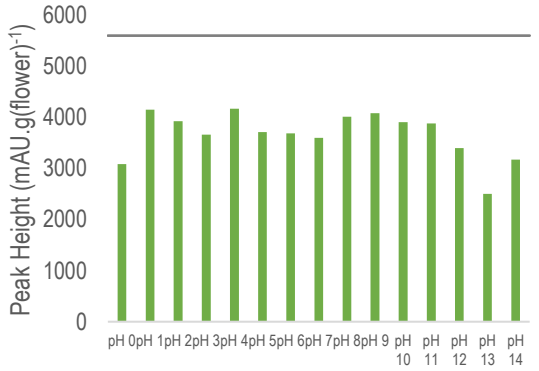


Figure 4-9: Height of Peak G in C. officinalis *extracts prepared with aqueous acid, base, and hydroethanolic solvents. The line represents the height of Peak A in a hydroethanolic extract.*

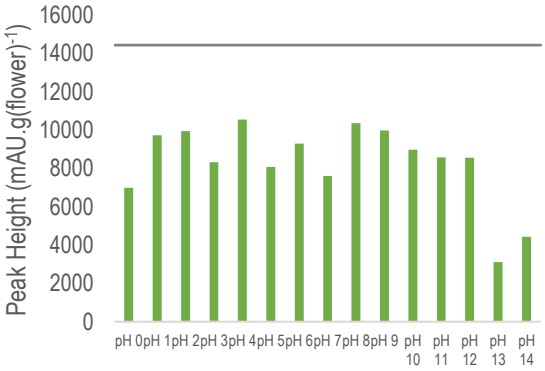


Figure 4-10: Height of Peak H in C. officinalis *extracts prepared with aqueous acid, base, and hydroethanolic solvents. The line represents the height of Peak A in a hydroethanolic extract.*

The total peak area of extracts prepared with aqueous acid or base was investigated. Figure 4-11 shows the total peak area of *C. officinalis* extracts prepared with hydroethanolic and aqueous acid and base solvents. It can be seen that extracts prepared with aqueous acid and base exhibit decreased total peak area when compared with a hydroethanolic extract prepared under similar conditions. These decreases in total peak area mirror the results obtained when analysing individual peaks. *C. officinalis* extracts prepared with pH 14 water demonstrate a substantially higher total UHPLC peak area than other aqueous extracts; however, analysis of the individual UHPLC chromatogram (Figure 4-6) shows that the higher total peak area is due to a large number of unretained compounds and contains fewer retained metabolites when compared with extracts prepared with either hydroethanolic or pH 2-12 aqueous solvents. Results observed in individual peak analyses and total peak area analysis indicate that the use

of aqueous acid and base does not result in equivalent or increased phytochemical extraction when compared with traditional hydroethanolic solvents.

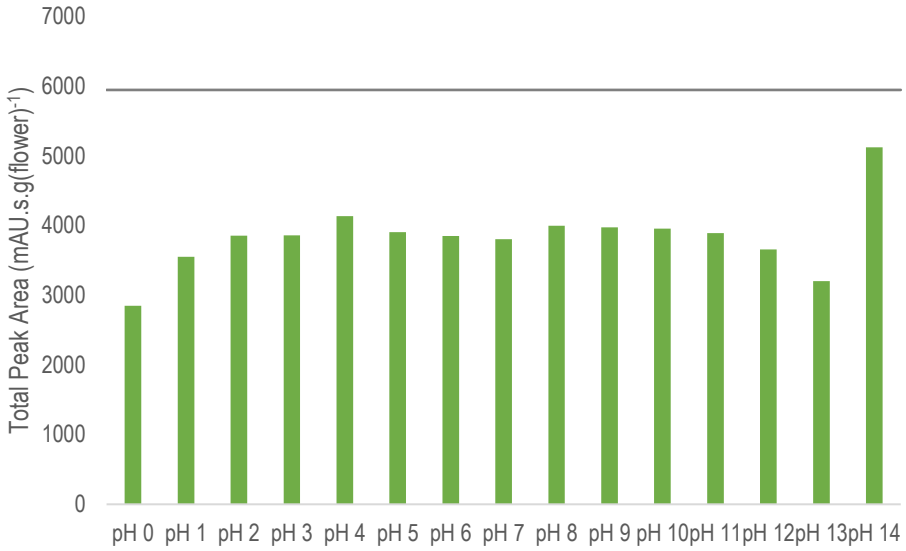


Figure 4-11: Total peak areas of C. officinalis *extracts prepared with pH 0-14 and hydroethanolic solvents. The line represents the total peak area in a hydroethanolic extract.*

Chemiluminescence response. SIA-CL assays were conducted with a view to determining the antioxidant activity of *C. officinalis* extracts prepared with aqueous acid and base solvents. Figure 4-12 shows the chemiluminescence response of *C. officinalis* extracts prepared with hydroethanolic and aqueous acid and base solvents. It can be seen that extracts prepared with aqueous acid and base had similar chemiluminescence response when compared with extracts prepared with hydroethanolic solvents. Apparent increases in chemiluminescence response were observed in extracts prepared with pH 0, 8, 9, 10, 12, and 13 solvents; however, these were not found to be significant. Significant decreases were observed in extracts prepared with pH 2 (21020 ± 3637.7 mV.s.g(flower)$^{-1}$), pH 6 (22723 ± 1872.6 mV.s.g(flower)$^{-1}$), and pH 14 (19694 ± 1628.6 mV.s.g(flower)$^{-1}$) when compared to an extract prepared with hydroethanolic solvents (28780 ± 2534.1 mV.s.g(flower)$^{-1}$). SIA-CL results obtained for this assay may not be indicative of the chemiluminescence response of the extract due to the presence of differing NaCl concentrations that occurred as a result of neutralisation of the extract prior to analysis. Fujimori *et al.* [195] observed an approximate 10% enhancement in permanganate chemiluminescence with a salinity of 3.4%. This suggests that the CL results obtained shown in Figure 4-12 may not be indicative of the antioxidant activity of the extracts due to the large variation in salt concentrations at each pH.

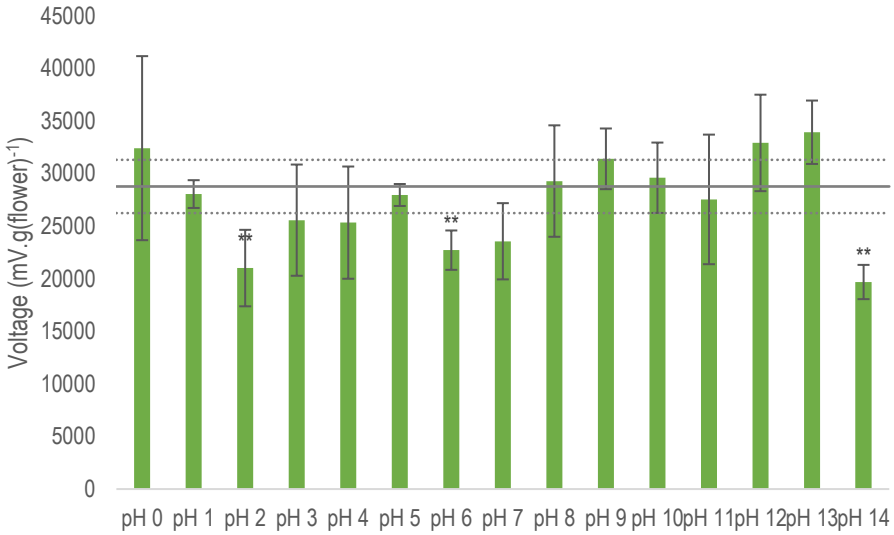


Figure 4-12: SIA-CL analysis of C. officinalis *extracts prepared with aqueous acid and base solvents. Data is presented as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance ($p<0.05$) when compared with an extract prepared with hydroethanolic solvents under similar conditions. The line represents the chemiluminescence response of a hydroethanolic extract, with dashed lines representing the error margin.*

Radical Scavenging Activity. DPPH assays were conducted with a view to determining the radical scavenging activity of *C. officinalis* extracts prepared with aqueous acid and base. Figure 4-13 shows the ability of *C. officinalis* extracts prepared with hydroethanolic and aqueous acid and base to scavenge stable DPPH radicals. It can be seen that extracts prepared with pH 0-11 water exhibit decreased radical scavenging activity when compared with an extract prepared with hydroethanolic solvents (Figure 4-13). This decrease is significant for extracts prepared with aqueous pH 0 (16.441 ± 3.0207%), pH 1 (17.478 ± 5.427%), pH 3 (28.189 ± 8.4928%), pH 4 (31.432 ± 7.0479%), pH 5 (24.227 ± 2.3255%), pH 10 (18.451 ± 0.90062%), and pH 12-14 (24.326 ± 5.4081%, 3.1530 ± 1.9494%, and -0.71199 ± 2.8332% respectively) solvents when compared with an extract prepared with hydroethanolic solvents (49.992 ± 4.42%). Extracts prepared with aqueous pH 2, pH 6-9, and pH 11 water also exhibit decreased radical scavenging activity; however, these results are not significant. The decreases in DPPH radical scavenging observed indicate that aqueous acid and base solvents do not extract equivalent quantities of radical scavenging phytochemicals from *C. officinalis*.

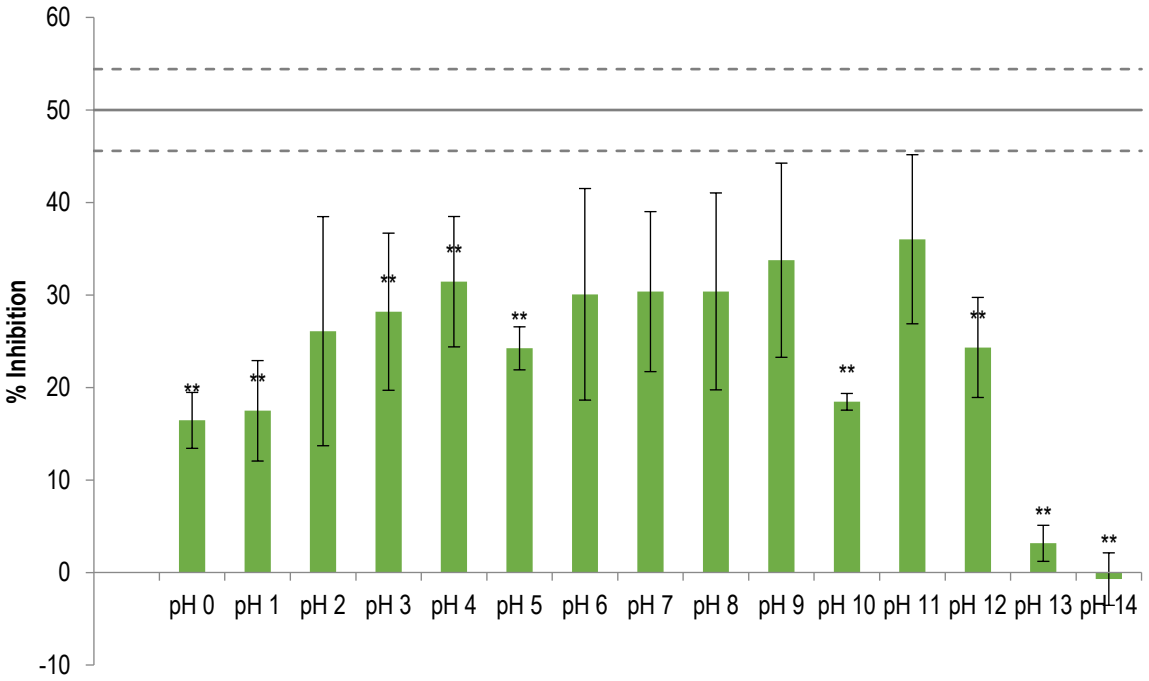


Figure 4-13: Radical scavenging activity of C. officinalis *extracts prepared with acidic and basic water. Data is presented as means ± standard deviation of replicates (n=3). Asterisks indicate statistically significant differences ($p<0.05$) when compared with extracts prepared with hydroethanolic solvents. The line represents the radical scavenging activity of a hydroethanolic extract, with dashed lines representing the error margin.*

Phenolic content. Folin-Ciocalteu assays were conducted with a view to determining the total phenolic content of *C. officinalis* extracts prepared with aqueous acid and base. Figure 4-14 shows the phenolic content of *C. officinalis* extracts prepared with aqueous acid and base. It can be seen that extracts prepared with relatively low concentrations of acid and base (pH 3-6 and 8-11) exhibit similar phenolic content to an extract prepared with neat water. Extracts prepared with higher concentrations of acid (pH 0-2) presented decreases in phenolic content, whereas extracts prepared with pH 12 and pH 13 solvents presented increases in phenolic content. This was in line with expectations, as phenolic functionalities exhibit slightly acidic behaviour in aqueous solutions [196]. The use of an acidic solvent results in protonation of the phenolic moiety, thereby reducing solute polarity and decreasing the aqueous solubility of flavonoids and other phenol-containing metabolites. Similarly, the use of basic solvents would result in deprotonation of the phenolic moiety, increasing solute-solvent interactions and increasing solubility of phenol-containing metabolites such as flavonoids. Extracts prepared with pH 14 solvents presented a significant decrease in phenol content when compared with extracts prepared with pH 7 water. It was hypothesised that this was the result of degradation due to the extremely high pH, which agreed with UHPLC results that indicated significant decreases in metabolite concentration and increases in unretained compounds. Extracts prepared with aqueous acid (pH 0-6), water (pH 7), and some

aqueous bases (pH 8-11 and 14) exhibited significantly lower phenolic content than an aqueous ethanol extract prepared under similar conditions. Extracts prepared with pH 12 and pH 13 water exhibited similar phenolic content to an extract prepared with aqueous ethanol; however, given the UHPLC, SIA-CL, and DPPH assay results discussed previously, it can be hypothesised that this increase is due to degradation of plant material rather than an increase in metabolite extraction. Additionally, these concentrations of base are hazardous and are therefore precluded from use in 'green' alternative solvents under Anastas' 'Green Chemistry' principles [86]. This result indicates that aqueous acid and base solvents do not produce extracts with equivalent phenolic content to traditional hydroethanolic solvents.

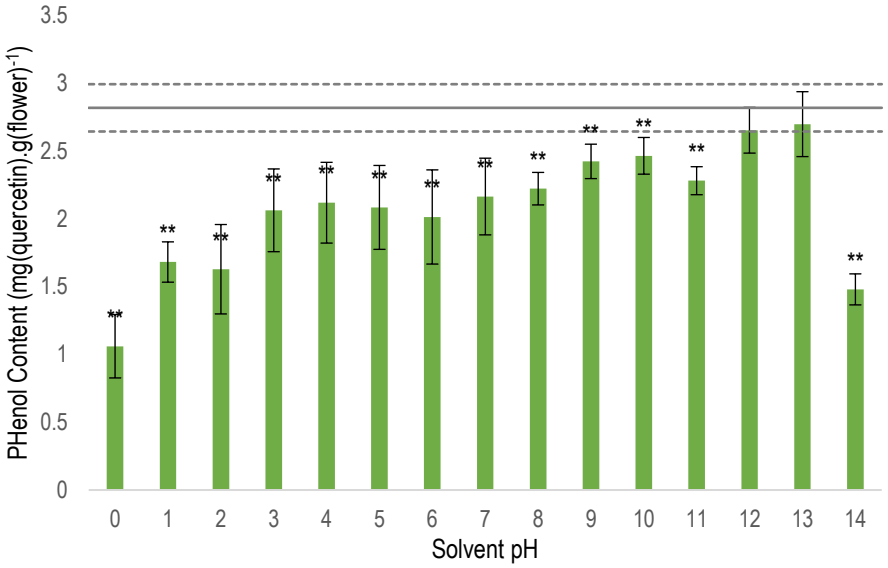


Figure 4-14: Phenolic content of C. officinalis *extracts prepared with aqueous acid and base as determined by Folin-Ciocalteu's assay. Data is presented as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance ($p<0.05$) when compared to a hydroethanolic extract prepared under similar conditions. The line represents the phenolic content radical scavenging activity of a hydroethanolic extract, with dashed lines representing the error margin.*

4.4.2 Supercritical Fluid Extracts.

Experiments using supercritical CO_2 as a solvent were conducted with a view to utilising the chemical properties of CO_2 in both a supercritical state (for metabolite extraction) and at ambient conditions (for solvent removal) to increase the extraction of plant metabolites from *C. officinalis*. Operating conditions were selected to be within instrument limitations. SCF extraction of *C. officinalis* flower heads yielded a thick, oily residue (Figure 4-15) that was found to solvate in 50% v/v ethanol/hexane (Figure 4-16); however, this was not explored further as the hazards presented by the ethanol/hexane mixture precluded Anastas' principles of green chemistry [86] discussed previously. Secondary liquid/liquid extraction of this residue with aqueous HCl (pH 3, Figure 4-17), aqueous NaOH (pH 12), deionised water, and acetonitrile

yielded 4 extracts. Liquid/liquid extracts prepared with aqueous acid and base, and deionised water were all identical in appearance. Extraction of the SF residue with aqueous NaOH resulted in the formation of an opaque yellow solid that when dried (Figure 4-18) exhibited soap-like (surfactant) properties when solubilised in water. It was hypothesised that this solid resulted from saponification of fatty acids and oils present in the SCF residue such as faradiol-3-myristic and faradiol-3-palmitic fatty acid esters which have previously been identified in *C. officinalis* flowers by Zitterl-Eglseera *et al.* [197]. SIA-CL, DPPH, and Folin-Ciocalteu assays were not conducted following the UHPLC results obtained as the difficulty in residue solvation and subsequent lack of metabolites observed in liquid/liquid extracts suggested that SF was not a viable alternative solvent.

Figure 4-15: C. officinalis *SCF residue in amberglass vial*

Figure 4-16: Secondary liquid/liquid extract of C. officinalis *SCF residue using ethanol/hexane*

Figure 4-17: Secondary liquid/liquid extract of C. officinalis *SCF residue using aqueous HCl*

Figure 4-18: Dried precipitate from secondary liquid/liquid extraction of C. officinalis *SCF residue using aqueous NaOH*

UHPLC Analysis of Secondary liquid/liquid extractions. UHPLC analyses were conducted with a view to determining the phytochemical composition of *C. officinalis* extracts prepared with SCF solvents. Figure 4-19 shows the phytochemical composition of liquid/liquid extracts prepared from supercritical *C. officinalis* extracts. It can be seen that aqueous extracts of the SCF residue yielded

negligible quantities of plant metabolites; indeed, secondary extracts prepared using DI water yielded a trace metabolite eluting at 13 minutes. Similarly, secondary extracts prepared using aqueous acid yielded approximately six trace metabolites. Secondary extracts prepared using aqueous base yielded observable quantities of unretained metabolites similar to those observed in extracts prepared with hydroethanolic solvents. Secondary extracts prepared with acetonitrile yielded a number of strongly retained metabolites; these were hypothesised to be long chain alkanes and similar fatty acids, as supercritical CO_2 extraction of plant material has been shown to preferentially extract lipophilic compounds , with poor extraction of polar components due to the lack of polarity [198]. All secondary extracts of SCF residues yielded negligible plant metabolites when compared to extracts prepared with traditional hydroethanolic solvents. This may be attributed to the higher temperature of the SCF resulting in phenolic degradation; however, it is more likely that the SCF was not suitable for phenolic extractions as work by Bajerova *et al.* [27] has previously found that SCF extraction methods are poorly suited to phenolic extraction.

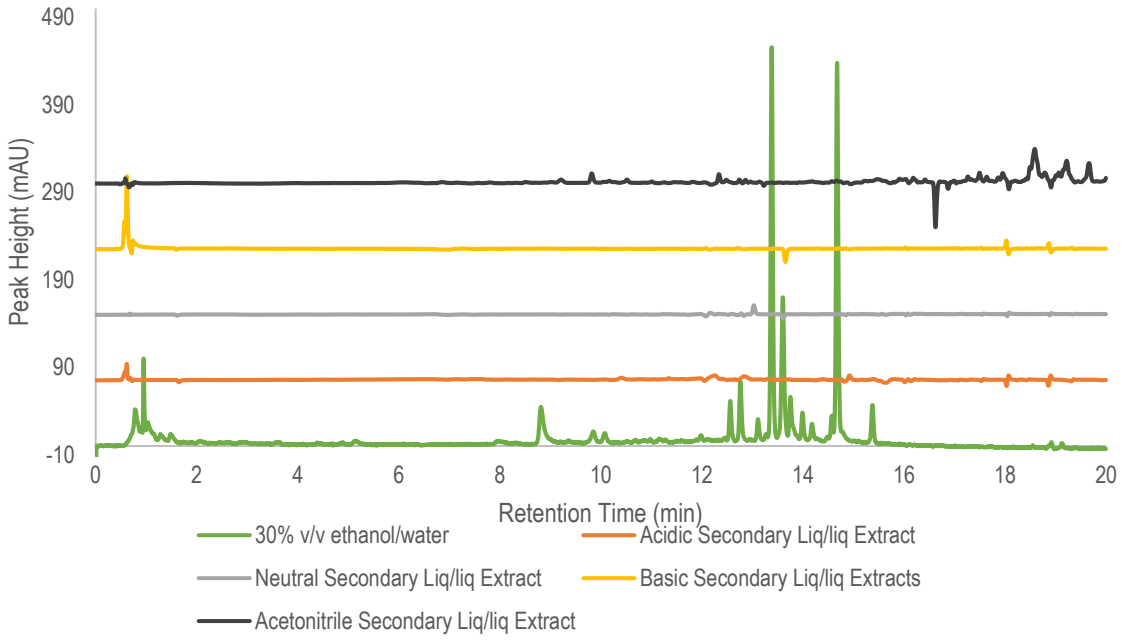


Figure 4-19: UHPLC chromatograms of secondary liquid/liquid extracts prepared from C. officinalis *SCF residues. Sequential chromatograms are offset vertically by 80 mAU.*

The total peak areas of the UHPLC chromatographs were calculated. Analysis of the total peak area of the secondary liquid/liquid extracts demonstrate that aqueous secondary liquid/liquid extracts exhibit significantly lower total peak areas (17.289 mAU.s.g(flower)$^{-1}$, 8.7701 mAU.s.g(flower)$^{-1}$, and 14.000 mAU.s.g(flower)$^{-1}$ for acidic, neutral, and basic extracts respectively) when compared with extracts prepared with a hydroethanolic solvent (150.24 mAU.s^{-1}). The use of acetonitrile as a solvent increased

the total peak area (34.549 mAU.s^{-1}) when compared with the aqueous solvents; however, this is still below the total peak area observed in extracts prepared with a hydroethanolic solvent. This result agrees with research by Garcia-Risco *et al.* [179] which found that SCF extracts of *C. officinalis* exhibited lower antioxidant activity when compared to extracts prepared with 50% v/v ethanol/water.

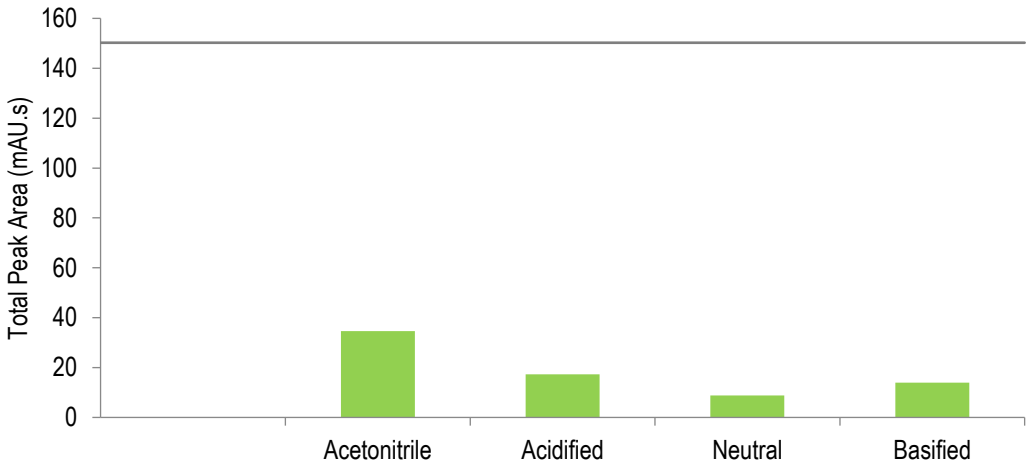


Figure 4-20: Total peak areas of secondary liquid/liquid extracts prepared from SCF residues of C. officinalis *flowers. The line represents the total peak area of a traditional hydroethanolic extract.*

4.4.3 Natural Deep Eutectic Solvent Extracts.

Experiments using NaDES as a solvents were conducted with a view to utilising the increased hydrogen bonding of sugars, acids, and glycerol to increase the extraction of plant metabolites from *C. officinalis.* It was observed during solvent preparation that water was incorporated into the NaDES at a molar ratio ranging from 1:10 water:NaDES (for glucose/glycerol) to 2:1 water:NaDES (for fructose/glucose/citric NaDES), as the final weight of solvent was higher than the sum of the individual sugars and acids used. The total water weight is listed in Appendix 3. Previous work by Choi *et al.* [69] demonstrated that water forms an integral part of some NaDES. Sucrose/fructose, sucrose/glucose, sucrose/glycerol, glucose/citric and glucose/glycerol NaDES precipitated or solidified at room temperature and were excluded from further testing. Solvents prepared with citric acid/sugar combinations changed colour through the dehydration process. It was hypothesised that this was a caramelisation reaction as reported by Chen *et al.* [199] who found that caramelisation reactions can occur in acidic glucose solutions at relatively low temperatures (75-95 ^{O}C). NaDES prepared from sugar/sugar mixtures exhibited visually higher viscosities when compared with sugar/acid, sugar/glycerol, and acid/glycerol composites. This high viscosity resulted in difficulties when handling the extract, and was hypothesised to contribute to the

observed decreases in phytochemical content (likely due to the decreased diffusivity observed with increasing solvent viscosities [167]).

UHPLC Analysis. UHPLC analyses were conducted with a view to determining the phytochemical composition of *C. officinalis* extracts prepared with NaDES. Peaks were selected at 0.8, 8.5, 11.1, 11.2, 11.8, and 12.8 minutes for individual analysis due to clear peak resolution and antioxidant activity determined in Chapter 3.4.1 and labelled A-F respectively (Figure 4-21). Extracts prepared with sugar/sugar and sugar/glycerol composites displayed extracts with similar phytochemical composition to hydroethanolic *C. officinalis* extracts (Figure 4-22). Extracts prepared with sugar/citric acid NaDES exhibited significantly different peaks (Figure 4-23). These were hypothesised to be the result of caramelisation reactions as the reaction conditions used to prepare the sugar/acid NaDES were similar to research by Chen *et al.* [199] who found that temperatures of 75 ^{O}C were sufficient to cause caramelisation in aqueous acidic glucose solutions, forming compounds such as 5-hydroxymethoxylfurfuran. Co-elution between selected peaks was observed between compounds formed during caramelisation and those typically extracted from *C. officinalis*.

Figure 4-24 shows the heights of peak A in *C. officinalis* extracts prepared with NaDES. It can be seen that extracts prepared with NaDES exhibit decreased Peak A heights when compared with extracts prepared with hydroethanolic solvents. These decreases were significant for extracts prepared with glycerol/citric and sucrose/citric NaDES when compared with extracts prepared with hydroethanolic solvents under similar conditions. Extracts prepared with fructose/glucose and fructose/glycerol NaDES exhibit an apparent decrease in Peak A height; however, these were not found to be significant. Decreases in peak heights were observed for later-eluting compounds.

Large variation in peak heights were observed for extracts prepared with fructose/citric and fructose/glucose/citric NaDES. These were observed in all peaks selected for analysis. It is likely that these large errors are due to co-elution of caramelisation products resulting in decreased peak resolution. Fructose/glycerol and fructose/glucose NaDES extracts exhibited reasonable reproducibility and similar concentrations of polar metabolites to hydroethanolic extracts, but exhibited reduced content of later-eluting compounds (Figure 4-25).

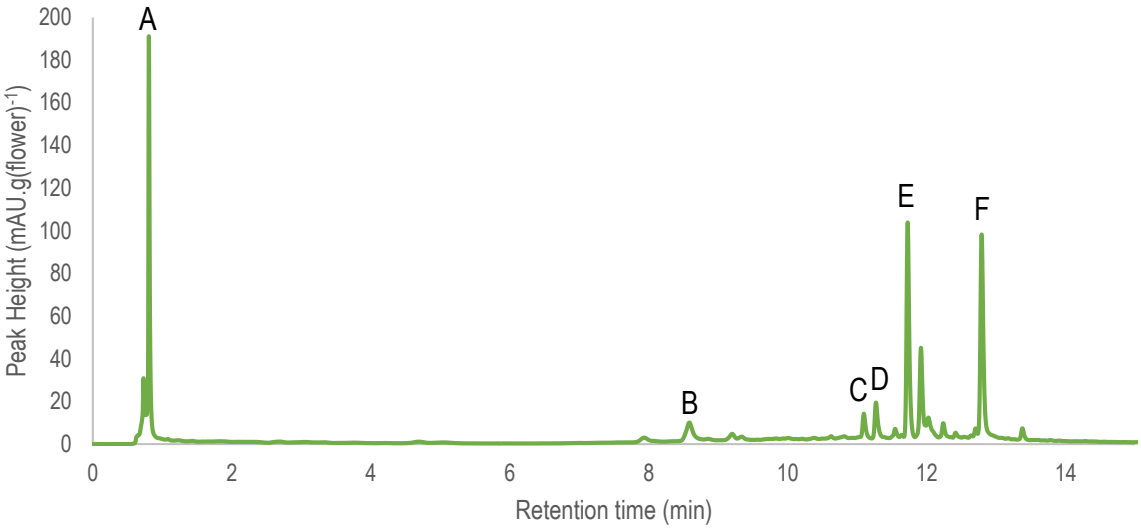


Figure 4-21: UHPLC chromatogram of hydroethanolic C. officinalis *extract. Peaks were selected at 0.8, 8.5, 11.1, 11.2, 11.8, and 12.8 minutes for individual analysis and labelled Peak A-F respectively.*

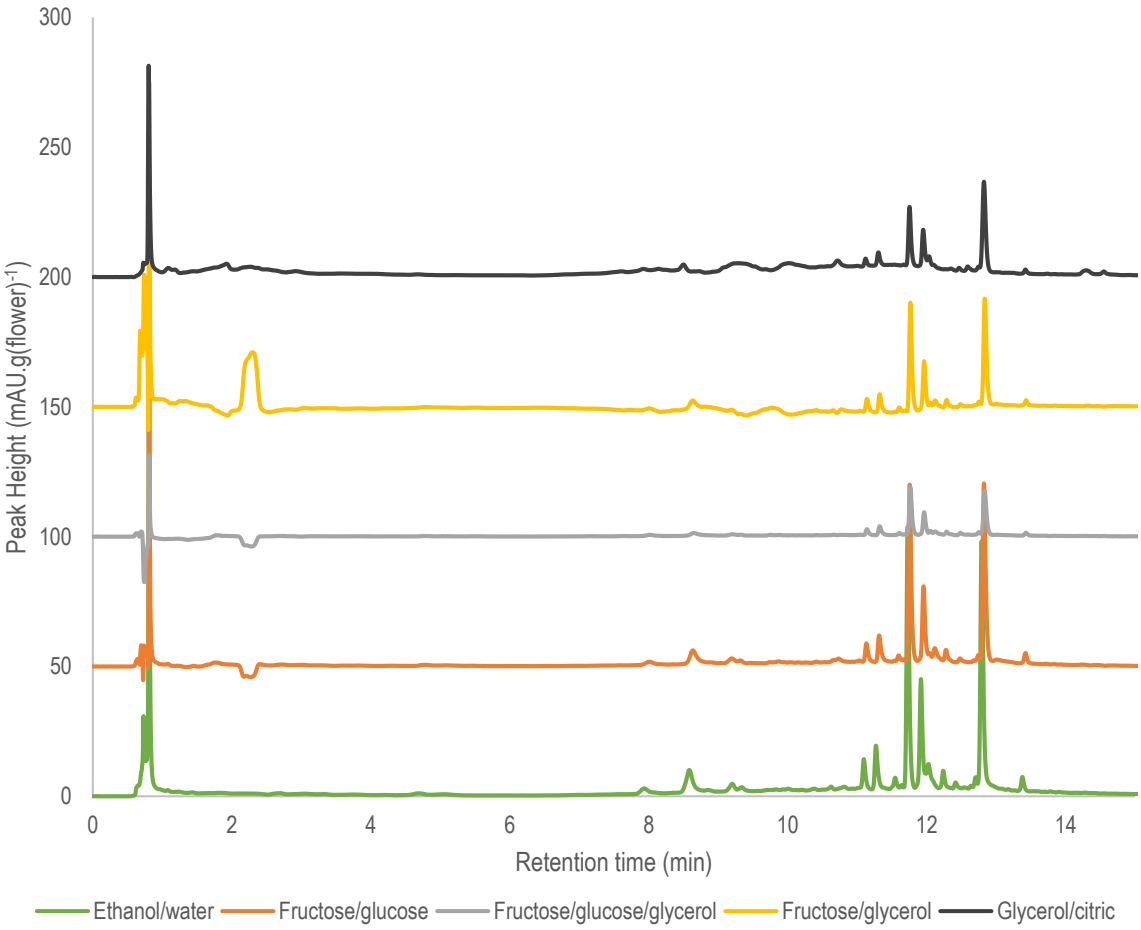


Figure 4-22: UHPLC chromatograms of C. officinalis *extracts prepared with hydroethanolic solvents and NaDES. Sequential chromatograms are offset vertically by 50 mAU.g(flower)$^{-1}$.*

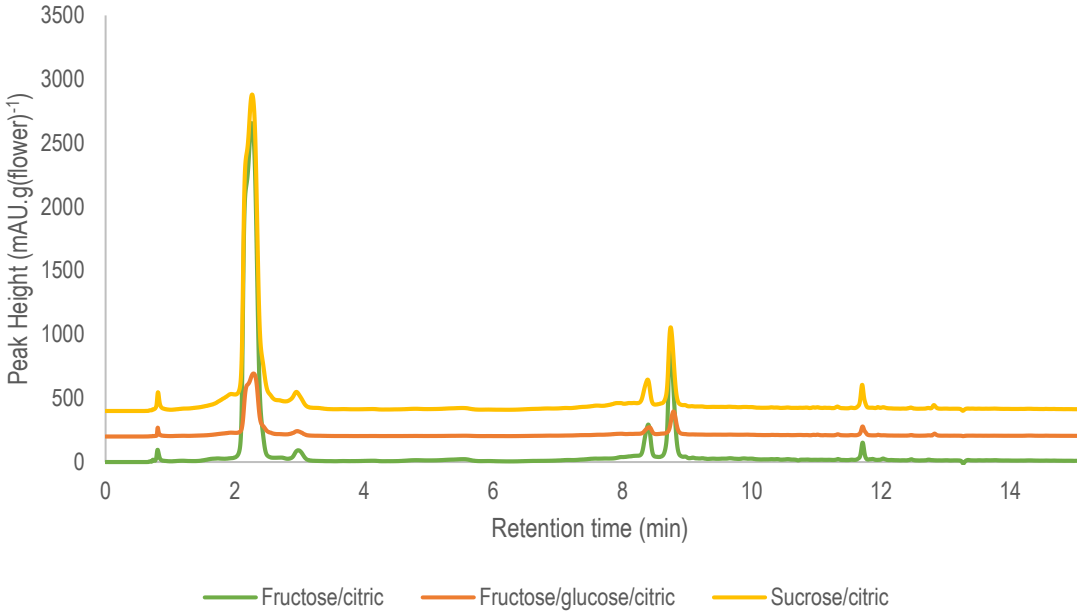


Figure 4-23: UHPLC chromatograms of C. officinalis *extracts prepared with NaDES. Sequential chromatograms are offset vertically by 250mAU.*

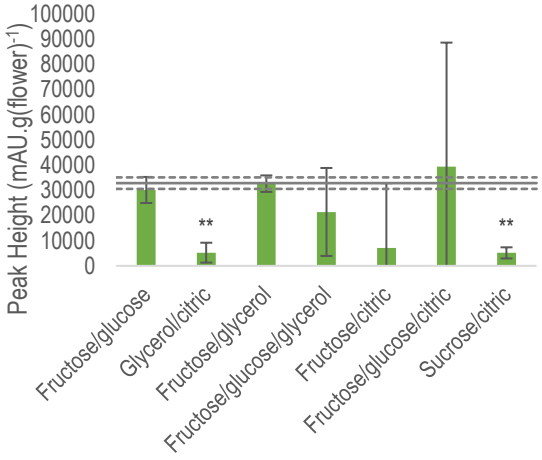


Figure 4-24: Peak A heights in C. officinalis *extracts prepared with NaDES. Data is presented as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with extracts prepared with hydroethanolic solvents. The line represents the Peak A height of hydroethanolic extracts, with dashed lines representing the error margin.*

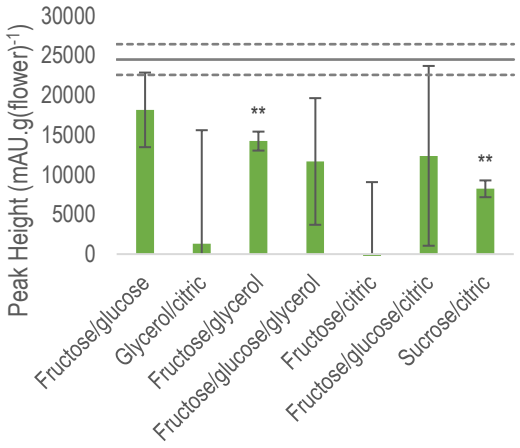


Figure 4-25: Peak F heights in C. officinalis *extracts prepared with NaDES. Data is presented as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with extracts prepared with hydroethanolic solvents. The line represents the Peak F height of hydroethanolic extracts, with dashed lines representing the error margin.*

UHPLC total peak areas were calculated. Extracts prepared with NaDES exhibited decreased total peak area when compared with hydroethanolic extracts. Extracts prepared with NaDES containing citric acid exhibited large variation between replicates; this was hypothesised to be due to the presence of UV-

active caramelisation products such as 5-hydroxymethoxylfurfural [199]. Extracts prepared with sugar/sugar or sugar/glycerol NaDES exhibited significant decreases in total peak area.

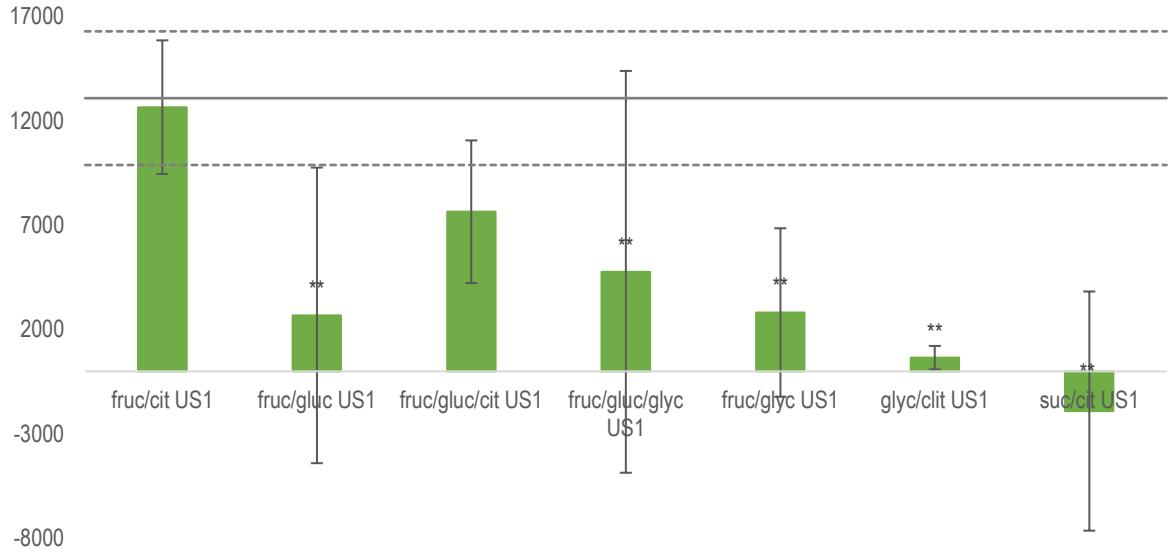


Figure 4-26: Total UHPLC peak area of C. officinalis *extracts prepared with NaDES. Data is presented as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with extracts prepared with hydroethanolic solvents. The line represents the total peak area of hydroethanolic extracts, with dashed lines representing the error margin.*

Chemiluminescence response. SIA-CL assays were conducted with a view to determining the total antioxidant activity of *C. officinalis* extracts prepared with NaDES. Figure 4-27 shows the chemiluminescence response of *C. officinalis* extracts prepared with NaDES containing sugars, acids, glycerol, and water. It can be seen that the chemiluminescence of various eutectic mixtures varies depending on the NaDES used. All *C. officinalis* extracts prepared with NaDES presented equivalent or increased chemiluminescence when compared with hydroethanolic extracts prepared under similar conditions. This was observed to be significantly higher in extracts prepared with sucrose/citric acid (93525 ± 3786.1 mV.s.g(flower)$^{-1}$), fructose/citric acid (89458 ± 4490.2 mV.s.g(flower)$^{-1}$), fructose/glycerol (128220 ± 16782 mV.s.g(flower)$^{-1}$), fructose/glucose/citric acid (120460 ± 900.44 mV.s.g(flower)$^{-1}$), and fructose/glucose (72833 ± 752.59 mV.s.g(flower)$^{-1}$) NaDES when compared with hydroethanolic extracts (68038 ± 2555.3 mV.s.g(flower)$^{-1}$). The greatest increases in chemiluminescence were observed for extracts prepared with fructose/glycerol and fructose/glucose/citric acid solvents. Fructose/glucose/glycerol and glycerol/citric acid extracts presented equivalent chemiluminescence to hydroethanolic extracts. Chen *et al.* [199] found that products from caramelisation reactions in acidic aqueous glucose solutions exhibited antioxidant activity, with increasing sugar concentrations exhibiting increased antioxidant activity. Thus, the significant increase

in chemiluminescence observed in extracts prepared with sucrose/citric, fructose/citric, and fructose/glucose/citric composites are likely due to the presence of caramelisation products that exhibit antioxidant activity.

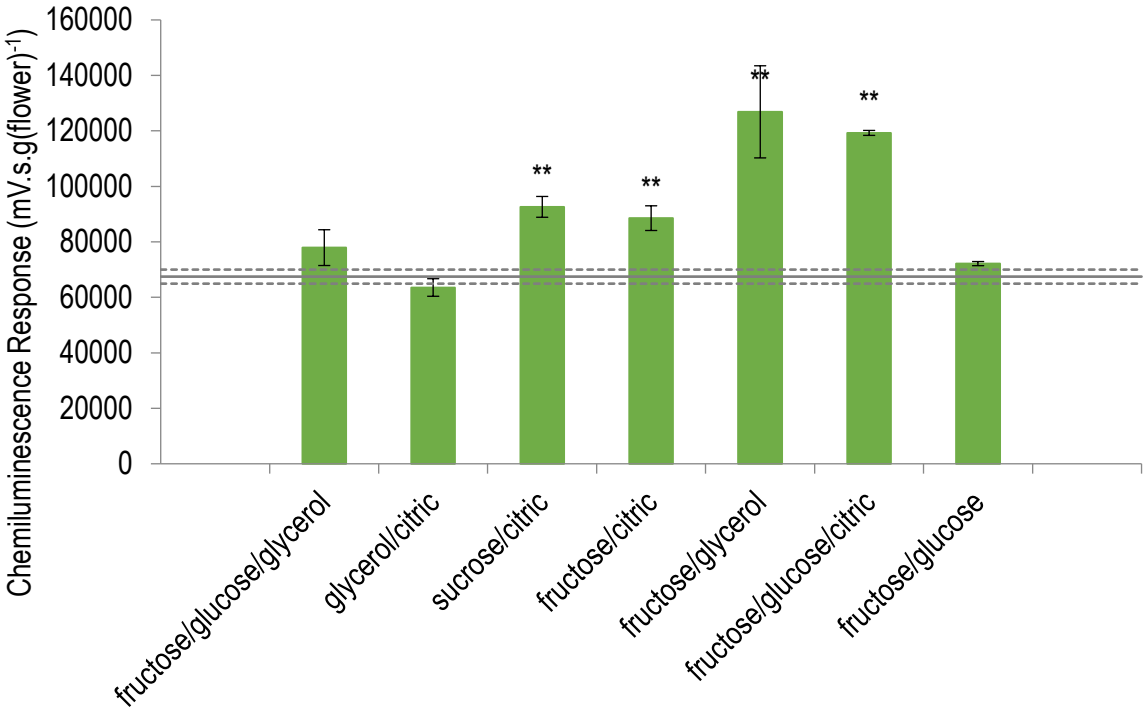


Figure 4-27: Chemiluminescence response of C. officinalis *extracts prepared with NaDES. Data is presented as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with hydroethanolic solvents under similar conditions. The line represents the chemiluminescence response of hydroethanolic extracts, with dashed lines representing the error margin.*

Radical scavenging activity. DPPH assays were conducted with a view to determining the radical scavenging activity of *C. officinalis* extracts prepared with NaDES. Figure 4-28 shows the radical scavenging activity of *C. officinalis* extracts prepared with NaDES containing sugars, acids, glycerol, and water. It can be seen that NaDES extracts prepared using fructose/glucose/glycerol and fructose/glucose/citric acid were observed to give slightly higher radical scavenging activity when compared with hydroethanolic extracts prepared under similar conditions; however, this result was not significant. NaDES extracts prepared using glycerol/citric, fructose/citric, sucrose/citric, fructose/glucose, and fructose/glycerol were observed to give lower radical scavenging activity when compared with hydroethanolic extracts (54.939 ± 8.8579%). This decrease was significant for extracts prepared with fructose/citric (26.304 ± 9.204%) and fructose/glucose (27.708 ± 10.847%) NaDES. Previous research by Bolling *et al.* [200] has previously shown that presence of citric acid can affect the end point of the DPPH radical scavenging assay by up to 30%, and as such the presence of citric acid may influence the

overall radical scavenging activity observed. Additionally, Chen *et al.* [199] found that caramelisation products formed in acidic aqueous glucose solution display DPPH radical scavenging activity which may further alter the radical scavenging activity exhibited by the phytochemicals extracted from *C. officinalis*. It was observed that extracts prepared with composites that contained glycerol demonstrated similar radical scavenging activity to hydroethanolic extracts prepared in a similar manner.

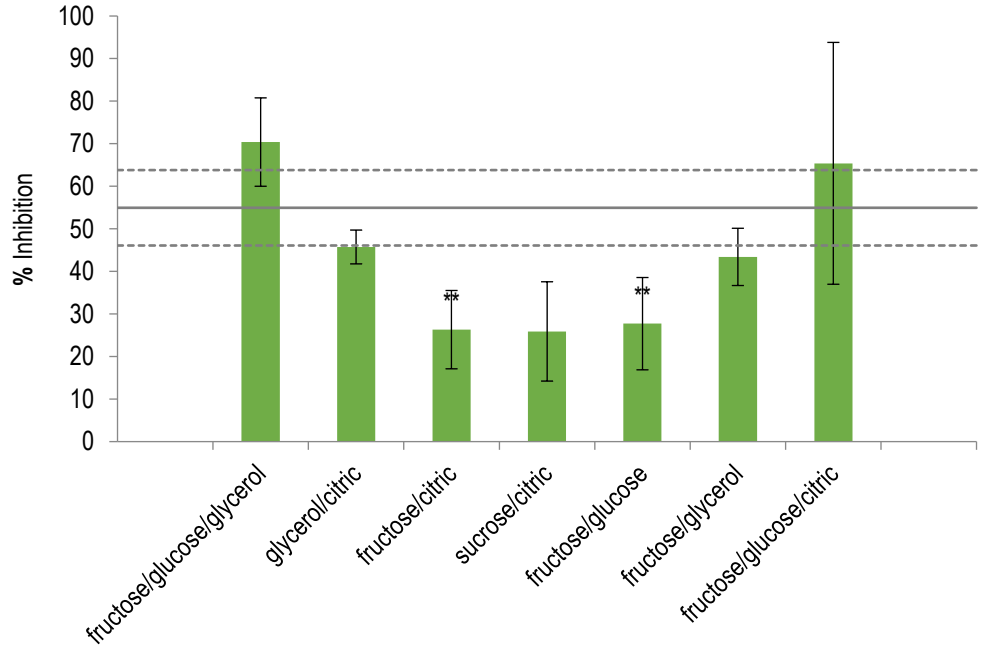


Figure 4-28: Radical scavenging activity of C. officinalis *extracts prepared with NaDES by reaction with DPPH radicals. Data is presented as means ± standard deviation of replicates (n=9). Asterisks indicate statistical significance ($p<0.05$) when compared with hydroethanolic extracts prepared under similar conditions. The line represents the radical scavenging activity of hydroethanolic extracts, with dashed lines representing the error margin.*

Phenolic Content. Folin-Ciocalteu assays were conducted with a view to determining the total phenolic content of *C. officinalis* extracts prepared with NaDES. Figure 4-29 shows the phenolic content of *C. officinalis* extracts prepared with NaDES containing sugars, acids, glycerol, and water. It can be seen that there is significantly lower phenolic content observed in extracts prepared with fructose/glucose/glycerol (5.759 ± 0.73938 mg(GAE).g(flower)$^{-1}$), glycerol/citric (4.8949 ± 0.32675 mg(GAE).g(flower)$^{-1}$) and fructose/glycerol (6.2793 ± 1.0851 mg(GAE).g(flower)$^{-1}$) solvents when compared with hydroethanolic extracts prepared under similar conditions (8.4079 ± 0.56619 mg(GAE).g(flower)$^{-1}$). Extracts prepared with fructose/glucose/citric NaDES exhibited slightly higher phenolic content, however this was not significant. Significant increases in phenolic content were observed for extracts prepared with sucrose/citric (3.7942 ± 0.40969 mg(GAE).g(flower)$^{-1}$), fructose/citric (3.6797 ± 0.54783 mg(GAE).g(flower)$^{-1}$) and fructose/glucose (9.4744 ± 0.87755 mg(GAE).g(flower)$^{-1}$)

NaDES. Citric and tartaric acids were found to have no significant effect on the Folin-Ciocalteu assay used (Appendix 3).

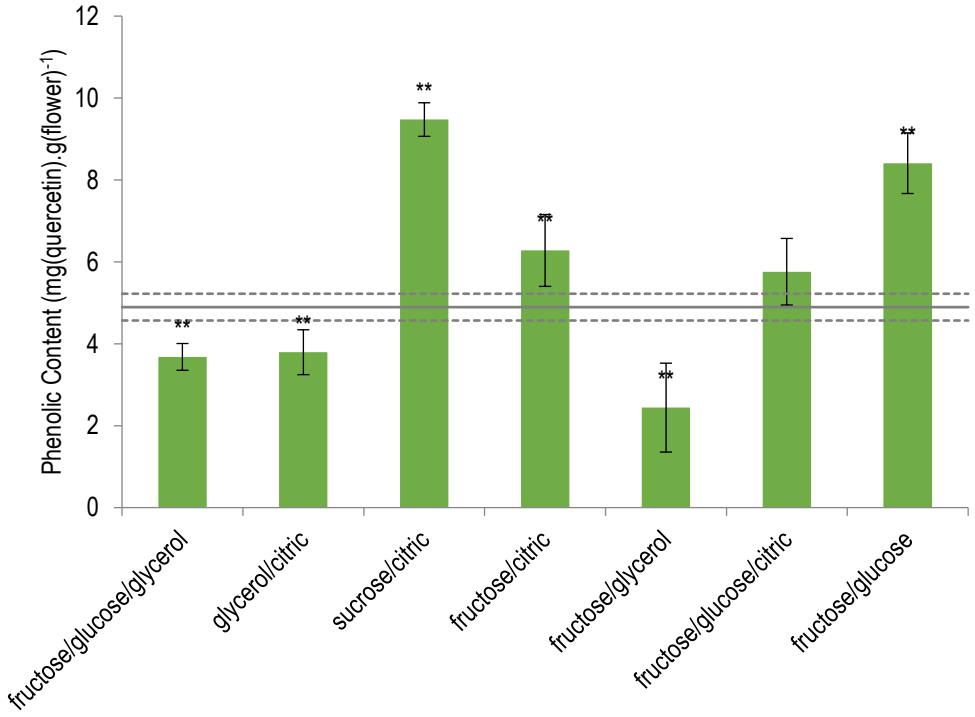


Figure 4-29: Total phenolic content of C. officinalis *extracts prepared with NaDES as determined by Folin-Ciocalteu assays. Data is presented as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with a hydroethanolic extract prepared under similar conditions. The line represents the total phenolic content of hydroethanolic extracts, with dashed lines representing the error margin.*

4.5 SUMMARY

Aqueous acids and bases were investigated as a potential replacement for aqueous ethanol solvents in the extraction of plant metabolites from *C. officinalis. C. officinalis* extracts prepared with aqueous acids or bases as solvents exhibited decreased phytochemical content and radical scavenging activity when compared with a traditional hydroethanolic solvent extract prepared under identical conditions. UHPLC analysis of reference extracts exhibited a number of phytochemicals that were absent in extracts prepared with aqueous acids or bases. Extracts prepared with pH 0-13 demonstrated reduced total phytochemical content. The large increase in total phytochemical content observed in pH 14 extracts was hypothesised to be the result of degradation of *C. officinalis* plant material. This decrease in phytochemical content was also observed in the decreased radical scavenging activity of the extracts. Folin-Ciocalteu assays demonstrated an increase in phenolic content with concentrated base as a solvent; however, these concentrations were hazardous and likely due to degradation of flower material rather than an increase in metabolite extraction. It was hypothesised that the overall decrease in total peak area and extracted compounds were due to the lack of an organic component with a low dielectric constant which reduced the extraction of less-polar phytochemicals. It can be concluded that neither aqueous acid nor base are adequate replacements for hydroethanolic solvents.

Supercritical CO_2 was investigated as a potential 'green' solvent for the extraction of *C. officinalis* metabolites. It was found that even though supercritical CO_2 could be used as a green solvent, the resulting SCF residue was only soluble in 'non-green' solvents. UHPLC analysis of secondary liquid/liquid extracts with aqueous and acetonitrile solvents demonstrated that negligible metabolites could be recovered from the SCF residue. It can be concluded that supercritical CO_2 is not an adequate replacement for hydroethanolic solvents.

C. officinalis extracts prepared with sugar/sugar and sugar/glycerol NaDES demonstrated similar phytochemical composition to hydroethanolic extracts prepared under similar conditions. NaDES extracts prepared from sugar/acid mixtures exhibited equivalent or greater antioxidant activity (Figure 4-27) and radical scavenging activity (Figure 4-28) when compared with hydroethanolic extracts prepared under similar conditions. However, these NaDES prepared exhibited low-temperature caramelisation products which have previously been reported to exhibit antioxidant and radical scavenging activity [199] which eliminated sugar/acid NaDES as potential alternative solvents. NaDES containing glycerol also exhibited equivalent or greater antioxidant activity and DPPH radical scavenging activity and did not exhibit caramelisation products. It can therefore be concluded that NaDES prepared from naturally occurring

compounds such as sugar/glycerol and acid/glycerol show potential as replacement solvents for the extraction of phytochemicals from *C. officinalis* when compared to hydroethanolic solvents. However, the low workability due to the observed high NaDES viscosities precluded their use as novel solvents.

5 GLYCEROL-BASED COMPOSITES AS SOLVENTS FOR PHYTOCHEMICAL EXTRACTIONS

5.1 CHAPTER SYNOPSIS

This chapter explores of the use of glycerol composites prepared with chaotropic and kosmotropic salts, organic acids, and sugars as alternative 'green' solvents for ultrasonic extraction of phytochemicals from *C. officinalis*. Dried *C. officinalis* flowers were used as a model plant material due to their high content of antioxidant and polyphenolic phytochemicals. Total phytochemical content and individual phytochemical concentrations were determined by UHPLC-DAD analysis. Total antioxidant activity, radical scavenging activity, and total phenolic content were determined by $KMnO_4$ chemiluminescence, DPPH, and Folin-Ciocalteu assays respectively. UHPLC-DAD, DPPH, Folin-Ciocalteu, and SIA-CL analyses showed that *C. officinalis* extracts prepared with glycerol composites were observed to contain higher quantities of extracted components when compared with extracts prepared with neat glycerol. Strong correlations were observed between individual and total peak areas, chemiluminescence, DPPH radical scavenging activity, phenol content, and chaotropicity for extracts prepared with sugar/glycerol composites.

5.2 INTRODUCTION

Glycerol (Figure 5-1) is a trihydric alcohol that occurs naturally in certain organisms [201] and is also a common by-product of biofuel synthesis [202]. It is considered nonhazardous due to its low vapour pressure, flammability, and toxicity [203]. Glycerol forms very strong and long-lasting hydrogen bonds when compared with ethanol or water [204]. The influence of these hydrogen bonds is demonstrated by their low temperature behaviours; glycerol and ethanol both display polymer-like glass transitions [205, 206]. The hydrogen bonding strength is also reflected in the solvent viscosities; glycerol is substantially more viscous than either water or ethanol (934 mPa.s, 0.890 mPa.s, and 1.074 mPa.s respectively [194]). The dielectric constant of glycerol (46.5 a.u.) is greater than that of ethanol (25.3 a.u.) and less than that of water (80.1 a.u.) [194]. Glycerol contains a number of hydrogen bonding sites that allow for up to six intermolecular interactions (3 HBD and 3 HBA). The use of pure glycerol as a solvent for extraction of plant metabolites has received limited attention; however, heated aqueous glycerol solvents have been used as replacements to hydroalcoholic solvents in the extraction of polyphenols from *Olea europaea* leaf [207].

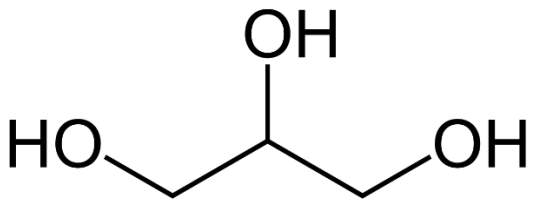


Figure 5-1: The molecular structure of glycerol (1,2,3-propanetriol)

Chaotropic and kosmotropic substances are known to influence diffusion and solubility in aqueous solutions. Chaotropic salts are weakly electronegative ionic compounds that cause perturbation of hydrogen bonds within a solvent through bonding with solute molecules or modifying the properties of the solvent itself [208]. In cells, this disruption of hydrogen bonding can result in lysing of enzymes and cell membranes, which can increase intracellular diffusion. The addition of chaotropic agents has been suggested to increase the solubility of nonpolar functionalities in aqueous systems by reducing the hydrophobic effect that occurs between nonpolar functionalities and water [208]. Examples of chaotropic salts include LiCl and $MgCl_2$. Conversely, kosmotropic compounds decrease the solubility of nonpolar compounds in aqueous systems [209]. Previous research by Madrona *et al.* [210] has shown that the addition of kosmotropic salt to aqueous systems can influence intermolecular interactions of coagulants in the treatment of wastewater, resulting in increased coagulation at high saline levels. Examples of kosmotropic salts include KCl and NaCl. Naser *et al.* [211] previously prepared DES using K_2CO_3/glycerol composites that showed similar physical properties to other DES. Abbott *et al.* [212] found that sodium salts formed homogenous liquids with glycerol that did not display eutectic behaviour, but did show similar physical properties to DES. It was hypothesised that salt/glycerol composites could therefore be used as alternative 'green' solvents for phytochemical extraction, with the degree of chaotropicity or kosmotropicity influencing the hydrogen bonding properties of the solvent and therefore the solvent's ability to extract phytochemicals.

Table 5-1: Calculated chaotropicities of salt/glycerol composites using data from Cray et al. [213]. *Units are $kJ.kg^{-1}.mol^{-1}$.*

Salt Concentration (‰ mol/mol)	*LiCl/glycerol*	*NaCl/glycerol*	*KCl/glycerol*	*$MgCl_2$/glycerol*
0‰	6.340	6.340	6.340	6.340
20‰	6.746	6.120	6.114	7.420
40‰	7.152	5.900	5.888	8.500
60‰	7.558	5.680	5.662	9.580
80‰	7.964	5.460	5.436	10.66
100‰	8.370	5.240	5.210	11.74

Protonation of compounds through the addition of acid is a common technique in liquid-liquid extraction to increase the solubility of organic compounds that contain acidic functional groups in a nonpolar liquid phase [214]. Weak acids are molecules with acidic functionalities that partially dissociate in aqueous systems (as opposed to strong acids which dissociate completely). The addition of acid can be used to protonate compounds that contain basic functional groups such as amines in order to increase their solubility in aqueous phases [214]. The addition of weak acid at sufficiently high concentrations is also known to influence the extraction of compounds in aqueous systems through acid hydrolysis. Acid hydrolysis has previously been used in the extraction of pectins from a range of plant materials including cacao husk [215]. Pectic polysaccharides are present in the primary cell wall and middle lamellar in plant cells [216]. Acid hydrolysis cleaves the less water-soluble pectic polysaccharides into smaller, more soluble pectin molecules. Weak acids such as citric acid have shown efficacy in increasing the extraction of pectin [217]. Examples of weak acids include citric acid and tartaric acid.

Sugars have been found to affect the stability of macromolecules in aqueous systems through chaotropic or kosmotropic effects [218], which in turn affect the strength of hydrophobic inter- and intramolecular interactions [208]. Sugar molecules have a large number of hydrogen bonding sites available that allow for multiple intermolecular interactions. Simple sugars such as sucrose, glucose, and fructose have been used to increase the stability of phenolic compounds in apple puree during long term storage [219]. There is limited research on the effect of sugar addition on extraction of phytochemicals for plant material. Fructose exists *in aquo* as a mixture of five different isomers; α-furanose, β-furanose, α-pyranose, β-pyranose, and ketohexose [220]. Glucose similarly exists *in aquo* as α-D-glucopyranose, β-D-glucopyranose, α-D-glucofuranose, β-D-glucofuranose, and as an open chain [221]. The most abundant Fructose isomers in aquo are β-furanose and β-pyranose. The most abundant glucose isomers in aquo are β-D-glucopyranose and α-D-glucopyranose [222].

Table 5-2: Calculated chaotropicities of sugar/glycerol composites using data from Cray et al. [213]. *Units are kJ.kg^{-1}.mol^{-1}.*

Sugar Concentration (‰ mol/mol)	*Sucrose/glycerol*	*Fructose/glycerol*	*Glucose/glycerol*
0‰	6.340	6.340	6.340
1‰	6.333	6.345	6.341
8‰	6.285	6.377	6.350
12‰	6.257	6.395	6.354
20‰	6.202	6.431	6.364

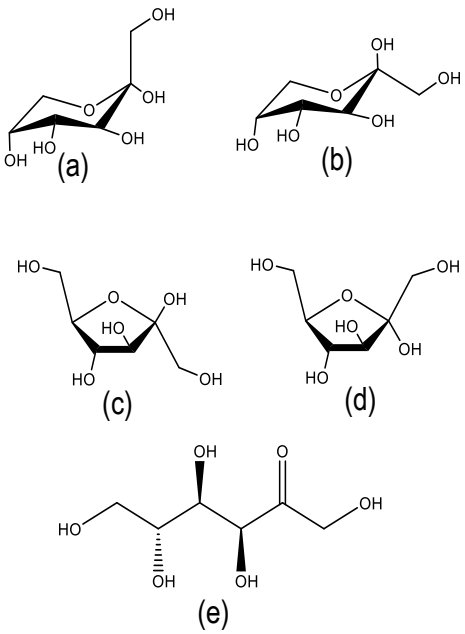


Figure 5-2: Isomers of fructose as a) α- furanose, b) β- furanose, c) α-pyranose, d) β-pyranose, and e) ketohexose [220].

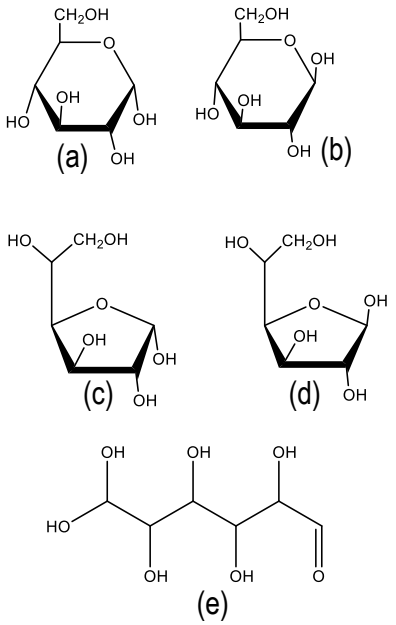


Figure 5-3: Isomers of glucose as a) α-D-glucofuranose, b) β-D-glucofuranose, c) α-D-glucopyranose, d) β-D-glucopyranose, and e) open-chain form [221].

This chapter presents investigations into the use of glycerol composites as alternative 'green' solvents in the extraction of *C. officinalis* metabolites. LiCl and $MgCl_2$ were chosen as additives due to their well-characterised chaotropic nature [213]. NaCl and KCl were chosen as additives due to their well-characterised kosmotropic nature [213] and low hazard. It was hypothesised that the addition of LiCl or $MgCl_2$ to the glycerol solvent could cause denaturation of the plant cell membrane through chaotropic effects. By denaturing the plant cell membranes, diffusion of intracellular glycerol would be increased which in turn would increase the extracellular diffusion of phytochemicals into glycerol. NaCl and KCl salts were also tested to compare the effect of kosmotropic agents to chaotropic agents and it was hypothesised that kosmotropic salts would not enhance phytochemical extraction. Citric and tartaric acids were chosen as additives due to their environmentally benign properties (such as low cost and hazard), and the favourable effect of citric acid upon the extraction of pectin [215, 217], and it was hypothesised that the addition of organic acids to glycerol would cause increased degradation of the plant cell structure through acid hydrolysis, allowing for increased metabolite diffusion into the glycerol solvent. Additionally, the addition of acid was hypothesised to result in protonation of basic (deprotonated) phenol moieties, increasing hydrogen bonding interactions between phenolic phytochemicals and the glycerol solvent. Sucrose, fructose, and glucose were chosen as additives due to their low hazard, large number of hydrogen bonding sites, and relative abundance, and it was hypothesised that the addition of sugar to the glycerol solvent would increase the number of available hydrogen bonding sites present in the solvent, allowing for greater intermolecular interaction with the metabolites in *C. officinalis*. Given that glycerol is

an effective extraction medium for phytochemicals, it was hypothesised that the addition of inorganic salts, organic acids, and sugars to glycerol would influence the intermolecular interactions between the solvent and the desired phytochemicals to further improve extraction yields.

5.3 EXPERIMENTAL

Calendula officinalis samples. Dried *C. officinalis* flower heads were used as supplied. Flowers were grown in the Adelaide Hills, South Australia on a certified biodynamic farm (Jurlique International, certified under the National Association for Sustainable Agriculture, Australia), hand-picked and air-dried in sheds. Dried *C. officinalis* was ground using a commercial grinder (Sunbeam Coffee Grinder) and stored in sealed plastic containers in darkness at ambient conditions prior to use.

Chemicals and Reagents. Ethanol (99.96%), NaCl (99%), and KCl (99%) were purchased from Chem-Supply (Gillman, SA, AUS). Tartaric and citric acids (Food grade, McKenzie's Foods), sucrose (Food grade, CSR Sugar Australia), fructose (Food grade, Fruisana) and glucose (Food grade, Glucodin) were purchased from local supermarkets. Centrifuge vials (10 mL, polypropylene) and storage vials (3 mL, polypropylene) were purchased from Sarstedt (Nümbrecht, DE). Plastic syringes (1 & 5 mL, polypropylene) and cotton wool were purchased from Livingstone (Rosebery, NSW, AUS). Glycerol (99.5%) was supplied by Jurlique International (Mount Barker, SA, AUS). LiCl (>99%) was purchased from Sigma-Aldritch (St Louis, MO, USA). $MgCl_2$ was purchased from Merck (Kilsyth, VIC, AUS). Deionised water was purified to 18 MΩ using a Thermo Fisher Scientific (Waltham, MA, USA) Barnstead™ E-Pure™ water system.

Preparation of hydroethanolic extract. Hydroethanolic solvent was prepared by titrating ethanol with deionised water to 30% v/v. Extracts were prepared in triplicate by combining *C. officinalis* (0.15 g) with solvent (3 g) with subsequent ultrasonication (Elmasonic S30, 120 min) and centrifugation (Clements Orbital 325, 3000 rpm, 30 min). Extracts were then filtered through a syringe packed with clean cotton wool. Process blanks were prepared and analysed concurrently using identical techniques. Samples and blanks were diluted with hydroethanolic solvent prior to analysis.

Inorganic glycerol composite extract preparation. LiCl, KCl, NaCl, and $MgCl_2$ were added to glycerol to a final concentration of 20, 40, 60, 80, and 100‰ mol/mol. Solvents were heated (70 ^{O}C) with stirring until a clear, homogenous solution formed. Composites were then stored in darkness under ambient conditions until use. Extracts were prepared in triplicate by combining *C. officinalis* (0.15 g) with the salt/glycerol composites (3 g) with subsequent ultrasonication (Elmasonic S30, 120 min) and centrifugation (Clements Orbital 325, 3000 rpm, 30 min). Extracts were then filtered through a syringe packed with clean cotton wool. Process blanks were prepared concurrently. Extracts were stored in

darkness at ambient conditions prior to analysis. Samples and blanks were diluted with hydroethanolic solvent (30% v/v) prior to analysis to adjust viscosity.

Organic glycerol composite extract preparation. Citric acid, tartaric acid, sucrose, glucose, and fructose were added to glycerol to a final concentration of 1, 8, 12, 20, 40, and 60‰ mol/mol. Solvents were heated (70 ^{O}C) with stirring until a clear, homogenous solution formed. Heating yielded 30 different composites. Composites were then stored in darkness under ambient conditions until use. Extracts were prepared in triplicate by combining *C. officinalis* (0.25 g) with the inorganic glycerol composites (5 g) with subsequent ultrasonication (Elmasonic S30, 120 min) and centrifugation (Clements Orbital 325, 3000 rpm, 30 min). Extracts were then filtered through a syringe packed with clean cotton wool. Filtration yielded 90 different samples. Process blanks were prepared concurrently. Extracts were stored in darkness at ambient conditions prior to analysis. Samples and blanks were diluted with hydroethanolic solvent (30% v/v) prior to analysis to adjust viscosity.

Chemiluminescence response. SIA-CL assays were conducted using the method detailed in Chapter 2.3.

Radical Scavenging Activity. DPPH assays were conducted using the method detailed in Chapter 2.4. A reference standard of methanolic quercetin (25 μM) was run concurrently.

Phenolic Content. Folin-Ciocalteu assays were conducted using the method detailed in Chapter 2.5. Reference standards of aqueous gallic acid (10, 20, 30, 40, and 50 mg/mL) were run concurrently.

Statistical Testing. F-testing and T-testing to determine statistical significance was conducted using the method outlined in Chapter 2.6.

5.4 Results & Discussion

5.4.1 Salt/glycerol Composites

Experiments were conducted with a focus on altering the glycerolic solubility of extracted components on the basis of chaotropic and kosmotropic effects. Peaks at 0.8, 8.5, 11.1, 11.2, 11.7, 11.9, and 12.8 min were chosen for further analysis due to clear resolution and antioxidant activity determined in Chapter 3.4.1 (Figure 5-4).

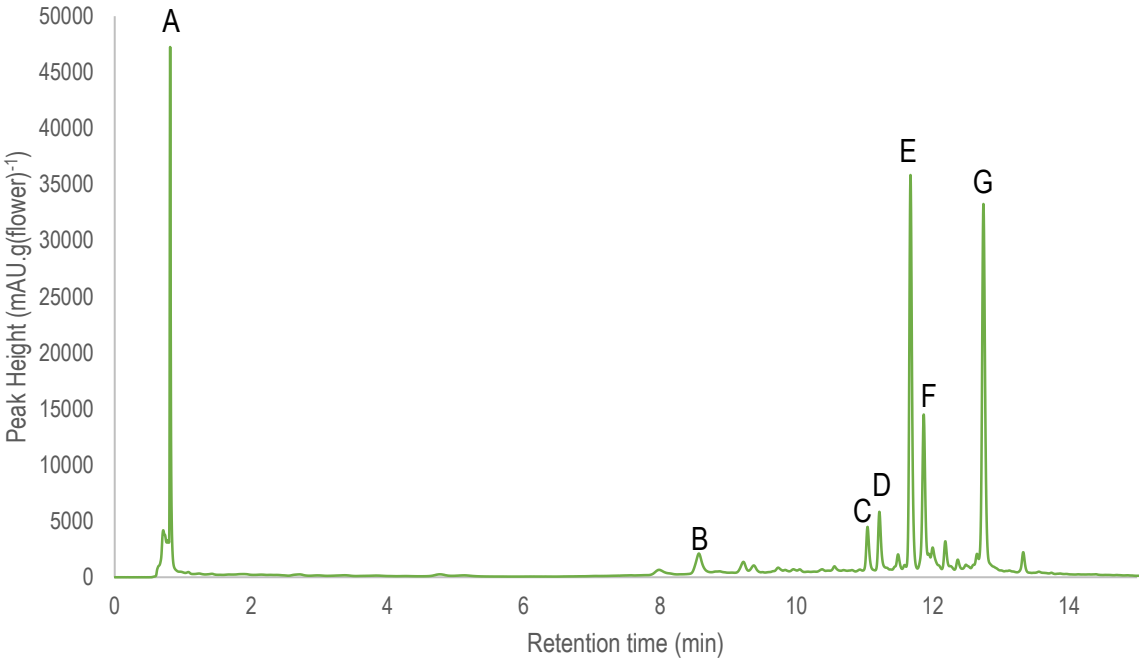


Figure 5-4: UHPLC chromatogram of C. officinalis *extracts prepared with glycerol solvent. Peaks at 0.8, 8.5, 11.1, 11.2, 11.7, 11.9, and 12.8 min were chosen for individual analysis and labelled A-G respectively.*

UHPLC Analysis. Experiments were conducted with a view to determining the concentration of metabolites in *C. officinalis* extracts prepared with salt/glycerol composites. Figure 5-5 shows the phytochemical composition of extracts prepared with a 20‰ mol/mol LiCl/glycerol composite and a hydroethanolic solvent (30% v/v), and it can be seen that the phytochemical composition of an extract prepared with LiCl/glycerol is similar to an extract prepared with hydroethanolic solvents. There is an observable shift in retention time in extracts prepared with hydroethanolic solvent (30% v/v); this was hypothesised to be due to different solvent matrices. Extracts prepared with NaCl/glycerol, KCl/glycerol, and $MgCl_2$/glycerol composites also demonstrated similar phytochemical compositions and similar shifts in retention time.

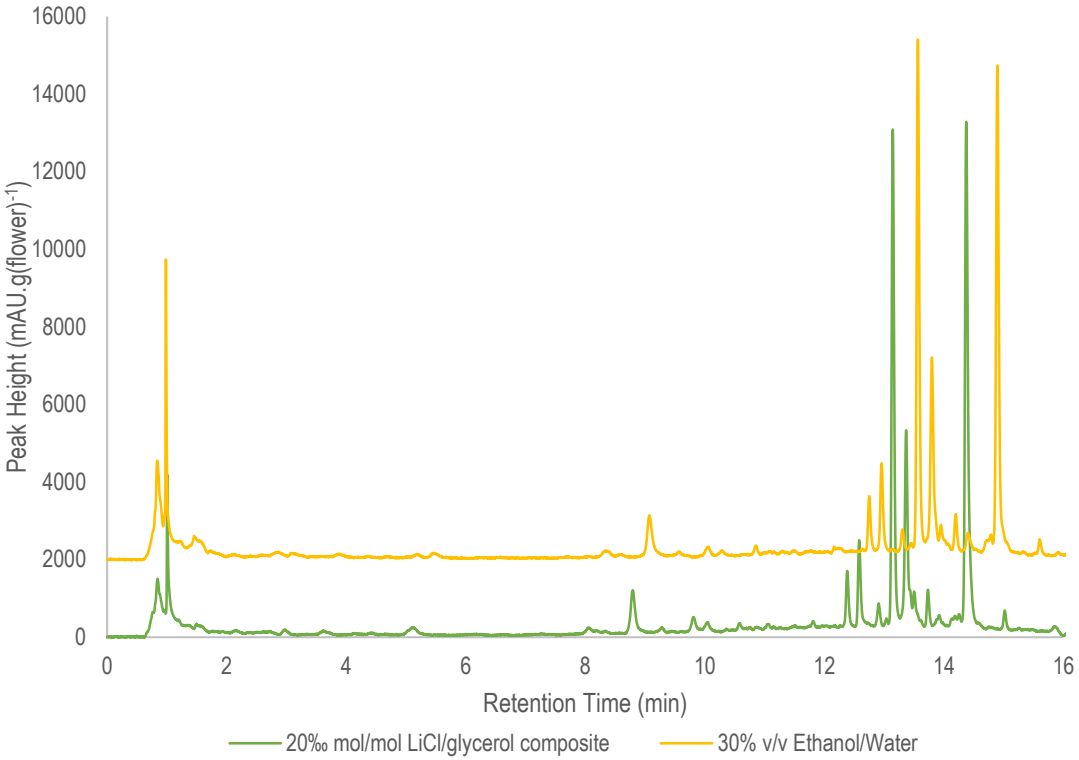


Figure 5-5: UHPLC analysis of C. officinalis *extracts prepared with 20‰ mol/mol LiCl/glycerol composites and hydroethanolic solvents. Sequential chromatograms were offset by 2000 mAU.g(flower)$^{-1}$).*

UHPLC analyses were conducted with a view to quantify the height of individual peaks in *C. officinalis* extracts prepared with salt/glycerol composites. Figure 5-6 shows the peak heights for Peak A in *C. officinalis* extracts prepared with salt/glycerol composites. It can be seen that the height of Peak A decreased in extracts prepared with LiCl/glycerol, NaCl/glycerol, KCl/glycerol, and $MgCl_2$/glycerol composites. These decreases were observed to be significant for extracts prepared with 40‰ and 60‰ mol/mol LiCl/glycerol, 40‰, 80‰, and 100‰ mol/mol NaCl/glycerol, and 100‰ mol/mol KCl/glycerol composites, with extracts prepared with composites containing higher concentrations of salt generally exhibiting lower peak heights. Maximum decreases in Peak A height were observed for extracts prepared with 60‰ mol/mol LiCl (1930.5 ± 112.30 mAU.g(flower)$^{-1}$), 100‰ mol/mol NaCl (1945.9 ± 532.09 mAU.g(flower)$^{-1}$), and 20‰ mol/mol KCl (1512.6 ± 523.45 mAU.g(flower)$^{-1}$) when compared with extracts prepared with neat glycerol (4738.6 ± 1734.6 mAU.g(flower)$^{-1}$). An apparent increase in Peak A heights were observed in extracts prepared with 20‰ and 40‰ mol/mol $MgCl_2$/glycerol composites; however, these increases were not found to be significant. Decreases in Peak A height were observed in extracts prepared with 60-100‰ mol/mol $MgCl_2$/glycerol composites, and were significant for extracts prepared with 100‰ mol/mol $MgCl_2$/glycerol (2088.3 ± 526.03 mAU.g(flower)$^{-1}$) when compared with extracts prepared with neat glycerol. A poor correlation (R=0.22) was observed

between Peak A heights and solvent chaotropicity, indicating that solvent chaotropicity is not a good indicator for the extraction of unretained plant metabolites

Decreases were also observed in later eluting compounds. Figure 5-7 shows the peak heights for Peak B in *C. officinalis* extracts prepared with salt/glycerol composites. It can be seen that the height of Peak B decreased in extracts prepared with LiCl/glycerol, NaCl/glycerol, KCl/glycerol, and $MgCl_2$/glycerol composites. These decreases were observed to be significant for extracts prepared with 40-100‰ mol/mol LiCl, 20-100‰ mol/mol NaCl, and 100‰ mol/mol $MgCl_2$. Decreases observed in extracts prepared with KCl/glycerol composites were not found to be significant. Maximum decreases were observed in extracts prepared with 100‰ mol/mol LiCl (666.48 ± 189.93 mAU.g(flower)$^{-1}$), 100‰ mol/mol NaCl (664.53 ± 172.54 mAU.g(flower)$^{-1}$), and 100‰ mol/mol $MgCl_2$ (718.72 ± 200.57 mAU.g(flower)$^{-1}$) when compared with extracts prepared with neat glycerol (1150.1 ± 279.55 mAU.g(flower)$^{-1}$). Apparent increases in Peak B height were observed in extracts prepared with 20-40‰ mol/mol $MgCl_2$; however, these were not found to be significant. All salt/glycerol composite extracts demonstrated similar behaviour, with extracts prepared with higher salt concentrations exhibiting larger decreases in Peak B height. No correlation (R= 0.04) was observed between Peak B heights and solvent chaotropicity, indicating that solvent chaotropicity is not a good indicator for the extraction of plant metabolites. Similar significant decreases and poor correlations were observed in peaks C-G (Appendix B), indicating that the use of salt/glycerol composites inhibits phytochemical extraction irrespective of solvent chaotropicity or kosmotropicity.

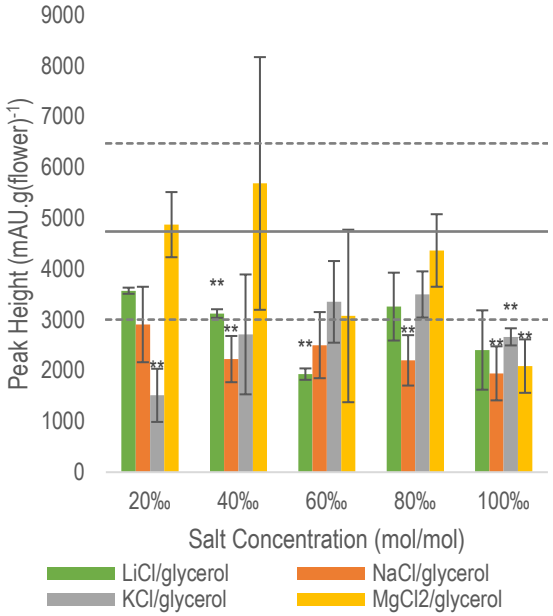


Figure 5-6: Peak heights for Peak A in C. officinalis *extracts prepared with salt/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the height of Peak A in glycerolic extracts, with dashed lines representing the error margin.*

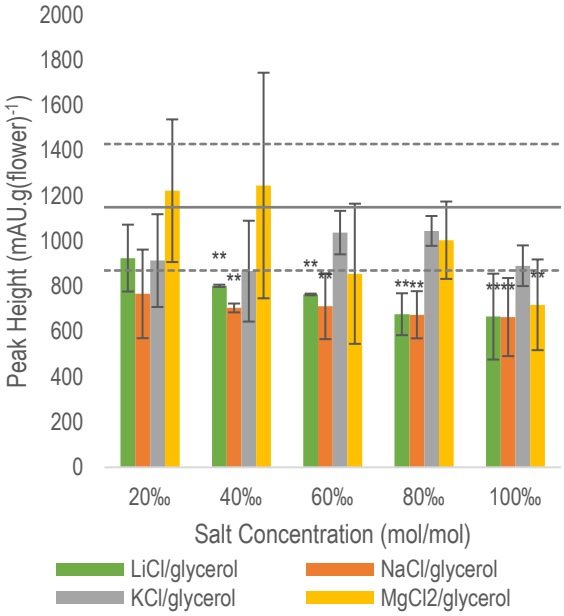


Figure 5-7: Peak heights for Peak B in C. officinalis *extracts prepared with salt/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the height of Peak B in glycerolic extracts, with dashed lines representing the error margin.*

Total Peak Area. Experiments were conducted with a view to determining the total peak area of *C. officinalis* extracts prepared with salt/glycerol solvents. Figure 5-8 shows the total peak areas of that *C. officinalis* extracts prepared with chaotropic salts. Figure 5-9 shows the total peak areas of that *C. officinalis* extracts prepared with kosmotropic salts. It can be seen from Figure 5-8 that 20‰ LiCl/glycerol extracts appear to give a slight increase when compared with an extract prepared from pure glycerol; however, this result is not significant. Similarly, 20‰ $MgCl_2$/glycerol extracts give an apparent increase when compared with extracts prepared in a similar fashion from pure glycerol, but were also not significant. Extracts prepared with kosmotropic salts (Figure 5-9) and glycerol appear to give slight decreases in total peak area. 20‰ KCl/glycerol extracts appear to show a slight decrease when compared with an extract prepared from pure glycerol; however, this result is not significant. 20‰ NaCl/glycerol extracts demonstrate a significant decrease (5721 ± 1436 mAU.s.g(flower)$^{-1}$) when compared with extracts prepared in a similar fashion from pure glycerol (2239 ± 475.8 mAU.s.g(flower)$^{-1}$). When comparing salt/glycerol extracts only, the total peak area presents very weak to moderate negative correlations (R=-0.67, -0.05, -0.63, and -0.29 for LiCl/glycerol, NaCl/glycerol, KCl/glycerol, and $MgCl_2$/glycerol composites respectively) with the chaotropicity of the solvent, with the more chaotropic

$MgCl_2$/glycerol solvent showing a greater increase in phytochemical extraction than the less chaotropic LiCl/glycerol solvent. Figure 5-10 is given as an example of this trend. Full information is available in Appendix 4. From Figure 5-10, it can be inferred that increasing salt concentrations result in a decrease in total peak area.

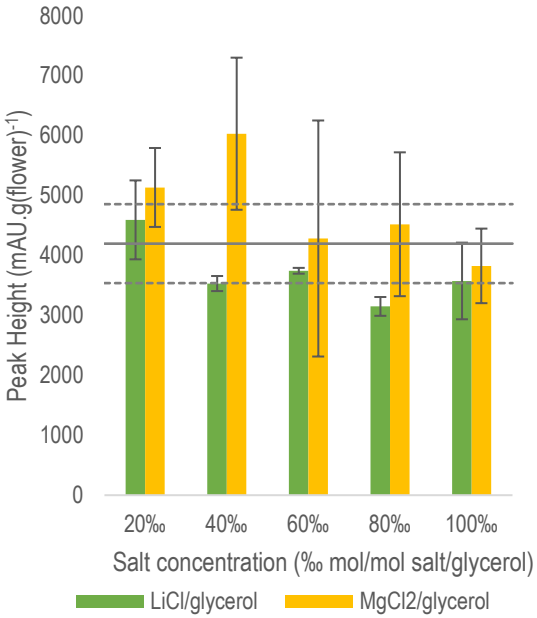


Figure 5-8: Total peak areas of C. officinalis *extracts prepared with chaotrope/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the total peak area of glycerolic extracts, with dashed lines representing the error margin.*

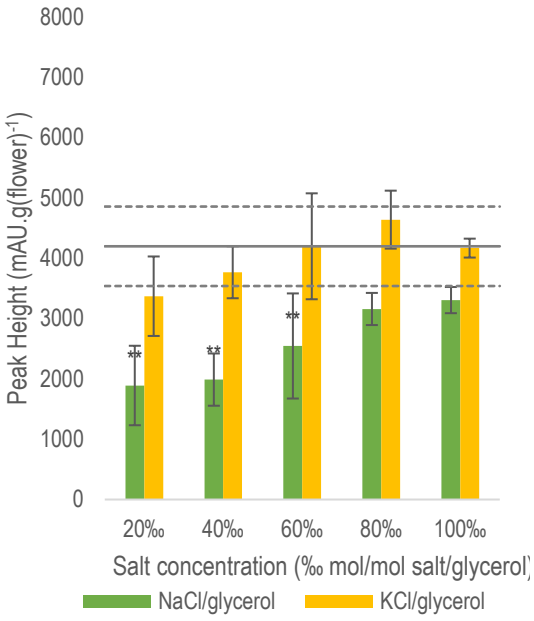


Figure 5-9: Total peak areas of C. officinalis *extracts prepared with kosmotrope/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the total peak area of glycerolic extracts, with dashed lines representing the error margin.*

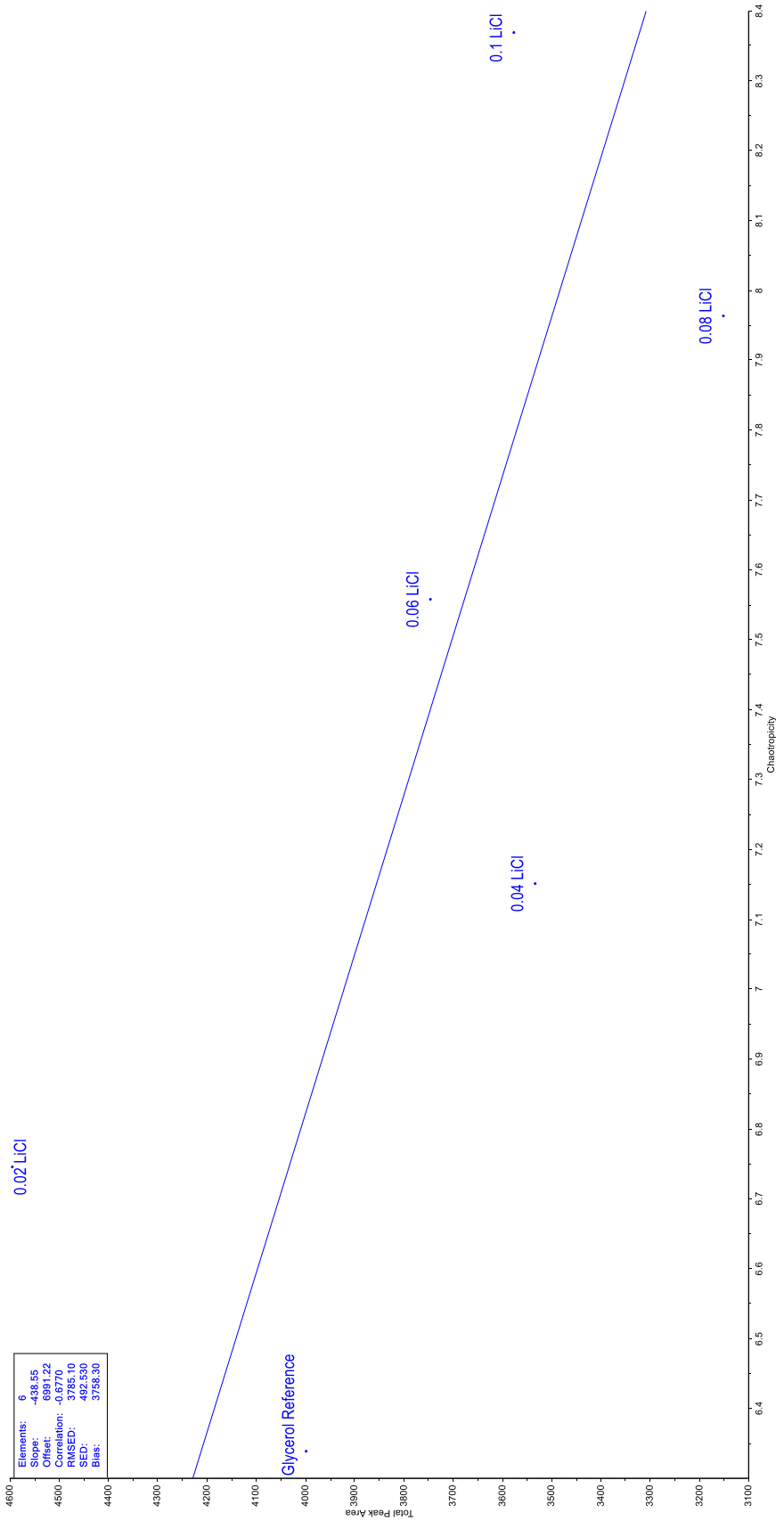


Figure 5-10: Unscrambler® correlation between total peak area and chaotropicity of C. officinalis *extracts prepared with LiCl/glycerol composites. Data is presented as means of replicates (n=3).*

Chemiluminescence response. Experiments were conducted with a view to determining the chemiluminescence response of *C. officinalis* extracts prepared with salt/glycerol composites. Figure 5-11 shows the chemiluminescence response of *C. officinalis* extracts prepared with salt/glycerol composites. It can be seen that there is a significant decrease in chemiluminescence response for extracts prepared using glycerol containing 20‰ LiCl (289.4 ± 70.70 V.s.g(flower)$^{-1}$) or 20‰ NaCl (258.52 ± 38.009 V.s.g(flower)$^{-1}$) when compared with pure glycerol (414.44 ± 70.7 V.s.g(flower)$^{-1}$). An apparent decrease is also observed in extracts prepared with glycerol containing 20‰ KCl, however this result is not significant. A significant decrease was observed in extracts prepared with 80‰ KCl/glycerol. It can also be seen that increasing concentrations of salt do not result in an increase in chemiluminescence response irrespective of the chaotropicity or kosmotropicity of the salt/glycerol composite used. Hindson *et al*. [150] found that sodium hexametaphosphate enhances the chemiluminescence emission through the formation of "cage-like" structures from hexametaphosphate oligomers to minimise non-radiative relaxation pathways for the Mn(II)* intermediate. The observed decrease in chemiluminescence was hypothesised to be due to cationic species interacting with the phosphate oligomers present in the chemiluminescence reaction, leading to an increase in non-radiative Mn(II)* relaxation and a subsequent loss of chemiluminescence. This distortion of the true chemiluminescence response of the prepared extracts would therefore increase with increasing salt concentrations and renders chemiluminescence a poor measure of antioxidant activity for salt/glycerol composite extracts.

Unscrambler analysis demonstrated strong to very strong correlations between the chaotropicity of kosmotrope/glycerol composites (R=0.68 and 0.80 for NaCl/glycerol and KCl/ glycerol composites respectively) and the chemiluminescence response of the extracts. Conversely, a strong negative correlation (R= -0.79) was observed between the chaotropicity of chaotropic LiCl/glycerol composites and the chemiluminescence response of the extracts. Correlation graphs are available in Appendix 4. However, due to the matrix effects described earlier, these correlations are not reliable for inferring behaviours or trends in metabolite extraction.

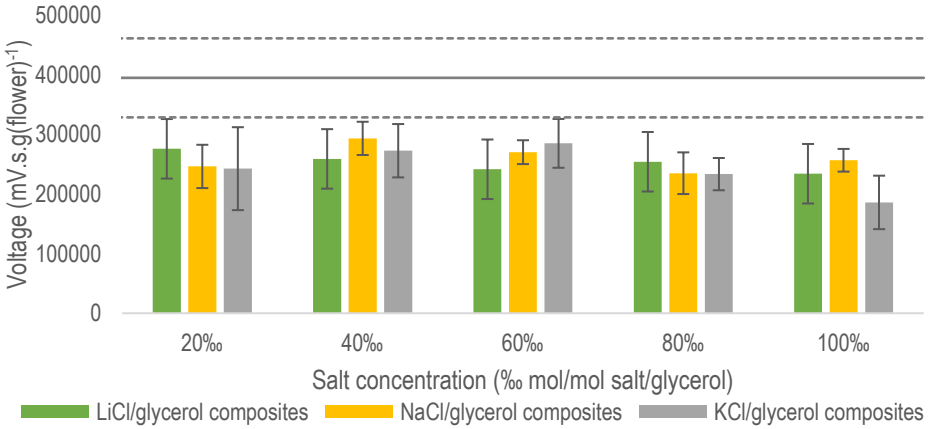


Figure 5-11: Chemiluminescence response of C. officinalis *extracts prepared with salt/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance ($p<0.05$) when compared with an extract prepared from neat glycerol. The line represents the chemiluminescence response of glycerolic extracts, with dashed lines representing the error margin.*

Radical Scavenging Activity. Experiments were conducted with a view to determining the total radical scavenging activity of *C. officinalis* extracts prepared with salt/glycerol composites. Figure 5-12 shows the radical scavenging activity of extracts prepared with salt/glycerol composites. It can be seen that extracts prepared with 20-60‰ mol/mol LiCl/glycerol composites appear to have slightly higher radical scavenging activity when compared with extracts prepared with neat glycerol. Extracts prepared with other salt/glycerol composites exhibit decreased radical scavenging activity. Strong correlations were observed when comparing the average radical scavenging activity of extracts prepared with kosmotrope/glycerol composites ($R=0.83$ for NaCl/glycerol and $R=0.70$ for KCl/glycerol) and the chaotropicity of the solvent. A moderate negative correlation ($R=-0.51$) was observed between the radical scavenging activity and chaotropicity of extracts prepared with LiCl/glycerol composites. Similarly to the correlations observed in Figure 5-10, it can be inferred that increasing salt concentrations results in a decrease in DPPH radical scavenging activity irrespective of the chaotropicity of the solvent.

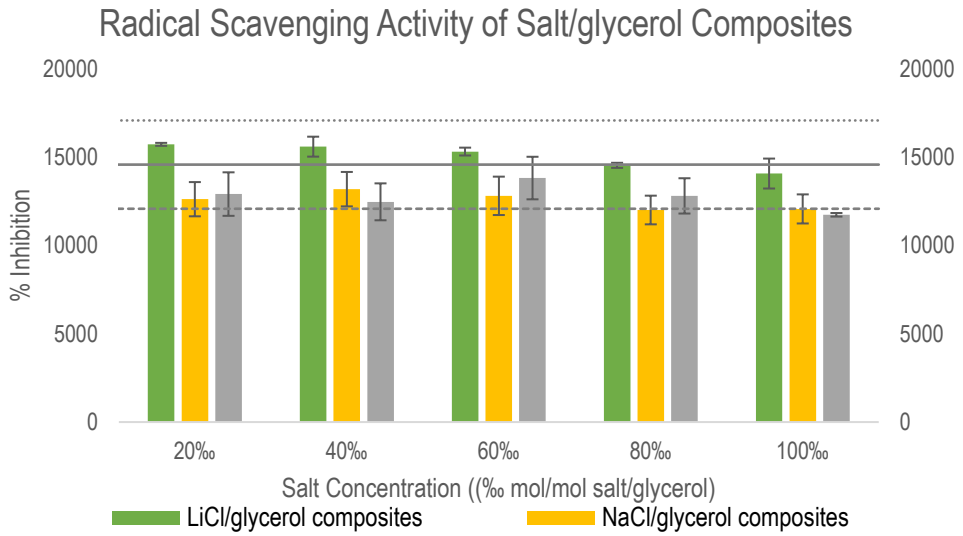


Figure 5-12: Radical scavenging activity of C. officinalis *extracts prepared with salt/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance ($p<0.05$) when compared with an extract prepared from neat glycerol. The line represents the radical scavenging activity of glycerolic extracts, with dashed lines representing the error margin.*

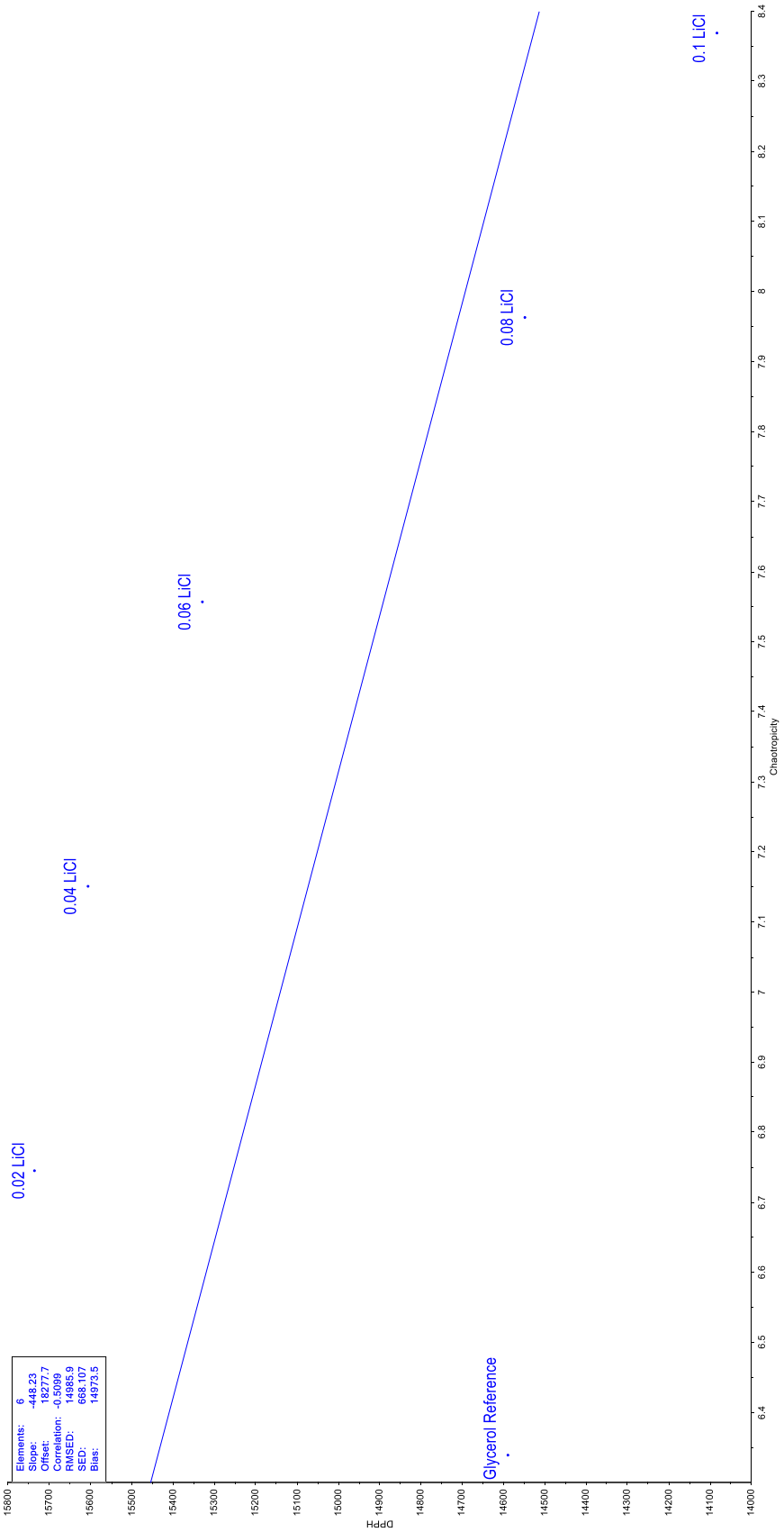


Figure 5-13: Unscrambler correlation between DPPH radical scavenging activity and chaotropicity of C. officinalis *extracts prepared with LiCl/glycerol composites. Data is presented as means of replicates (n=3).*

Phenolic Content. The total phenolic content of *C. officinalis* extracts prepared using LiCl/glycerol solvents were analysed with the Folin-Ciocalteu assay. Figure 5-14 shows the total phenolic content of *C. officinalis* extracts prepared with salt/glycerol composites. It can be observed that there is significantly lower phenolic content in extracts prepared from glycerol solvents containing 40‰, 80‰, and 100‰ mol/mol LiCl/glycerol when compared with neat glycerol extracts prepared in a similar fashion. A similar trend is observed in extracts prepared with $MgCl_2$/glycerol and NaCl/glycerol. Further information is available in Appendix 5.

Unscrambler® analysis (Figure 5-15) reveals very strong negative correlations between phenol content and chaotropicity of chaotrope/glycerol composites (R=-0.92 for LiCl/glycerol composites) and a strong correlation between phenol content and chaotropicity of kosmotropic NaCl/glycerol (R=0.75) and KCl/glycerol (R=0.70) composites. Similarly to the Unscrambler® analysis of total peak area and DPPH radical scavenging activity, these results infer that increasing salt content has a negative impact on the phenolic content observed in *C. officinalis* extracts prepared with salt/glycerol composites irrespective of the chaotropicity of the solvent.

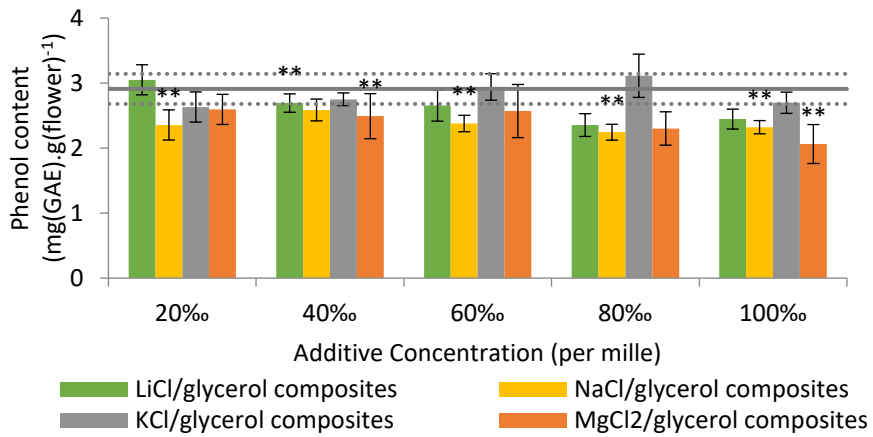


Figure 5-14: Total phenolic content of C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means ± standard deviations of replicates (n=9). Asterisks indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol. The line represents the phenolic content of glycerolic extracts, with dashed lines representing the error margin.*

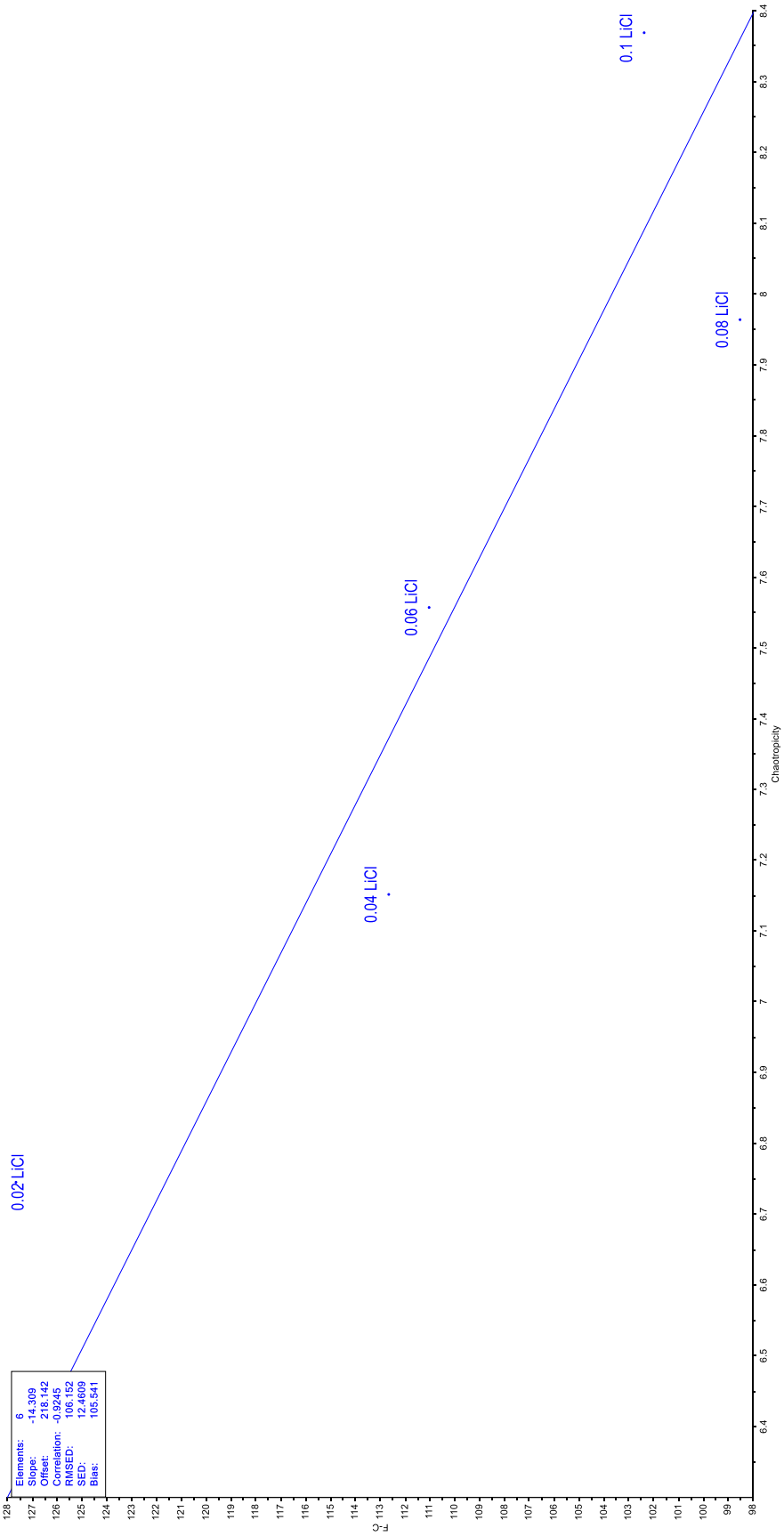


Figure 5-15: Unscrambler® correlation between total phenolic content of C. officinalis *extracts and chaotropicity of salt/glycerol composites. Data is presented as means of replicates (n=9).*

5.4.2 Acid/Glycerol Composites

Experiments were conducted with a focus on altering the glycerolic solubility of extracted components on the basis of dielectric coefficients of the solvents. Increased concentrations of acid to glycerol resulted in a visually more viscous solvent. In particular, extracts containing 20-60‰ mol/mol acid/glycerol or sugar/glycerol were significantly more viscous. The increased viscosity observed suggests that the addition of acid to glycerol results in an increase in hydrogen bonding strength. Peaks at 0.8 min, 8.5 min, 11.05 min, 11.2 min, 11.5 min, 11.65 min, 11.87 min, 12.75 min, and 13.3 min were chosen for further analysis due to clear resolution and antioxidant activity determined in Chapter 3.4.1 (Figure 5-4) and labelled peaks A-I respectively.

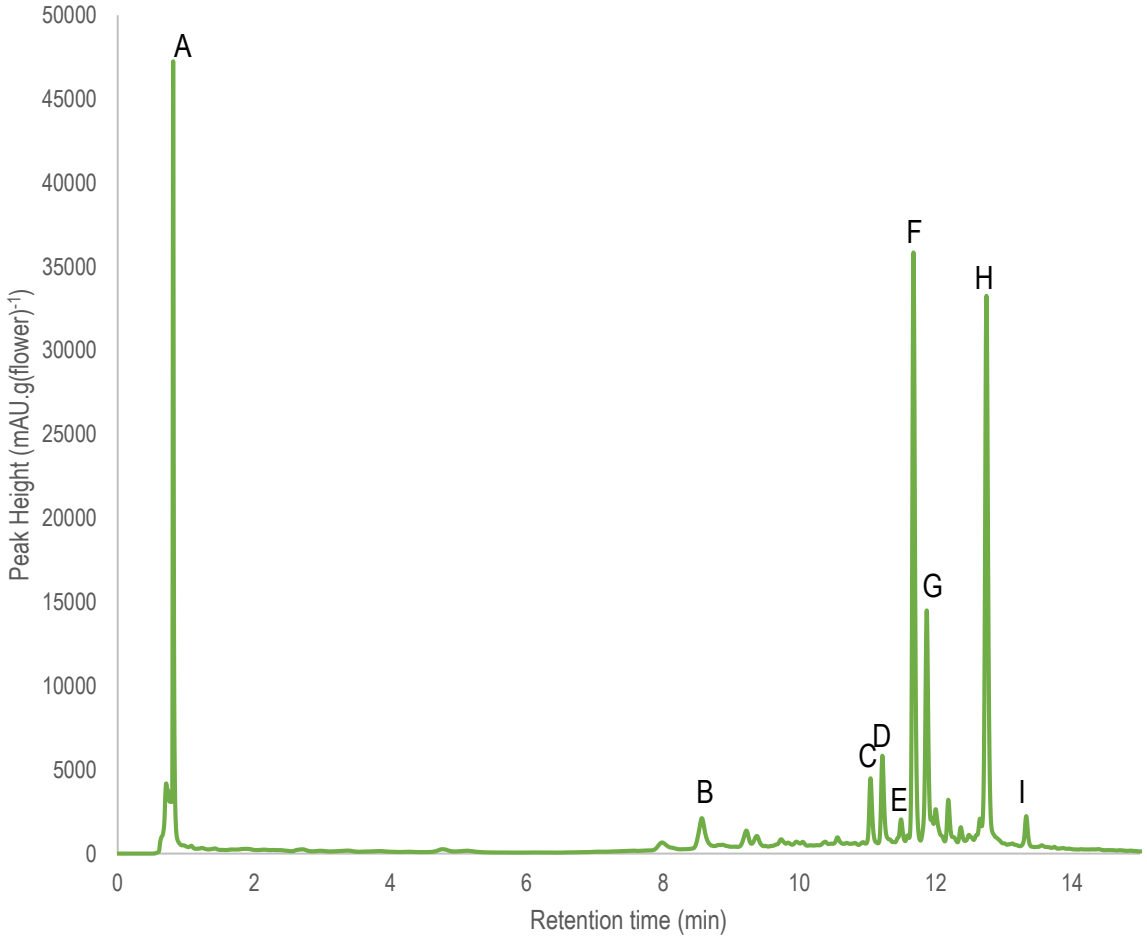


Figure 5-16: UHPLC chromatogram of C. officinalis *extracts prepared with glycerol solvent. Peaks at 0.8, 8.5, 11.1, 11.2, 11.5, 11.7, 11.9, 12.8, and 13.3 min were chosen for individual analysis and labelled A, B, C, D, E, F, G, H, and I respectively. Peak G was identified as Isorhamnetin by Johns* et al. *[223].*

UHPLC Analysis – Individual Peak Analysis. UHPLC analyses were conducted with a view to quantify the height of Peak A in *C. officinalis* extracts prepared with acid/glycerol composites. Figure 5-17

shows the peak heights for Peak A in *C. officinalis* extracts prepared with acid/glycerol composites. Extracts containing tartaric acid or citric acid exhibited increased peak heights for low-retention metabolites. Increases were determined to be significant for extracts prepared with 8-40‰ mol/mol tartaric acid/glycerol composites, with a maximum increase observed in extracts prepared with 8‰ mol/mol tartaric acid/glycerol (59282 ± 3360 mAU.g(flower)$^{-1}$) when compared to an extract prepared with neat glycerol (49154 ± 3063.4 mAU.g(flower)$^{-1}$). Increases in peak height were also observed in glycerol composites containing 1-8‰ mol/mol citric acid; however, these increases were not significant. Significant decreases in peak height were observed in extracts prepared with glycerol composites containing 60‰ mol/mol tartaric acid and 40-60‰ mol/mol tartaric acid. A strong negative correlation (R=-0.75) was observed between the height of Peak A and the dielectric constant of the acid/glycerol solvents (Appendix B). It was hypothesised that the unretained compounds contained basic functionalities such as amine moieties that, when protonated by the acid, resulted in increased solvent-metabolite interactions and subsequently increased extraction of polar compounds.

C. officinalis extracts prepared with acid/glycerol composites exhibited apparent increases in the height of less polar metabolites (Peak B) (Figure 5-18). These increases were significant for 8-40‰ mol/mol tartaric acid/glycerol, with a maximum increase observed at 20‰ mol/mol tartaric acid/glycerol (2384.1 ± 99.595 mAU.g(flower)$^{-1}$) when compared to extracts prepared with neat glycerol (1782.6 ± 183.44 mAU.g(flower)$^{-1}$). Increases in Peak B height were also observed in extracts prepared with 1-20‰ mol/mol citric acid/glycerol when compared with extracts prepared with neat glycerol; however, these increases were not found to be significant. Significant decreases were observed for 60‰ mol/mol tartaric acid/glycerol and 40-60‰ citric acid/glycerol composites.

UHPLC analyses conducted with a view to identifying the height of Peak G in *C. officinalis* extracts prepared with glycerol composites demonstrated that extracts containing citric acid or tartaric acid exhibited increased peak heights. Figure 5-19 shows the peak height of Peak G in *C. officinalis* extracts prepared with acid/glycerol composites. It can be seen that significant increases in the height of Peak G were observed for extracts prepared from glycerol composites containing 1‰, 8‰, and 20‰ mol/mol tartaric acid/glycerol, with a maximum increase observed in extracts prepared with 12‰ mol/mol tartaric acid/glycerol (16706 ± 308.53 mAU.g(flower)$^{-1}$) when compared with extracts prepared with neat glycerol (13911 ± 1163 mAU.g(flower)$^{-1}$). Similarly, increases in peak G height were also observed in glycerol composites containing 8-12‰ citric acid; however, these increases were not found to be significant. Significant decreases in peak G height were observed in extracts prepared with glycerol composites containing 60‰ tartaric or citric acids (11112 ± 471.54 mAU.g(flower)$^{-1}$ and

11845 ± 1003.2 mAU.g(flower)$^{-1}$ for tartaric and citric acid/glycerol composites respectively). Although increases can be observed in individual peaks, there was no observed change in relative peak heights between extracts prepared with tartaric acid/glycerol or citric acid/glycerol composites and extracts prepared with neat glycerol. This suggests that acid hydrolysis of glycosides to increase metabolite diffusion is not the mechanism by which an increase in metabolite content is achieved. However, significant increases in peak height were observed and may be attributed to increased hydrogen bonding between the glycerol composite and metabolites.

C. officinalis extracts prepared with acid/glycerol composites exhibited apparent increases in the heights of Peak I (Figure 5-20). These increases were significant for 8‰ and 20-40‰ mol/mol tartaric acid/glycerol, with a maximum increase observed at 20‰ mol/mol tartaric acid/glycerol (2201.6 ± 24.057 mAU.g(flower)$^{-1}$) when compared to extracts prepared with neat glycerol (1886.4 ± 151.83 mAU.g(flower)$^{-1}$). Increases in Peak I height were also observed in extracts prepared with 12‰ mol/mol citric acid/glycerol when compared with extracts prepared with neat glycerol; however, these increases were not found to be significant. Significant decreases were observed for 60‰ mol/mol tartaric acid/glycerol and 40-60‰ citric acid/glycerol composites. Similar increases in peak height were observed in glycerolic *C. officinalis* extracts prepared with acid/glycerol composites for Peaks C-F and H, with significant increases in extracts prepared with tartaric acid/glycerol composites for Peaks C-I, typically at 8-12‰ mol/mol PS/glycerol. Increases observed in extracts prepared with citric acid/glycerol composites for Peaks C-I were not found to be significant. Peak heights for Peaks C-I are available in Appendix B. Correlations between peak height and dielectric constant for peaks B-I were poor and ranged from very weak to moderate (R=-0.39, -0.05, -0.18, -0.05, -0.37, -0.39, and -0.53 for Peaks B, C, D, E, F, G, H, and I respectively). This indicates that the modification to the dielectric constant of the solvent appears to primarily affect highly polar metabolites and has little effect on the extraction of less polar metabolites. Correlation tables are available in Appendix B.

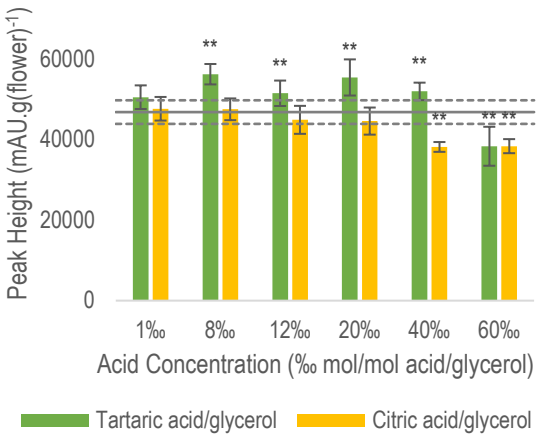


Figure 5-17: Peak heights for Peak A in C. officinalis *extracts prepared with acid/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the height of Peak A in glycerolic extracts, with dashed lines representing the error margin.*

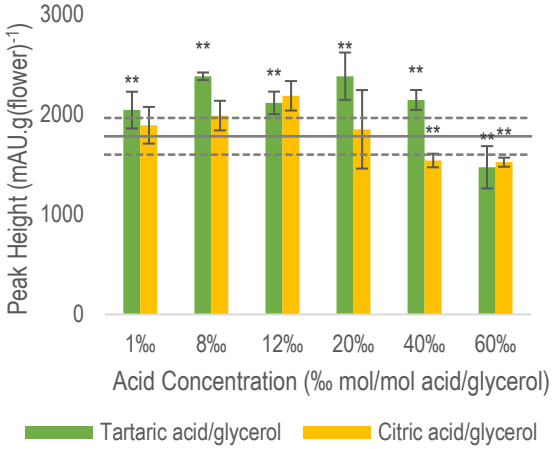


Figure 5-18: Peak heights for Peak B in C. officinalis *extracts prepared with acid/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the height of Peak B in glycerolic extracts, with dashed lines representing the error margin.*

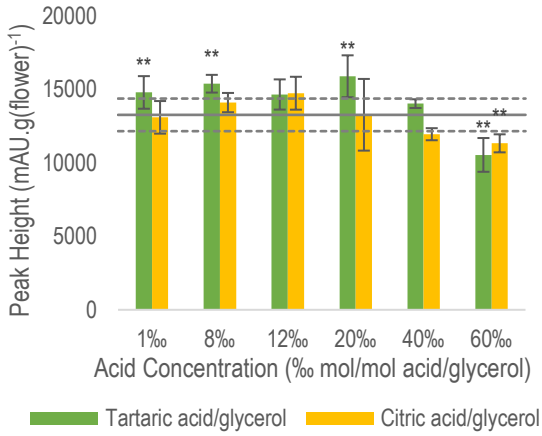


Figure 5-19: Peak heights for Peak G in C. officinalis *extracts prepared with acid/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the height of Peak G in glycerolic extracts, with dashed lines representing the error margin.*

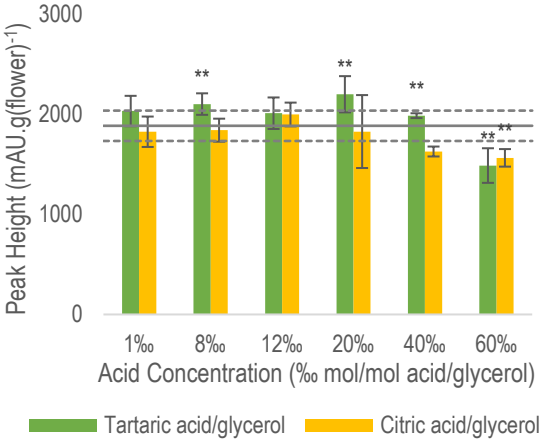


Figure 5-20: Peak heights for Peak I in C. officinalis *extracts prepared with acid/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the height of Peak G in glycerolic extracts, with dashed lines representing the error margin.*

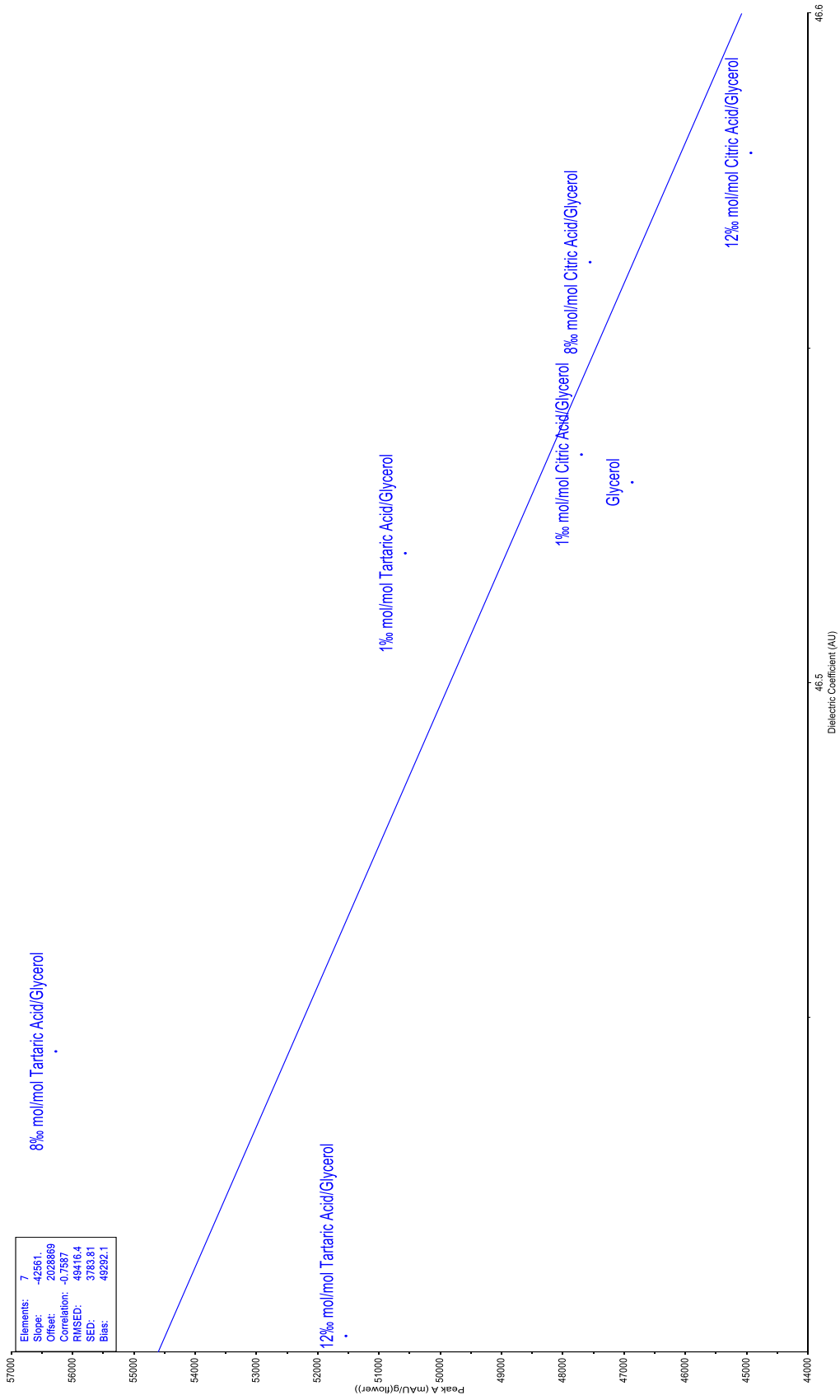


Figure 5-21: Example Unscrambler® correlation graph between average Peak A height and dielectric constants of C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as averages of replicates (n=3).*

Total Peak Area. Analyses of UHPLC chromatograms were conducted with a view to determining the total peak area presented in *C. officinalis* extracts prepared with acid/glycerol composites. Figure 5-22 shows the total peak area of *C. officinalis* extracts prepared with acid/glycerol composites and demonstrates that extracts prepared with 12‰ mol/mol citric acid/glycerol solvents appear to exhibit increased total peak area when compared with extracts prepared with glycerol, however this was not found to be significant. A significant decrease in total peak area can be observed in solvents with citric acid concentrations of 1‰ and 20-60‰ mol/mol (10411 ± 1032.1 mAU.s.g(flower)$^{-1}$, 10757 ± 570.23 mAU.s^{-1}.g(flower)$^{-1}$, 7960.1 ± 565.24 mAU.s.g(flower)$^{-1}$, and 8221.7 ± 1337.2 mAU.s.g(flower)$^{-1}$) when compared to extracts prepared with glycerol (12156 ± 1065.2 mAU.s^{-1}.g(flower)$^{-1}$). This decrease in peak area can be attributed to the increased hydrogen bonding of the citric acid/glycerol solvent limiting extracellular phytochemical diffusion through increased viscosity. Extracts prepared with tartaric acid/glycerol solvents demonstrate a significant increase in total peak area at 8‰ mol/mol (13920 ± 874.98 mAU.s^{-1}.g(flower)$^{-1}$) and 20‰ mol/mol (14205 ± 311.53) when compared with extracts prepared with glycerol (9618.3 ± 519.12 mAU.s^{-1}.g(flower)$^{-1}$), with relatively low levels of tartaric acid required (1‰ mol/mol) in order to obtain an apparent increase in total peak area. Higher quantities of tartaric acid (>20‰ mol/mol tartaric acid/ glycerol) result in an observable decrease in the total peak area of the extracts. Similarly to extracts prepared from citric acid/glycerol solvents, this can be attributed to the increasing viscosity of the tartaric acid/glycerol mixture that results in limited diffusion into the solvent matrix. There was a moderate negative correlation (R=-0.41) observed between total peak area and dielectric coefficient, indicating that dielectric coefficient was not likely to have an effect on the extraction of metabolites from *C. officinalis*.

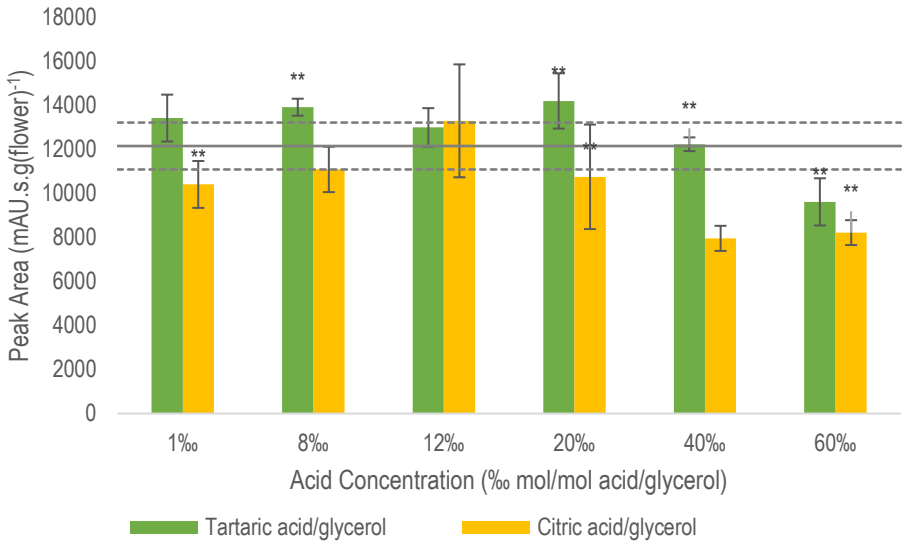


Figure 5-22: Total UHPLC peak area of C. officinalis *extracts prepared with acid/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance ($p<0.05$) when compared with extracts prepared with neat glycerol. The line represents the total peak area of glycerolic extracts, with dashed lines representing the error margin.*

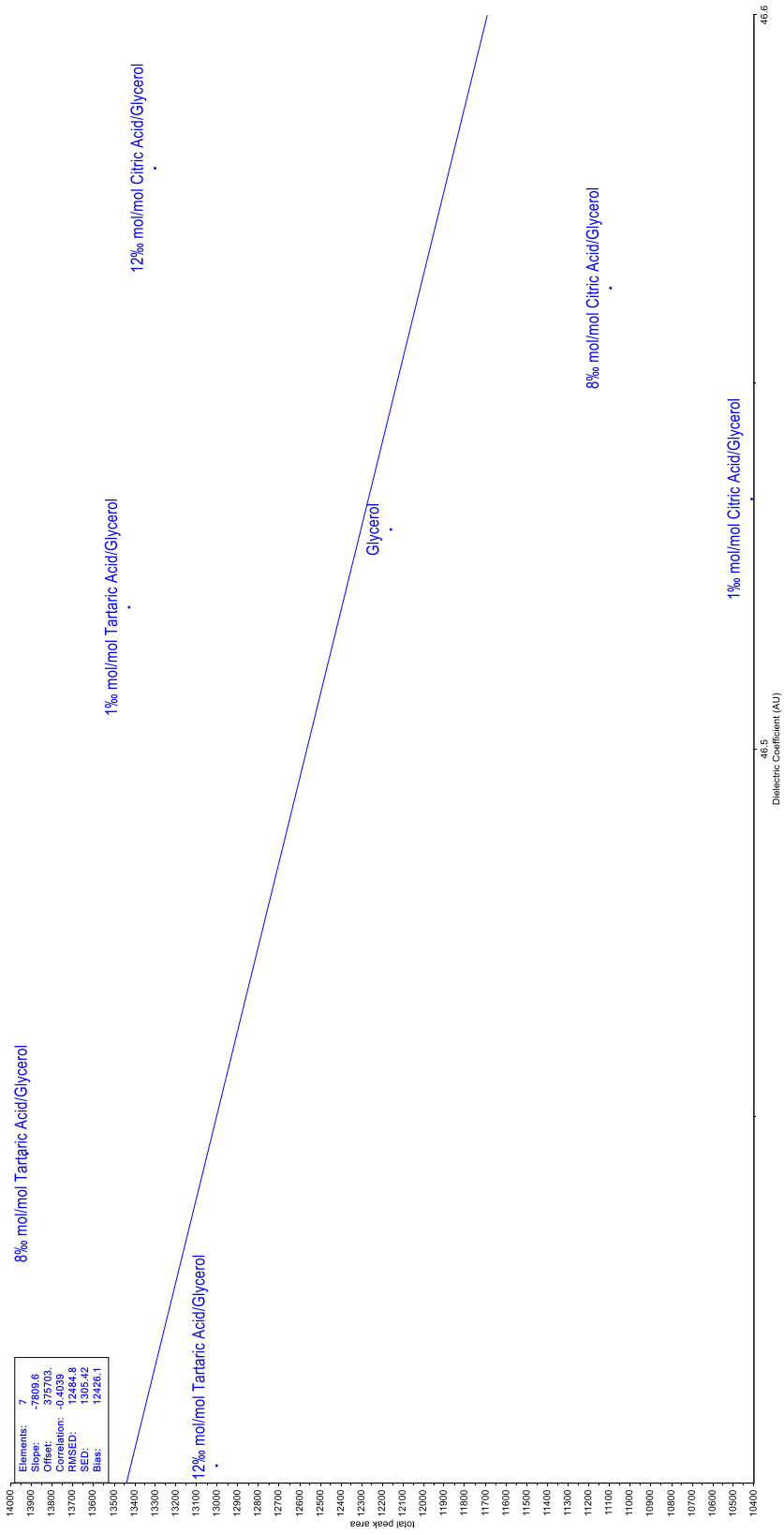


Figure 5-23: Unscrambler® correlation graph between total peak area and dielectric constants of C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as averages of replicates (n=3).*

Chemiluminescence response. SIA-CL assays were conducted with a view to identify the total antioxidant activity of *C. officinalis* extracts prepared with organic acid/glycerol and sugar/glycerol composites. Figure 5-24 shows the chemiluminescence response of *C. officinalis* extracts prepared with acid/glycerol composites. It can be observed that extracts prepared from 1-12‰ mol/mol citric acid/glycerol solvents exhibit an apparent increases in total antioxidant activity when compared to an extract prepared with glycerol (29133 ± 4924.2 mV.s.g(flower)$^{-1}$), and determined to be significant for 8‰ mol/mol citric acid/glycerol (34502 ± 642.29 mV.s.g(flower)$^{-1}$). Extracts prepared with tartaric acid were observed to behave in a similar manner, with significant increases in total antioxidant activity observed for concentrations of 8‰ mol/mol (42287 ± 7690.5 mV.s.g(flower)$^{-1}$) and 20‰ mol/mol tartaric acid/glycerol (36118 ± 2556.7 mV.s.g(flower)$^{-1}$). Higher concentrations of citric acid were observed to negatively impact on the observed increase in total antioxidant activity, with 40‰ mol/mol citric acid extracts having antioxidant activity similar to extracts prepared with pure glycerol and 60‰ mol/mol citric acid/glycerol presenting a significant decrease in chemiluminescence response (25196 ± 1050.4 mV.s.g(flower)$^{-1}$). It can be hypothesised that further increases in citric acid concentration will result in extracts with lower phytochemical content when compared with a glycerol extract. Similarly to extracts prepared using citric acid/glycerol, higher tartaric acid concentrations (40-60‰ mol/mol) result in a negative impact on observed increases in total antioxidant activity. This decrease was significant for extracts prepared with 60‰ tartaric acid/glycerol (16891 ± 3058.2 mV.s.g(flower)$^{-1}$). It was anticipated that citric acid and tartaric acid would display similar behaviours due to the similar carboxyl functionalities present on both molecules.

A moderate negative correlation (R=-0.41) was observed between the chemiluminescence response presented by *C. officinalis* extracts prepared and the dielectric constant of the acid/glycerol composites (Figure 5-25). This indicates that the dielectric constant of an acid/glycerol composite does not influence the ability of the solvent to extract metabolites with antioxidant activity.

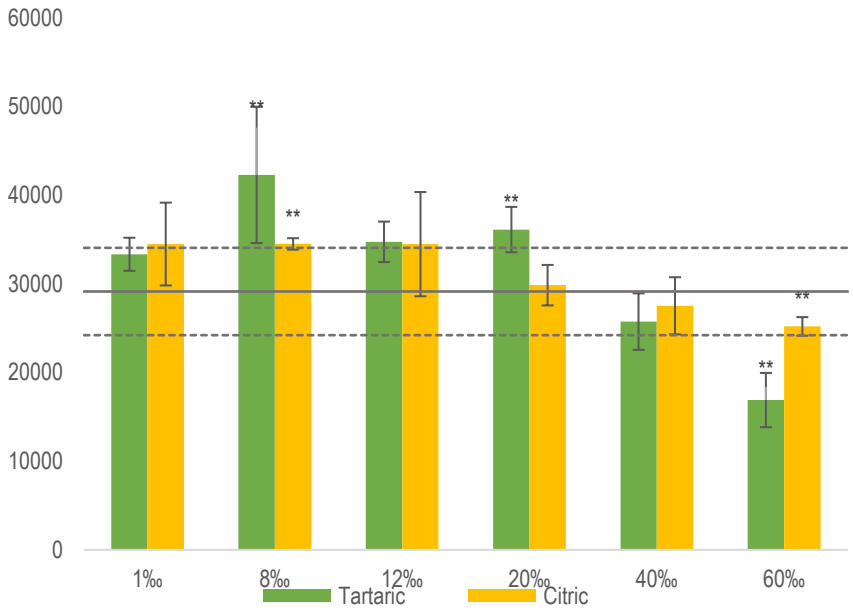


Figure 5-24: Chemiluminescence response of C. officinalis *extracts prepared with acid/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. The line represents the total peak area of glycerolic extracts, with dashed lines representing the error margin.*

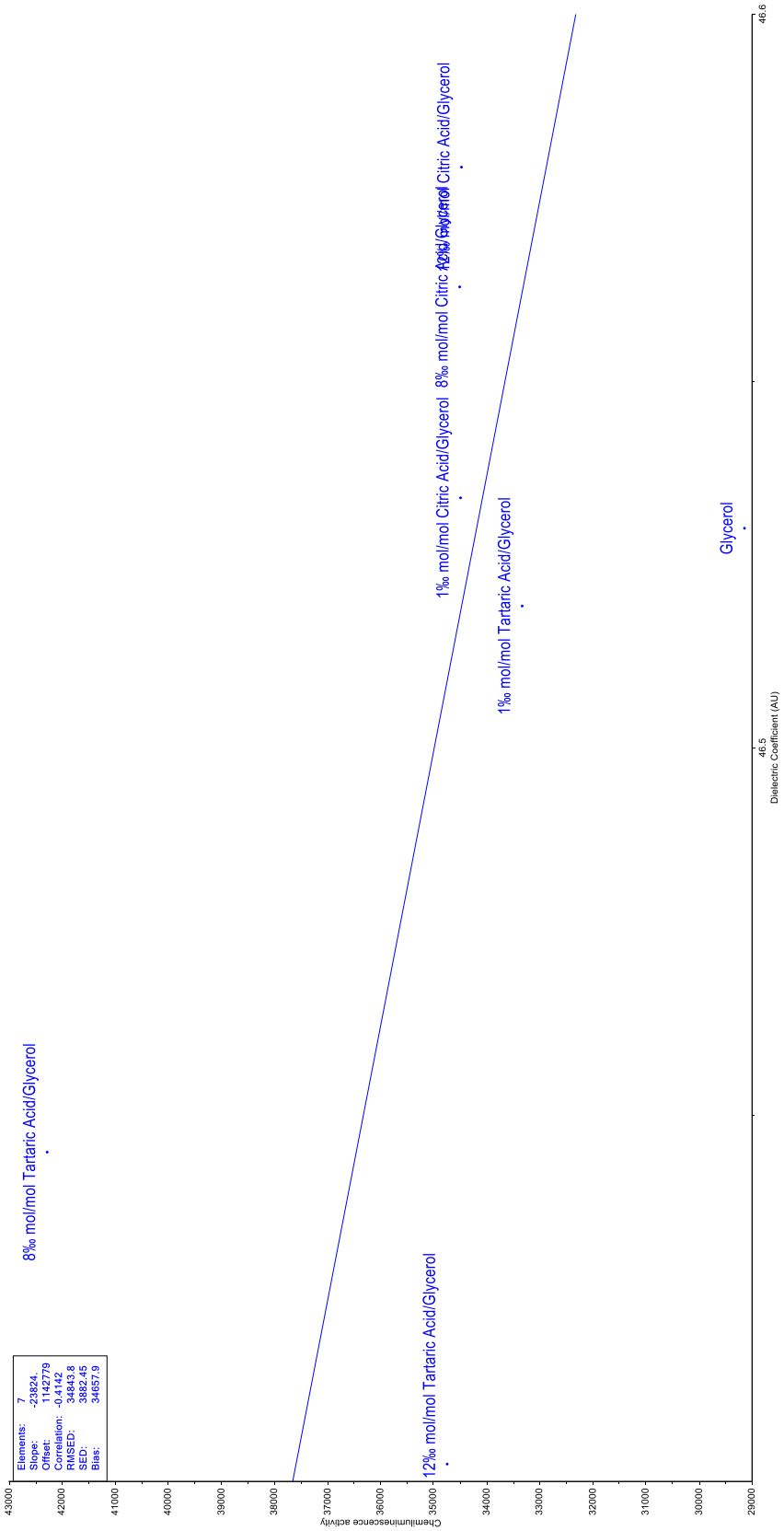


Figure 5-25: Unscrambler correlation between the chemiluminescence response of C. officinalis *extracts prepared with acid/glycerol solvents and the dielectric constant of the solvent composite. Data is expressed as means of replicates (n=3).*

Radical Scavenging Activity. DPPH analyses were conducted with a view to determining the radical scavenging activity of *C. officinalis* extracts prepared with glycerol composites. Figure 5-26 shows the radical scavenging activity of *C. officinalis* extracts prepared with acid/glycerol composites. It can be seen that extracts prepared with 1‰ tartaric acid/glycerol exhibit an apparent increase in radical scavenging activity when compared to an extract prepared from pure glycerol; however, this was not found to be significant. Other tartaric acid/glycerol and citric acid/glycerol composites did not present increased radical scavenging activity. Significant decreases in radical scavenging activity were observed for 20-60‰ mol/mol tartaric acid/glycerol and 12-60‰ mol/mol citric acid/glycerol composites. Citric acid has previously been observed to cause a decrease in DPPH radical scavenging of between 13-30% by Bolling *et al.* [200]. As tartaric acid and citric acid both contain carboxyl functionalities, it is likely that tartaric acid would also also exhibit a decrease in DPPH radical scavenging which agrees with experimental results. A poor negative correlation (R=-0.12) was observed between the chemiluminescence response presented by *C. officinalis* extracts prepared with acid/glycerol composites and the dielectric constant of the acid/glycerol composites (Figure 5-27). This indicates that the dielectric constant of an acid/glycerol composite does not influence the ability of the solvent to extract metabolites with radical scavenging activity.

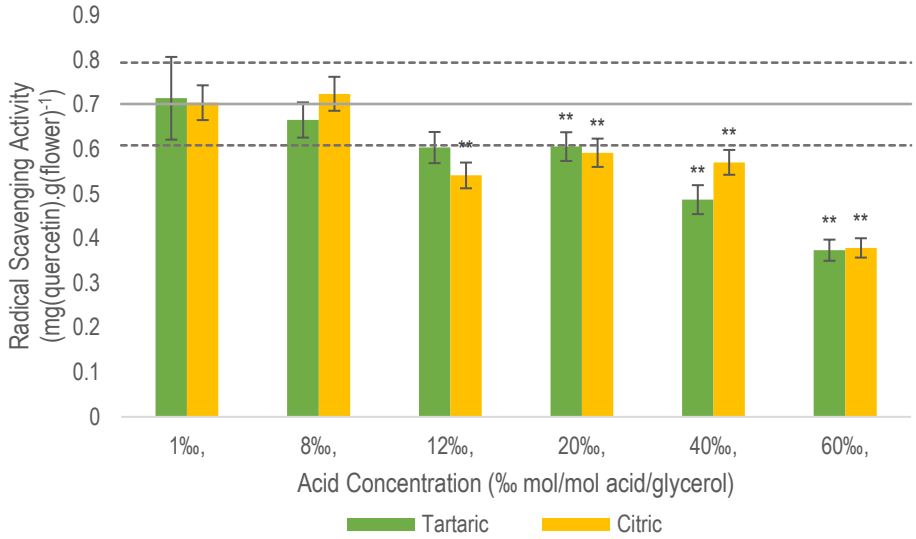


Figure 5-26: DPPH radical scavenging activity of C. officinalis *extracts prepared with acid/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=9). Asterisks indicate statistical significance (p<0.05) when compared with extracts prepared from neat glycerol. The line represents the total peak area of glycerolic extracts, with dashed lines representing the error margin.*

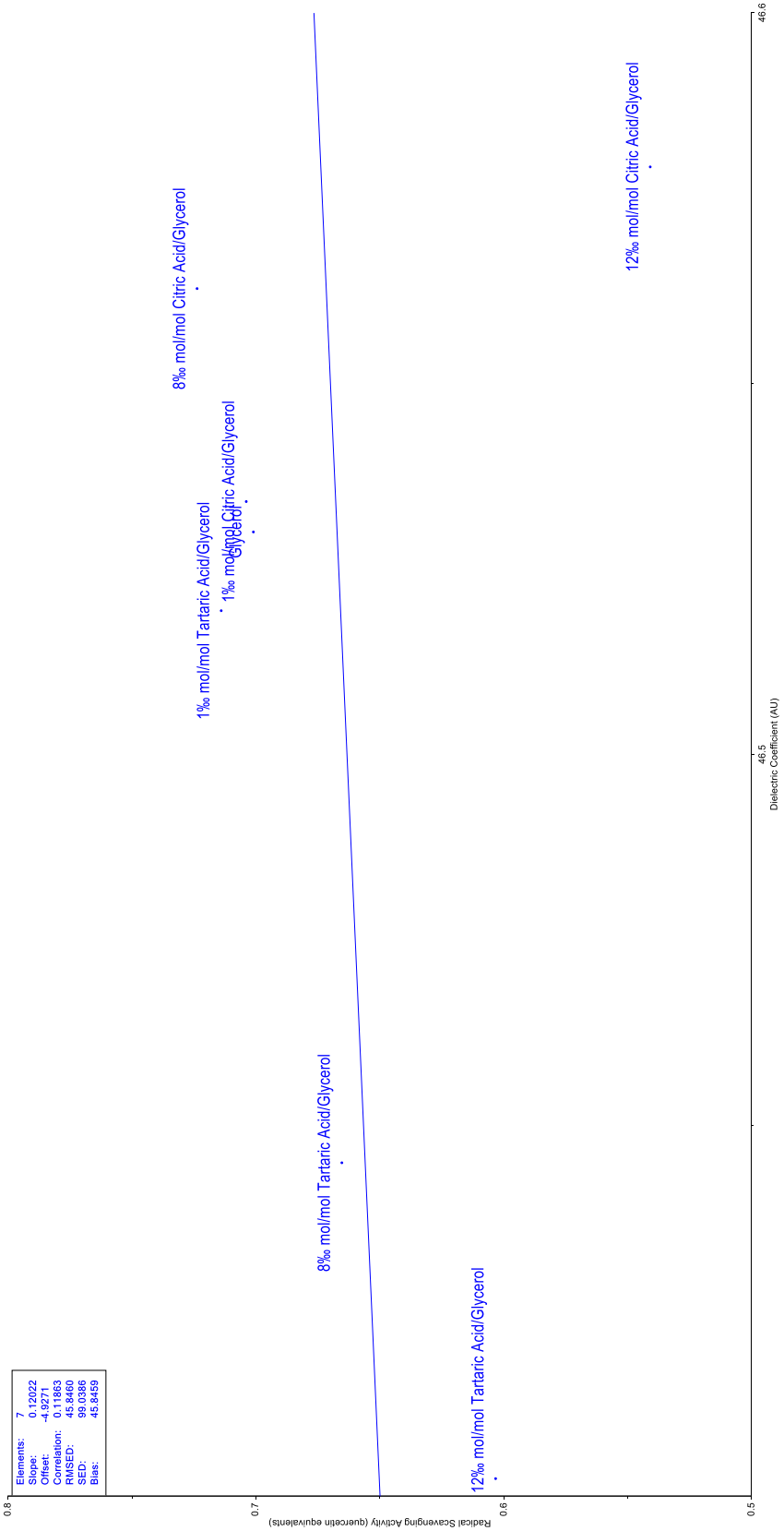


Figure 5-27: Unscrambler correlation between the DPPH radical scavenging activity of C. officinalis *extracts prepared with acid/glycerol solvents and the dielectric constant of the solvent composite. Data is expressed as means of replicates (n=3).*

Phenolic content. The phenolic content of *C. officinalis* extracts prepared with acid/glycerol composites were determined with the Folin-Ciocalteau assay. Figure 5-28 shows the phenolic content of *C. officinalis* extracts prepared with acid/glycerol composites. It can be seen that there is an apparent increase in phenolic content in C*. officinalis* extracts prepared with both tartaric acid/glycerol and citric acid/glycerol composites. Significant increases in phenol content can be observed in extracts prepared with 1-40‰ mol/mol tartaric acid/glycerol, with a maximum increase observed in extracts prepared with 20‰ mol/mol tartaric acid/glycerol (2.1698 ±0.17443 mg(GAE).g(flower)$^{-1}$) when compared with extracts prepared from neat glycerol (1.8289 ± 0.12883 mg(GAE).g(flower)$^{-1}$). Increases in phenolic content observed in extracts prepared with 8-12‰ mol/mol citric acid/glycerol were not significant. Extracts prepared with 60‰ mol/mol tartaric acid/glycerol and 40-60‰ mol/mol citric acid/glycerol composites exhibited significant decreases in phenol content. A moderate negative correlation (R=-0.52) was observed between the phenolic content presented by *C. officinalis* extracts prepared with acid/glycerol composites and the dielectric constant of the acid/glycerol composites (Figure 5-29), indicating that the dielectric constant of an acid/glycerol composite may influence the ability of the solvent to extract phenolic metabolites.

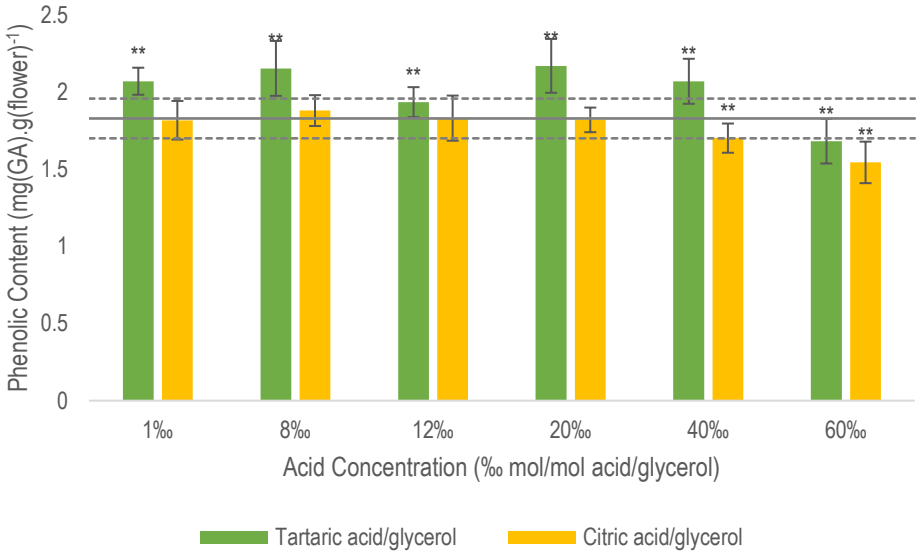


Figure 5-28: Total phenolic content of C. officinalis *extracts prepared with acid/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=9). Asterisks indicate statistical significance (p<0.05) when compared with extracts prepared from neat glycerol. The line represents the total peak area of glycerolic extracts, with dashed lines representing the error margin.*

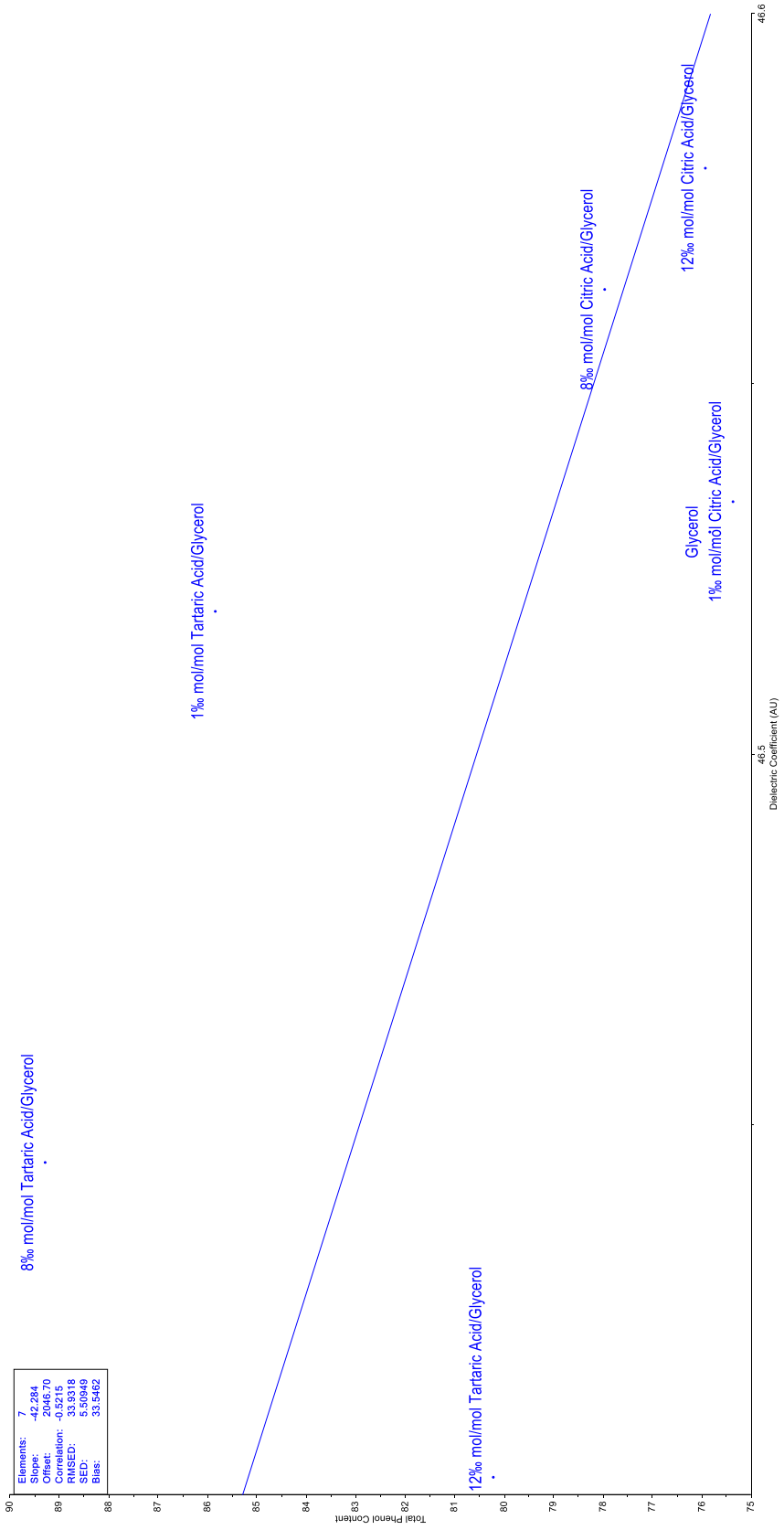


Figure 5-29: Correlation between total phenolic content of C. officinalis *extracts prepared with acid/glycerol composites and the dielectric constant of the composite. Data is expressed as means of replicates (n=9).*

5.4.3 Sugar/Glycerol Composites

Initial experiments focused on the use of sugar/glycerol composites with a view to increasing the phytochemical extraction through modification of the hydrogen bonding properties of the solvent. Increased concentrations of sugars to glycerol resulted in a visually more viscous solvent. In particular, extracts containing 20-60‰ mol/mol acid/glycerol or sugar/glycerol were significantly more viscous. Sugar/glycerol composites were observed to be more viscous than acid/glycerol composites. Sucrose/glycerol composites were the most viscous. The increased viscosity observed suggests that the addition of sugar to glycerol results in an increase in hydrogen bonding strength.

UHPLC Analysis - Individual Peak Areas. UHPLC analyses were conducted with a view to quantify the height of peak A in *C. officinalis* extracts prepared with sugar/glycerol composites. Figure 5-30 shows the height of Peak A in *C. officinalis* extracts prepared with sugar/glycerol composites. It can be seen that *C. officinalis* extracts prepared using 8-12‰ mol/mol fructose/glycerol exhibit significant increases in peak height (56046 ± 2401 mAU.g(flower)$^{-1}$ and 56345 ± 60.075 mAU.g(flower)$^{-1}$ respectively). Similarly, significant increases in the height of Peak A were observed in extracts prepared with 1-20‰ mol/mol glucose/glycerol composites (53374 ± 2922.3 mAU.g(flower)$^{-1}$, 57387 ± 1328.6 mAU.g(flower)$^{-1}$, 57984 ± 2147.7 mAU.g(flower)$^{-1}$, and 54037 ± 1787.1 mAU.g(flower)$^{-1}$ respectively). Decreases in peak height were observed for 40-60‰ mol/mol fructose/glycerol and glucose/glycerol. Extracts prepared with sucrose/glycerol composites did not display increased peak height, and demonstrated significant decreases in peak height at 20-60‰ mol/mol sucrose/glycerol (39986 ± 1459.1 mAU.g(flower)$^{-1}$, 33215 ± 769.7 mAU.g(flower)$^{-1}$, and 29854 ± 859.12 mAU.g(flower)$^{-1}$ respectively). A strong correlation (R=0.66) was observed between the average peak A heights and the chaotropicity of sugar/glycerol composites (Figure 5-34).

Similar behaviours were observed when analysing the height of peak B. Figure 5-31 shows the height of Peak B in *C. officinalis* extracts prepared with sugar/glycerol composites. It can be seen that significant increases to the height of Peak B can be observed for *C. officinalis* extracts prepared using 8-12‰ mol/mol fructose/glycerol (2228.5 ± 131.76 mAU.g(flower)$^{-1}$, and 2277 ± 80.857 mAU.g(flower)$^{-1}$ respectively) and 1-20‰ mol/mol glucose/glycerol composites (2297.9 ± 242.97 mAU.g(flower)$^{-1}$, 2391.6 ± 132.82 mAU.g(flower)$^{-1}$, 2494.7 ± 107.84 mAU.g(flower)$^{-1}$, and 2394.4 ± 66.592 mAU.g(flower)$^{-1}$ respectively). Significant decreases in peak height were observed for extracts prepared with 60‰ mol/mol fructose/glycerol. Sucrose did not display increased peak height,

and demonstrated significant decreases in peak height at 20-60‰ mol/mol sucrose/glycerol. A strong correlation (R=0.66) was observed between the average peak B heights and solvent chaotropicity in *C. officinalis* extracts prepared from sugar/glycerol composites (Appendix B).

The increase in peak heights observed in Figure 5-30 and Figure 5-31 were not reflected in later-eluting compounds. Figure 5-32 shows the peak height of Isorhamnetin in *C. officinalis* extracts prepared with sugar/glycerol composites. It can be seen that extracts prepared from glycerol composites that contained 1-12‰ fructose demonstrated apparent increases in peak height; however, these increases were not significant. Extracts prepared from glycerol composites that contained 1-20‰ glucose demonstrated an apparent increase in peak heights when compared with *C. officinalis* extracts prepared with neat glycerol, but were similarly found to be not significant. Significant decreases in peak height were observed at glucose concentrations of 40‰ and 60‰ mol/mol and fructose concentrations of 60‰ mol/mol. No increases in peak height were observed for extracts prepared with sucrose/glycerol composites; however, significant decreases in peak height were observed, for extracts prepared with 20-60‰ mol/mol sucrose/glycerol composites. A very strong correlation (R=0.96) was observed between isorhamnetin content and the chaotropicity of the sugar/glycerol composite used to prepare the *C. officinalis* extracts. Similarly, increases were not observed in Peak I heights shown in Figure 5-33. Significant decreases were observed in all extracts prepared with sugar/glycerol composites at concentrations of 20‰ mol/mol sugar/glycerol and higher.

Peaks C, D, E, F, and H displayed similar behaviours to peak B (Appendix B). Significant increases in peak height were only observed in tartaric acid/glycerol composites. Increases in peak height were also observed in extracts prepared with fructose/glycerol and glucose/glycerol; however, these increases were not found to be significant. Decreases in peak heights were observed in all extracts at 60‰ sugar/glycerol composites, and were typically found to be significant. Very strong correlations between chaotropicity and peak heights were observed (R=0.97, 0.92, 0.95, 0.97, and 0.92 for peaks C, D, E, F, and H respectively) using the guide by Evans [163]. Correlations for peaks D-F and H are available in Appendix 4. It was particularly interesting to note that for low sugar concentrations (typically 1-12‰), increases in peak heights could be observed in extracts prepared with fructose and glucose composites but not in extracts prepared with sucrose composites. Fructose and glucose were both observed by Cray *et al.* [218] to exhibit chaotropic behaviour, whereas sucrose exhibited kosmotropic behaviour. The results obtained indicate that the addition of chaotropic sugars to glycerol result in an increase in the extraction of polar metabolites but have little effect on the extraction of nonpolar metabolites.

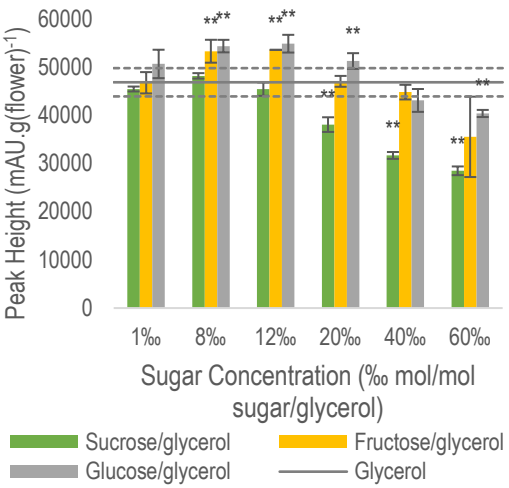


Figure 5-30: Peak A heights in C. officinalis *extracts prepared with sugar/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the total peak area of glycerolic extracts, with dashed lines representing the error margin.*

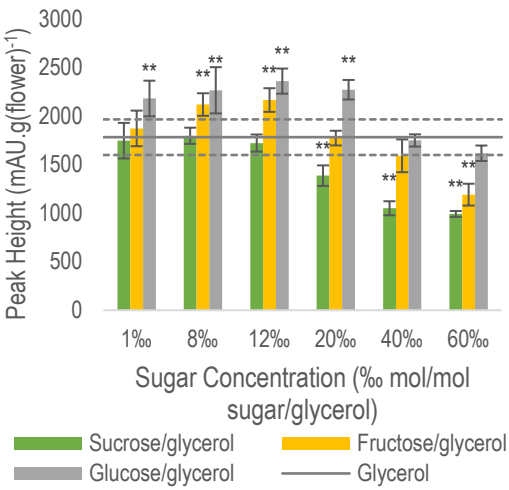


Figure 5-31: Peak B heights in C. officinalis *extracts prepared with sugar/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the total peak area of glycerolic extracts, with dashed lines representing the error margin.*

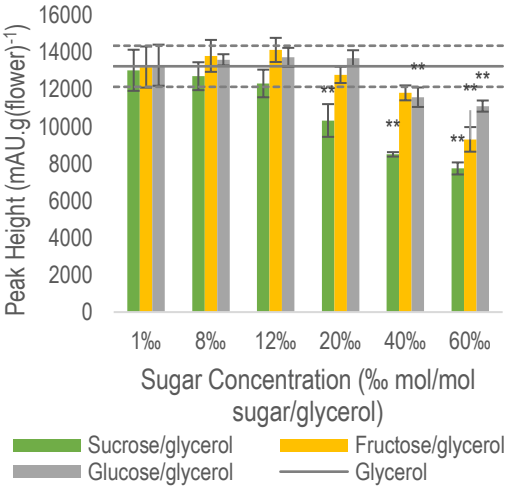


Figure 5-32: Peak G height in C. officinalis *extracts prepared with sugar/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the total peak area of glycerolic extracts, with dashed lines representing the error margin.*

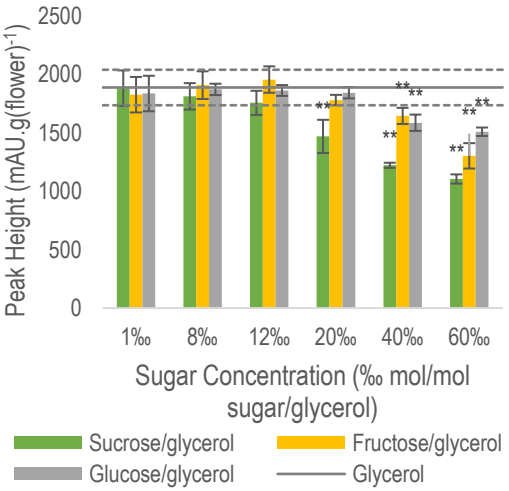


Figure 5-33: Peak I heights in C. officinalis *extracts prepared with sugar/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the total peak area of glycerolic extracts, with dashed lines representing the error margin.*

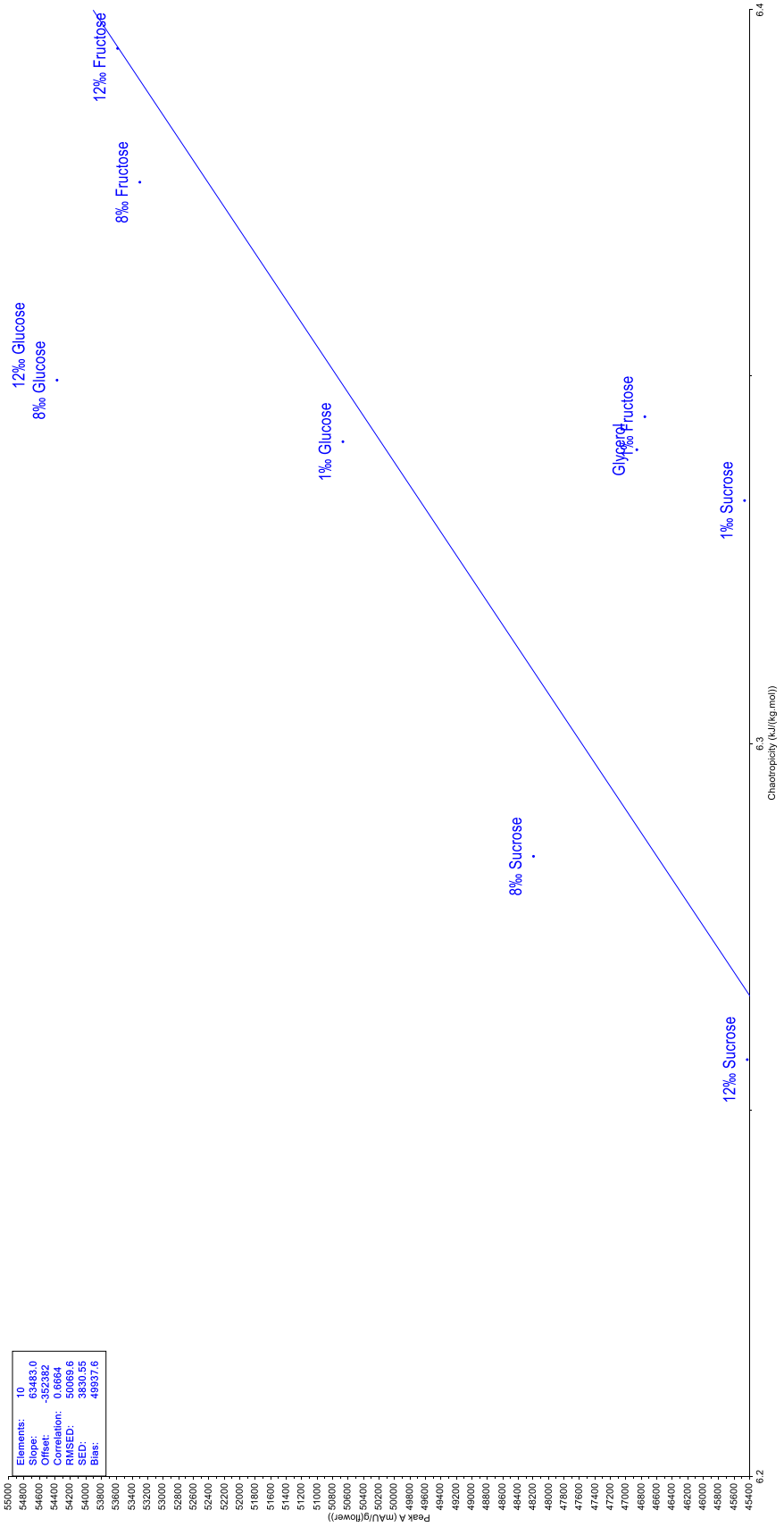


Figure 5-34: Unscrambler® correlation between Peak A height and chaotropicity of C. officinalis *extracts prepared with sugar/glycerol composites. Data is expressed as means of replicates (n=3).*

UHPLC Analysis – Total Peak Areas. UHPLC analyses were conducted with a view to determining the total peak area of *C. officinalis* extracts prepared with sugar/glycerol composites. Figure 5-35 shows the total peak areas of *C. officinalis* extracts prepared with sugar/glycerol composites. It can be seen that *C. officinalis* extracts prepared from glycerol containing 8‰ mol/mol and 12‰ mol/mol fructose demonstrate significantly higher total peak areas (13780 ± 388.75 mAU.s^{-1}.g(flower)$^{-1}$ and 13936 ± 299.75 mAU.s^{-1}.g(flower)$^{-1}$ respectively) when compared with extracts prepared in a similar fashion from neat glycerol (12269 ± 945.5 mAU.s^{-1}.g(flower)$^{-1}$. The greatest enhancement in phytochemical concentration can be observed in extracts prepared from glycerol containing 12‰ mol/mol fructose. A decrease in total peak area was observed for extracts prepared using 20-60‰ mol/mol fructose/glycerol solvents; however, this was not found to be significant. Similarly, increases in total peak area was observed for extracts prepared with 12‰ mol/mol glucose/glycerol (13733 ± 422.2 mAU.s^{-1}.g(flower)$^{-1}$) when compared with an extract prepared using neat glycerol.

Unscrambler™ analysis of total peak area (Figure 5-36) revealed a very strong correlation (R=0.81) between solvent chaotropicity and the UHPLC total peak area for C. officinalis extracts prepared with 0-12‰ mol/mol sugar/glycerol composites. It can therefore be inferred that the use of a more chaotropic solvent results in increased peak area for the extraction of *C. officinalis* metabolites.

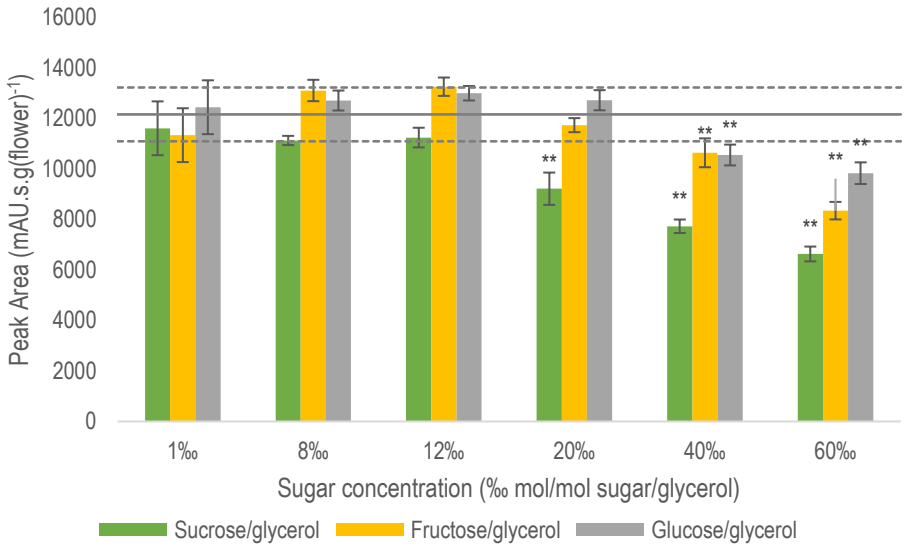


Figure 5-35: Total UHPLC peak area of C. officinalis *extracts prepared with sugar/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol. The line represents the total peak area of glycerolic extracts, with dashed lines representing the error margin.*

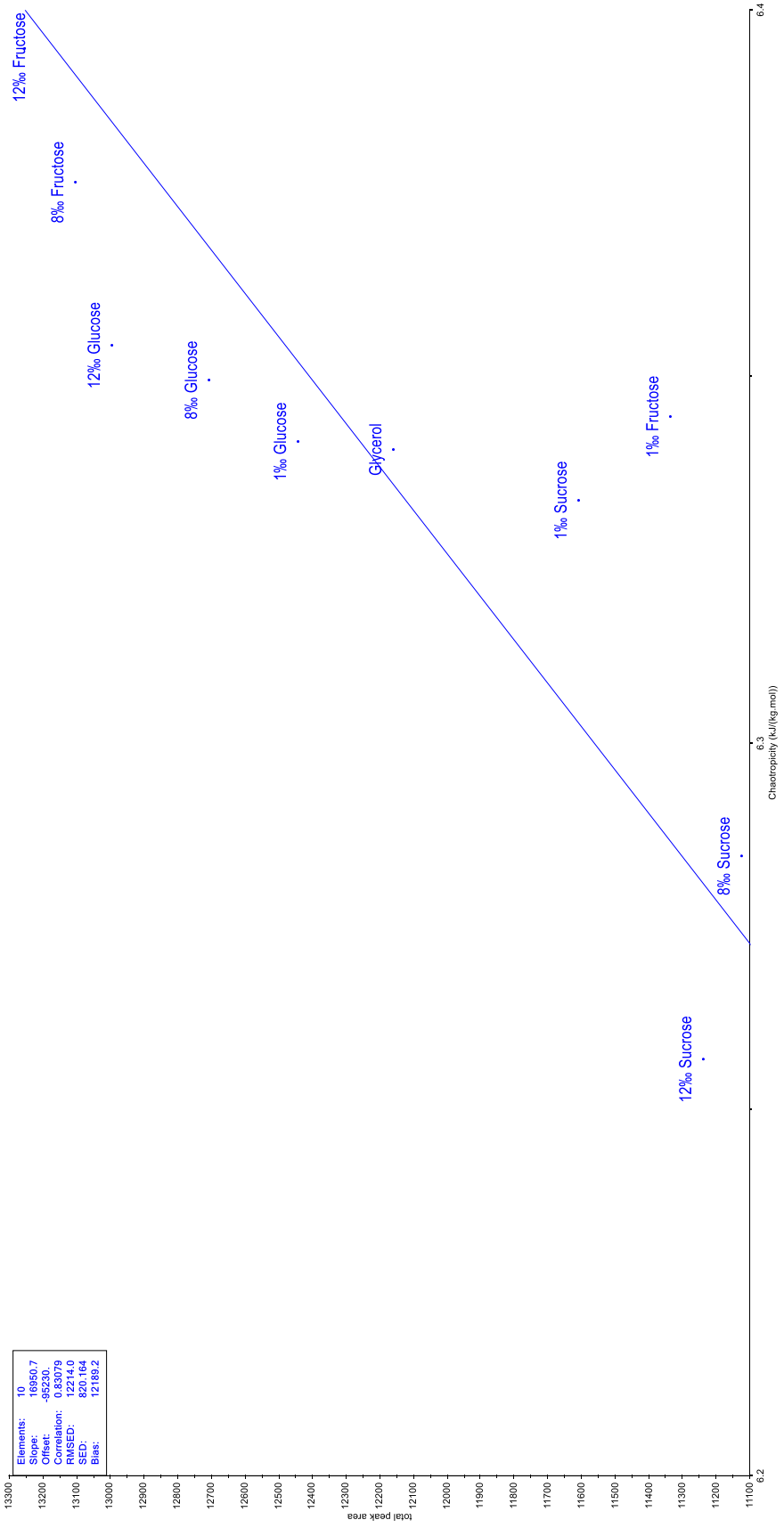


Figure 5-36: Unscrambler® correlation between total peak area and chaotropicity of C. officinalis *extracts prepared with sugar/glycerol composites. Data is expressed as means of replicates (n=3).*

Chemiluminescence response. SIA-CL assays were conducted with a view to identify the total antioxidant activity of *C. officinalis* extracts prepared with sugar/glycerol composites. Figure 5-37 shows the chemiluminescence response of *C. officinalis* extracts prepared with sugar/glycerol composites. It can be seen that Increases in total antioxidant activity were observed in extracts prepared with both fructose and glucose. Increases in total antioxidant activity were observed for extracts containing 8‰ and 12‰ fructose mol/mol (33610 ± 2288.7 mV.s.g(flower)$^{-1}$ and 31884 ± 3156.4 mV.s.g(flower)$^{-1}$ respectively); however, these increases were not found to be significant. Significant increases in total antioxidant activity were observed in extracts prepared with 8‰ (38403 ± 3365.6 mV.s.g(flower)$^{-1}$), 12‰ (38697±2120.4 mV.s.g(flower)$^{-1}$), and 20‰ (37121 ± 5140.3 mV.s.g(flower)$^{-1}$) mol/mol glucose/glycerol solvents. There was no increase in total antioxidant activity observed for extracts prepared with sucrose/glycerol composites; indeed, significant decreases were observed for extracts prepared with 8-60‰ mol/mol sucrose/glycerol composites (22416 ± 4121.1 mV.s.g(flower)$^{-1}$, 21147 ± 3453.7 mV.s.g(flower)$^{-1}$, 20757 ± 2651.7 mV.s.g(flower)$^{-1}$, 18287 ± 3451 mV.s.g(flower)$^{-1}$, and 13131 ± 3278.6 mV.s.g(flower)$^{-1}$ for 8‰, 12‰, 20‰, 40‰, and 60‰ mol/mol sucrose/glycerol respectively). Some chemiluminescence was observed during analysis of matrix blanks; this was anticipated as sucrose, fructose, and glucose exhibit chemiluminescence response [224]; however, this was significantly lower than the chemiluminescence of the *C. officinalis* extracts and was subtracted accordingly. Similarly to UHPLC, extracts prepared with low molarity (1-12‰ mol/mol) chaotropic fructose and glucose composites exhibited increases in chemiluminescence response (significant for glucose/glycerol composites), whereas extracts prepared with kosmotropic sucrose composites did not exhibit any increases in chemiluminescence response.

A strong correlation was observed between the chemiluminescence response of the extracts prepared with 0-12‰ mol/mol sugar/glycerol composites and the chaotropicity of the solvent (R=0.75). As permanganate chemiluminescence can be used as a measure of the antioxidant activity of a plant extract [225], it can therefore be inferred that the use of a more chaotropic solvent for the preparation of *C. officinalis* extracts results in increased antioxidant activity.

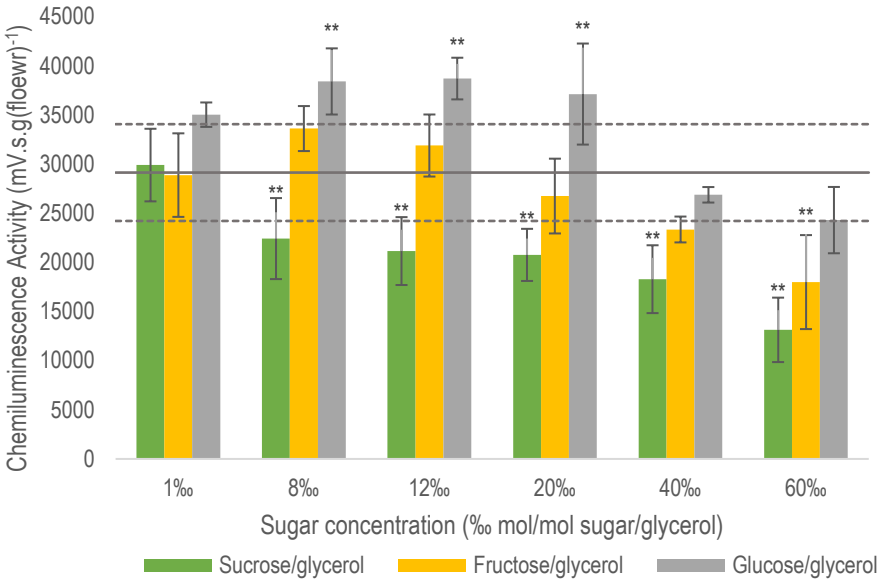


Figure 5-37: Chemiluminescence response of C. officinalis *extracts prepared with sugar/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=3). Asterisks indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. The line represents the chemiluminescence response of glycerolic extracts, with dashed lines representing the error margin.*

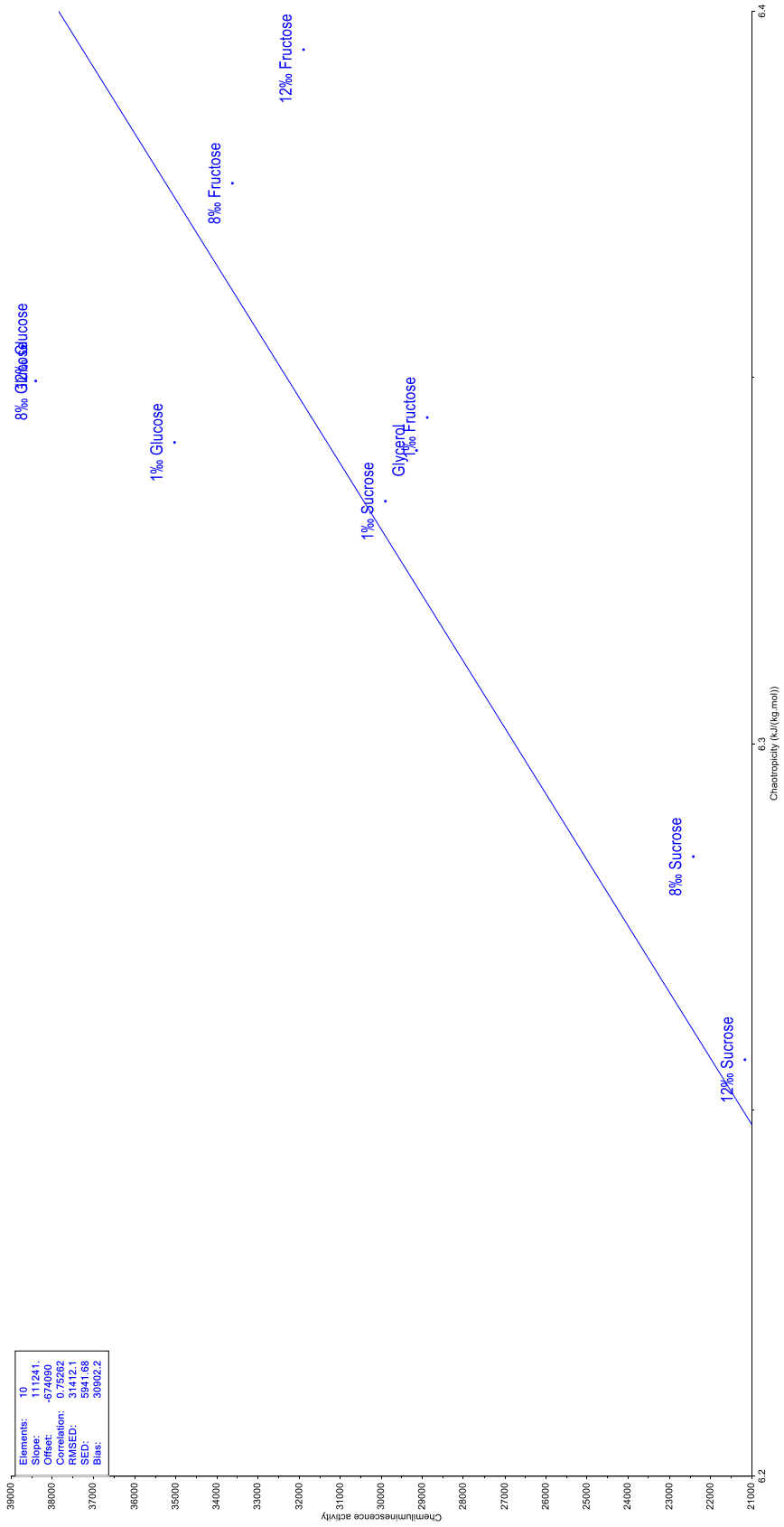


Figure 5-38: Unscrambler® Correlation between chemiluminescence response and solvent chaotropicity in C. officinalis *extracts prepared with sugar/glycerol composites. Data is presented as means of replicates (n=3).*

Radical Scavenging Activity. DPPH analyses were conducted with a view to determining the radical scavenging activity of *C. officinalis* extracts prepared with glycerol composites. Figure 5-39 shows the radical scavenging activity of *C. officinalis* extracts prepared with sugar/glycerol composites. It can be seen that extracts prepared with sucrose/glycerol composites did not present increased radical scavenging activity. Apparent increases in radical scavenging activity were observed in extracts prepared with 1-12‰ fructose/glycerol and 1-20‰ glucose/glycerol composites; however, these results were not found to be significant. Significant decreases in radical scavenging activity were observed in extracts prepared with 8-60‰ mol/mol sucrose/glycerol, 60‰ mol/mol fructose/glycerol, and 40-60‰ mol/mol glucose/glycerol.

A very strong [163] correlation (R=0.81) was observed between the radical scavenging activity of extracts prepared with sugar/glycerol composites and solvent chaotropicity (Figure 5-40). Similarly to correlations observed between solvent chaotropicity and peak heights and chemiluminescence, it can be inferred that the use of more chaotropic solvents results in increased DPPH radical scavenging activity.

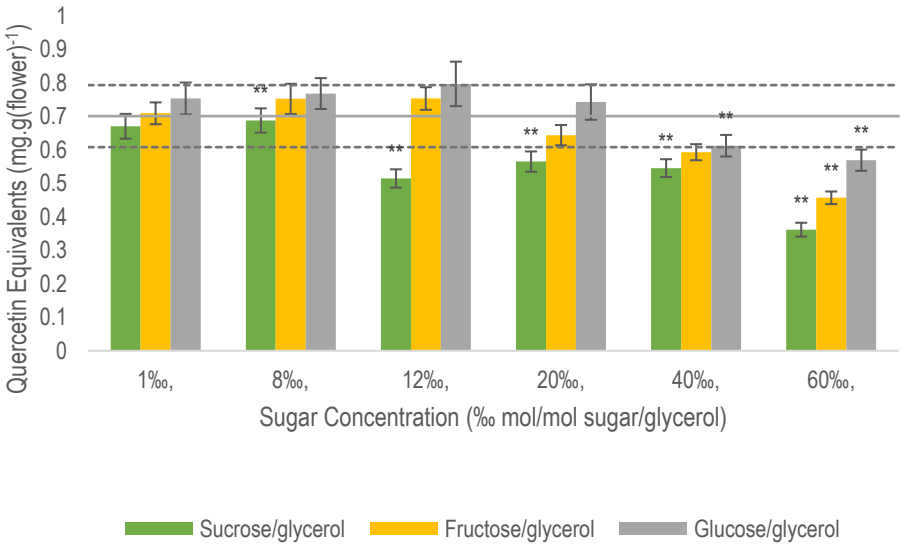


Figure 5-39: DPPH radical scavenging activity of C. officinalis *extracts prepared with sugar/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=9). Asterisks indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. The line represents the radical scavenging activity of glycerolic extracts, with dashed lines representing the error margin.*

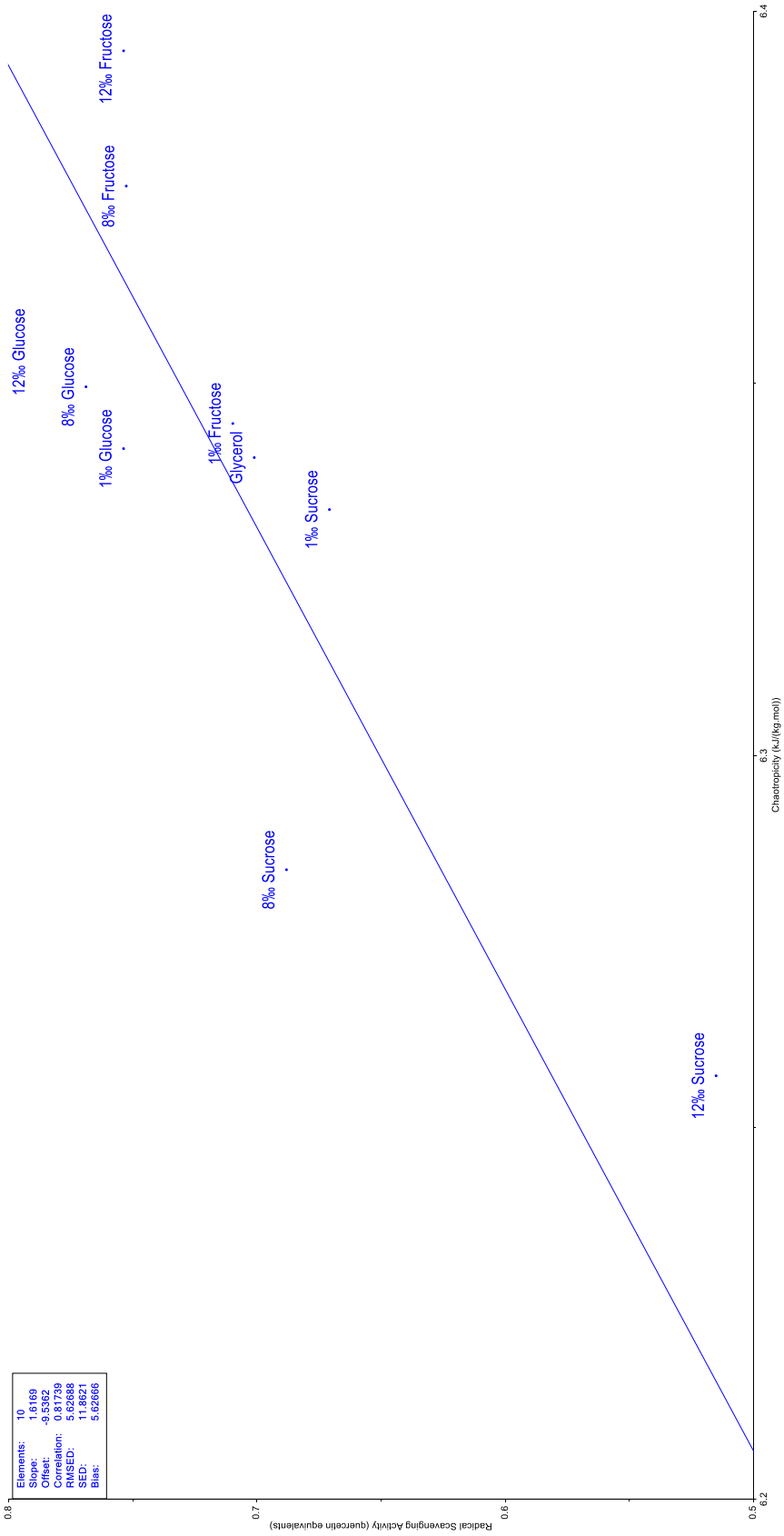


Figure 5-40: Unscrambler® correlation between DPPH radical scavenging activity and solvent chaotropicity in C. officinalis *extracts prepared with sugar/glycerol composites. Data is expressed as means of replicates (n=9).*

Phenolic content. Experiments were conducted with a view to determining the total phenolic content of *C. officinalis* extracts prepared with sugar/glycerol composites. Figure 5-41 shows the GAE content of *C. officinalis* extracts prepared with sugar/glycerol composites. It can be seen that significant increases in GAE can be observed in extracts prepared with 8-40‰ mol/mol fructose/glycerol composites, with a maximum increase observed at 12‰ mol/mol fructose/glycerol (2.4046 ± 0.23855 mg(gallic acid).g(flower)$^{-1}$) when compared with extracts prepared with neat glycerol (1.8289 ± 0.12883 mg(gallic acid).g(flower)$^{-1}$). Similarly, extracts prepared with 8-20‰ mol/mol glucose/glycerol composites exhibited significant GAE increases with a maximum increase observed at 20‰ mol/mol glucose/glycerol (2.0266 ± 0.18285 mg(gallic acid).g(flower)$^{-1}$) when compared with extracts prepared with neat glycerol. An apparent increase in phenolic content was observed in extracts prepared with 8‰ mol/mol sucrose/glycerol composites; however, this was not found to be significant. Significant decreases in phenol content were observed in 40-60‰ mol/mol glucose/glycerol composites and 20-60‰ mol/mol sucrose/glycerol composites.

Unscrambler analysis of the total phenolic content of *C. officinalis* extracts prepared with 0-12‰ mol/mol glycerol composites showed a strong (R=0.74) correlation between phenolic content and composite chaotropicity (Figure 5-42). The increases in phenolic content observed in *C. officinalis* extracts prepared with sugar/glycerol composites indicates that the use of more chaotropic solvents for the preparation of *C. officinalis* extracts result in an increased phenol content.

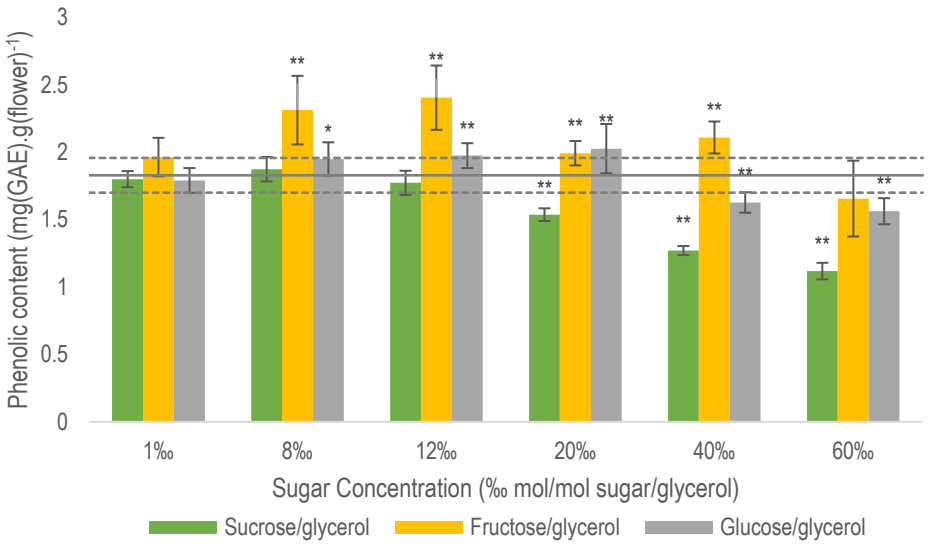


Figure 5-41: Total phenolic content of C. officinalis *extracts prepared with sugar/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=9). Asterisks indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. The line represents the phenolic content of glycerolic extracts, with dashed lines representing the error margin.*

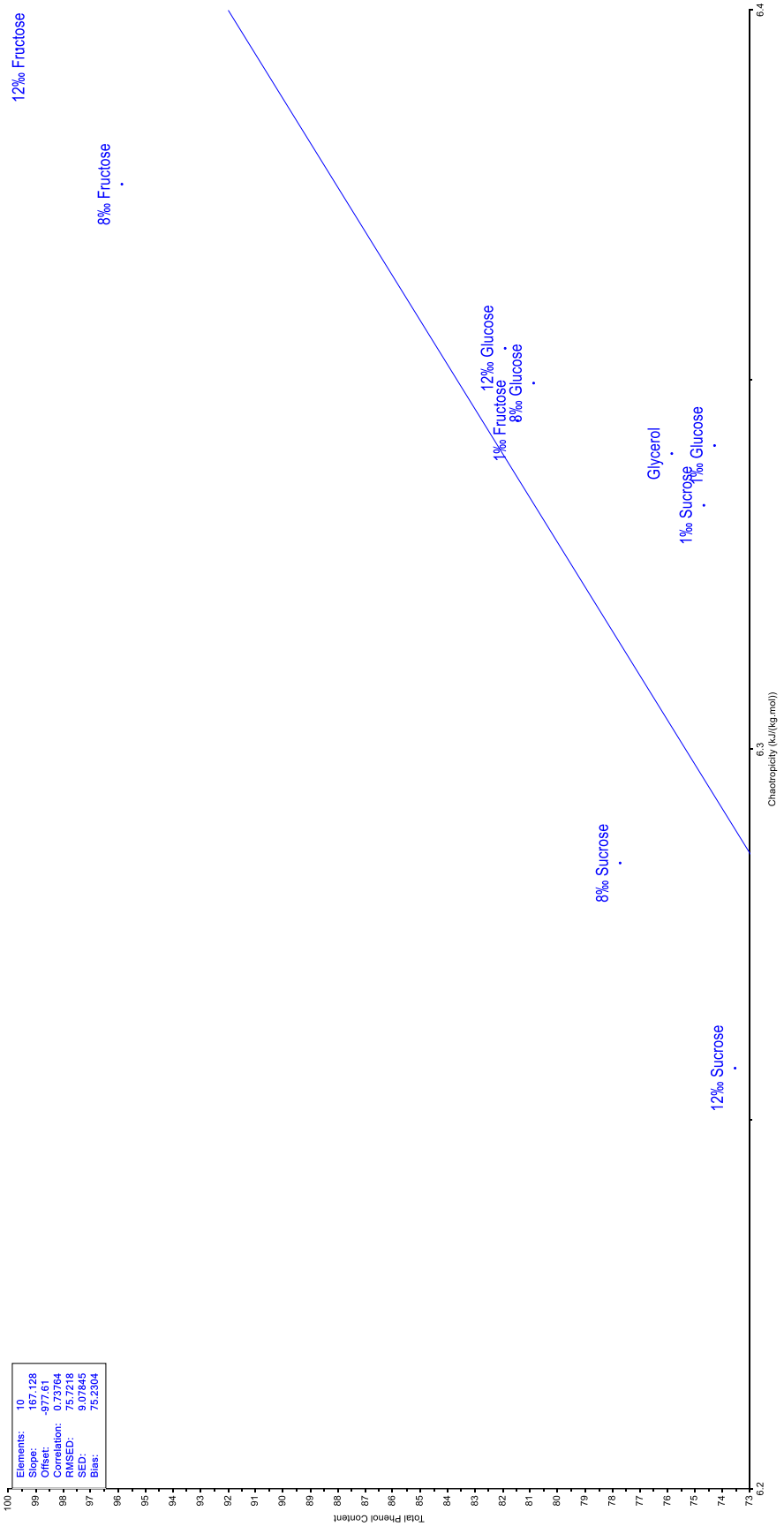


Figure 5-42: Correlation between phenolic content and chaotropicity of C. officinalis *extracts prepared with sugar/glycerol composites. Data is presented as means of replicates (n=9).*

From the above results, it can be observed that the preparation of *C. officinalis* extracts from sugar/glycerol composites result in increased total peak area, chemiluminescence, DPPH radical scavenging activity, and phenol content when compared with extracts prepared with neat glycerol. This may result from the increased hydrogen bonding present in acid/glycerol and sugar/glycerol composites when compared to neat glycerol.

Strong correlations exist between solvent chaotropicity and the phytochemical content of *C. officinalis* extracts prepared with sugar/glycerol composites. There are three non-exclusive explanations of this phenomenon; firstly, the use of chaotropic agents may result in the destabilisation of lipid bilayers [226] that make up the membrane of plant vacuoles [227] present in plant cells that are responsible for the storage of plant metabolites [228], resulting in increased diffusion of metabolites into the solvent. Secondly, as chaotropic agents are known to increase the solubility of relatively nonpolar compounds [226], the use of more chaotropic solvents may result in increased glycerolic solubility of *C. officinalis* metabolites. Thirdly, sucrose, fructose, and glucose have previously been shown to stabilise polyphenols in apple puree [219] and similarly could be reducing metabolite degradation compared to neat glycerol.

The use of sugar/glycerol composites is limited, however, by the increased viscosity observed at higher molar ratios (typically 20-60‰ mol/mol) that results in decreased diffusion of molecules through the solvent. An optimal additive for the preparation of glycerol composites will therefore have high chaotropicity and will not result in increased solvent viscosity.

5.5 SUMMARY

Glycerol composites prepared from chaotropic and kosmotropic salts, acids, and sugars were investigated as alternative solvents for the extraction of plant metabolites from *C. officinalis*. Extracts prepared from salt/glycerol composites exhibited total peak area, chemiluminescence, DPPH radical scavenging activity, and phenol content that decreased with increasing salt content. It can therefore be concluded that salt/glycerol composites are not suitable solvents for the extraction of plant metabolites from *C. officinalis.* Extracts prepared from acid/glycerol composites exhibited total peak area, chemiluminescence, DPPH radical scavenging activity, and phenol content that increased with increasing molar ratios of up to 12‰ mol/mol, with significant decreases observed at higher molar ratios. Similarly, *C. officinalis* extracts prepared from fructose/glycerol and glucose/glycerol composites exhibited total peak area, chemiluminescence, DPPH radical scavenging activity, and phenol content that increased with increasing molar ratios of up to 12‰ mol/mol, with significant decreases observed at higher molar ratios. Sucrose/glycerol composites did not exhibit increases, and displayed significant decreases at higher molar ratios. Strong correlations were observed between total peak area, chemiluminescence, DPPH radical scavenging activity, phenol content, and the chaotropicity of the sugar/glycerol composites. This suggests that solvent chaotropicity plays a role in increasing the total plant metabolite content. It can be hypothesised that a more chaotropic composite would further increase metabolite concentration in *C. officinalis* extracts.

6 POLYSORBATE-BASED COMPOSITE EXTRACTIONS

6.1 CHAPTER SYNOPSIS

This chapter explores the use of surfactant/glycerol and surfactant/water composites as solvents for the extraction of metabolites from *C. officinalis*. Dried *C. officinalis* flowers were used as a model plant material due to their high content of antioxidant and polyphenolic phytochemicals. Extracts were prepared using ultrasonic and maceration in polysorbate composites prepared from Eumulgin® 20 and Tween™ 80. Total phytochemical content and individual phytochemical concentrations were determined by UHPLC-DAD analysis. Total antioxidant activity, radical scavenging activity, and total phenolic content were determined by $KMnO_4$ chemiluminescence, DPPH, and Folin-Ciocalteau assays respectively. Results showed that *C. officinalis* extracts prepared with glycerolic polysorbate composites were observed to contain higher quantities of extracted components when compared with extracts prepared with neat glycerol. Similarly, assays showed that *C. officinalis* extracts prepared with aqueous polysorbate composites were observed to contain higher quantities of extracted components when compared with extracts prepared with neat water. Water/polysorbate composites were observed to give the greatest yields of extracted components.

6.2 INTRODUCTION

Polysorbates (PS) are non-ionic surfactants composed of polyoxyethylene sorbitan fatty acid esters that contain both hydrophobic and hydrophilic groups. Structurally, polysorbates comprise a sorbitan core (highlighted red in figure), which has been ethoxylated (highlighted green in figure) and finally modified with a single saturated or unsaturated fatty acid (highlighted blue in figure), typically caproic, caprylic, capric, lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, or linolenic acid (Figure 6-1). The total number of repeating oxyethylene subunits across the 4 chains (W+X+Y+Z) is indicated in the name of the molecule. For example, Polysorbate 20 (PS20) contains a total of 20 oxyethylene subunits, however the distribution of the oxyethylene subunits across the various arms can vary. The name, however, does not indicate the acid, and often Polysorbates are sold as mixtures of fatty acids. Hydrophilicity is provided by the polyoxyethylene chains and hydrophobicity is provided by the fatty acid moiety 'tail.'

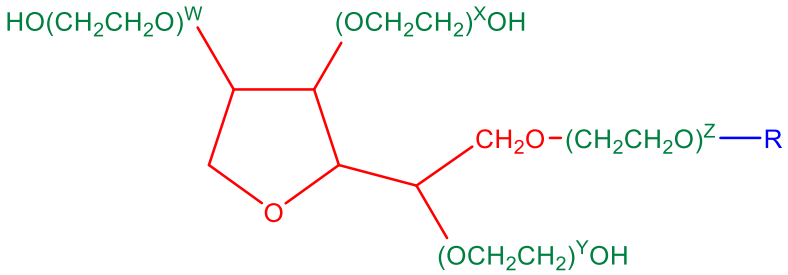


Figure 6-1: Structure of a PS ester - polyoxyethylene sorbitan fatty acid ester where (W+X+Y+Z) = 20 oxyethylene subunits and R is a fatty acid ester.

Polysorbates are composed of a mixture of esters with different polyoxyethylene chain lengths and fatty acid moieties, and can vary substantially in composition (Table 6-1). The percentage of fatty acid esters that are present in a polysorbate are defined in the European Pharmacopoeia but not in the United States Pharmacopoeia [229]. Table 6-1 demonstrates that PS20 is composed of smaller-chain fatty acid esters such as lauric, palmitic, and myristic acids and (on average) shorter polyoxyethylene chains (20 repeating units) when compared with PS80, which is primarily composed of oleic, linoleic, and palmitic fatty acid esters and longer polyoxyethylene chains (80 repeating units).

Table 6-1: Fatty acid ester structure and typical composition in polysorbate 20 and polysorbate 80 [229].

Acid	Structure	Percentage composition (%)	
		P20	*P80*
Linolenic	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$		≤4
Oleic	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	≤11	≥58
Stearic	$CH_3(CH_2)_{16}(COOH)$	≤7	≤6
Linoleic	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$	≤3	≤18
Palmitoleic	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$		≤8
Palmitic	$CH_3(CH_2)_{14}COOH$	7-15	≤16
Myristic	$CH_3(CH_2)_{12}COOH$	14-25	≤5
Lauric	$CH_3(CH_2)_{10}COOH$	40-60	
Capric	$CH_3(CH_2)_8COOH$	≤10	
Caprylic	$CH_3(CH_2)_6COOH$	≤10	
Caproic	$CH_3(CH_2)_4COOH$	≤1	

Polysorbates are used to stabilise proteins against aggregation and surface adsorption [230] or to solubilise compounds that have poor solubility (for example as oil-in-water emulsions [231] or micelles [232] for use in cosmetics [233], pharmaceutical drug delivery [234], and food (for example, polysorbate is used to improve the texture and melting resistance of icecream [235]). Pharmaceutical and food-grade polysorbates typically have fewer impurities than cosmetic or industrial grades and have been generally recognised as safe [236]. Research by Sharma *et al.* [237] has shown increased extraction of phenol content and antioxidant activity by aqueous non-ionic surfactant systems in apple, mango, and lemon fruit juices. Similar research by Hosseinzadeh *et al.* [238] has shown increased extraction by aqueous

non-ionic surfactant systems from apple pulp. There is, however, no research on the effect of surfactants on the extraction of metabolites from *C. officinalis*.

Polysorbates Eumulgin® 20 Tween™ 80 were determined by Cray *et al.* [213] to be highly chaotropic (+361 $kJ.kg^{-1}.mol^{-1}$ and +127$kJ.kg^{-1}.mol^{-1}$ for pure Tween® 20 and Tween™/ 80 respectively). Given the correlations between individual metabolite concentrations (including Isorhamnetin content), chemiluminescence response, radical scavenging activity, and phenolic content in *C. officinalis* extracts prepared with sugar/glycerol composites and solvent chaotropicity observed in Chapter 5, it was hypothesised that the addition of polysorbate to glycerol will increase the concentration of plant metabolites in *C. officinalis* extracts when compared with extracts prepared with neat glycerol. Viscosity was additionally observed to be detrimental to metabolite concentration and it can be hypothesised that water/polysorbate composites will also increase the concentration of plant metabolites in *C. officinalis* extracts when compared with extracts prepared with neat water.

6.3 EXPERIMENTAL

Calendula officinalis samples. Dried *C. officinalis* flower heads were used as supplied. Flowers were grown in the Adelaide Hills, South Australia on a certified biodynamic farm (Jurlique International, certified under the National Association for Sustainable Agriculture, Australia), hand-picked and air-dried in sheds. Dried *C. officinalis* was ground using a commercial grinder (Sunbeam Coffee Grinder, purchased commercially) and stored in sealed plastic containers in darkness at ambient conditions prior to use.

Chemicals and Reagents. Polysorbate 20 (as Eumulgin® SML 20) and Polysorbate 80 (as Tween™ 80-LQ-(SG)) were provided by Jurlique International (Mount Barker, SA, AUS). Centrifuge vials (10 mL, polypropylene) and storage vials (3 mL, polypropylene) were purchased from Sarstedt (Nümbrecht, DE). Plastic syringes (1 & 5 mL, polypropylene) and cotton wool were purchased from Livingstone (Rosebery, NSW, AUS). Glycerol (99.5%) was purchased from Chem-supply (Gillman, SA, AUS). Deionised water was purified to 18 MΩ using a Thermo Fisher Scientific (Waltham, MA, USA) Barnstead™ E-Pure™ water system.

Solvent Preparation.

Glycerolic polysorbate composites. PS20/glycerol composites were prepared by titrating PS20 with glycerol at ambient conditions to 1‰, 8‰, 12‰, and 20‰ mol/mol PS/glycerol with subsequent stirring for 12 hours. PS80/glycerol composites were similarly prepared by titrating PS80 with glycerol. PS20 and PS80 were treated as pure for the purposes of determining molar ratios.

Aqueous polysorbate composites. PS20/water composites were prepared by titrating PS20 with water to 1‰, 8‰, 12‰, and 20‰ mol/mol PS/water with subsequent heating (70 ^{O}C) and stirring for 3 hours until solution was homogenous. PS80/glycerol composites were similarly prepared by titrating PS80 with water. PS20 and PS80 were treated as pure for the purposes of determining molar ratios.

Extract Preparation.

Reference extracts. Glycerolic extracts were prepared in triplicate by combining *C. officinalis* (0.25 g) with glycerol (5 g) with subsequent ultrasonication (Elmasonic S30, 120 min) and centrifugation (Clements Orbital 325, 3000 rpm, 30 min). Extracts were then filtered through a syringe packed with clean

cotton wool. Process blanks were prepared and analysed concurrently using identical techniques. Samples and blanks were diluted with hydroethanolic solvent prior to analysis. Aqueous extracts were prepared in triplicate by combining *C. officinalis* (0.25 g) with deionised water (5 g) with subsequent ultrasonication (Elmasonic S30, 120 min) and centrifugation (Clements Orbital 325, 3000 rpm, 30 min). Extracts were then filtered through a syringe packed with clean cotton wool. Filtration yielded 3 different samples. Process blanks were prepared and analysed concurrently using identical techniques. Samples and blanks were diluted with hydroethanolic solvent prior to analysis.

Polysorbate composite extracts. Extracts were prepared in triplicate by combining *C. officinalis* (0.25 g) with the PS/glycerol composites (5 g) with subsequent ultrasonication (120 min) and centrifugation (3000 rpm, 30 min). Extracts were then filtered through a syringe packed with clean cotton wool. Process blanks were prepared concurrently. Extracts were stored in darkness at ambient conditions prior to analysis. Samples and blanks were diluted with hydroethanolic solvent prior to analysis to adjust viscosity.

UHPLC.

Chemiluminescence response. SIA-CL assays were conducted using the method detailed in Chapter 2.3.

Radical Scavenging Activity. DPPH assays were conducted using the method detailed in Chapter 2.4. A reference standard of methanolic quercetin (25 μM) was run concurrently.

Phenolic Content. Folin-Ciocalteu assays were conducted using the method detailed in Chapter 2.5. Samples were centrifuged (Clements Orbital 325, 3000 rpm, 5 min) prior to UV/Vis analysis. Reference standards of aqueous gallic acid (10, 20, 30, 40, and 50 mg/mL) were run concurrently.

Statistical Testing. F-testing and T-testing to determine statistical significance was conducted using the method outlined in Chapter 2.6.

6.4 RESULTS & DISCUSSION

Polysorbate composites of Eumulgin® 20 and Tween 80 were prepared at a range of concentrations (1‰, 8‰, 12‰, and 20‰ mol/mol) in both water and glycerol. PS/water composites contained concentrations of PS above the CMC for PS20 and PS80. When prepared in water, significant increases to viscosity were observed with increasing PS content, with PS80/water composites demonstrating much higher viscosity than PS20/water composites at a similar molarity. It was not possible to prepare 20‰ mol/mol PS80/water composites under the conditions used to prepare the remaining composites, as the composite viscosity was high enough that a laboratory stirrer – hot plate was unable to stir the mixture. As such, *C. officinalis* extracts were not prepared in 20‰ mol/mol PS80/water composites. In contrast, when the composites were prepared in glycerol, the higher concentrations of both polysorbates (12‰ and 20‰ mol/mol PS/glycerol) were observed to form emulsions. Despite this the composite material was used for extraction as prepared. It was observed that plant extracts from these emulsions separated during ultrasonic-assisted extraction, resulting in two phases with plant material present at the interface between phases. Blanks were also observed to partially separate, but not form two distinct phases as observed in the plant extracts. These phases were different colours when compared to each other, and different in colour to the glycerol and PS starting materials. This indicated that different metabolites may have been extracted into both glycerol and PSs, however to be consistent with the remaining samples, the phases were recombined for analysis.

UHPLC Analysis. UHPLC analyses were conducted with a view to determining the phytochemical composition of *C. officinalis* extracts prepared with glycerolic and aqueous PS composites. Figure 6-2 shows the phytochemical composition of *C. officinalis* extracts prepared with hydroethanolic, glycerolic, and aqueous PS20 composites. It can be seen that extracts prepared with PS composites exhibit similar phytochemical composition to extracts prepared with aqueous ethanol. An additional peak was observed at 15.6 minutes, indicating the presence of an additional compound. Peaks were selected at 0.8, 8.5, 11.1, 11.2, 11.5, 11.9, 12.8, 13.3, and 15.6 minutes for further analysis due to clear peak resolution and antioxidant activity determined in Chapter 3.4.1 and labelled peaks A-J respectively.

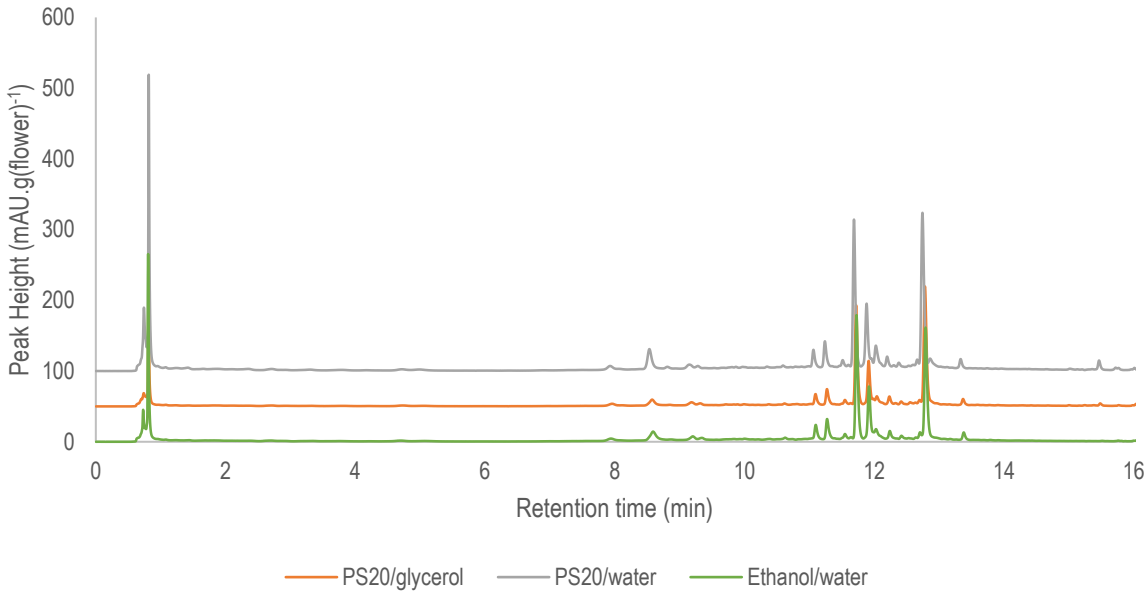


Figure 6-2: UHPLC chromatograms of C. officinalis *extracts prepared with aqueous and glycerolic PS20 composites, and hydroethanolic solvents. Sequential chromatograms are offset vertically by 50 mAU.g(flower)$^{-1}$.*

UHPLC experiments were conducted with a view to determining the peak heights of a number of representative metabolites extracted from *C. officinalis* flowers with PS/glycerol and PS/water composites. Figure 6-3 shows the peak heights for peak A at 1-20‰ mol/mol PS/glycerol mixtures. Figure 6-4 similarly shows the peak heights for peak A at 1-20‰ mol/mol PS/water mixtures. It can be seen that significant increases in Peak A height are exhibited in extracts prepared with 1‰ and 12‰ mol/mol PS20/glycerol (32812 ± 2227.7 mAU.g(flower)$^{-1}$ and 32859 ± 2216.9 mAU.g(flower)$^{-1}$ respectively) when compared with extracts prepared with neat glycerol (24420 ± 5016.2 mAU.g(flower)$^{-1}$). Extracts prepared with aqueous PS composites exhibited apparent decreases in Peak A height; however, these were not found to be significant. This result indicates that the addition of surfactants may increase the extraction of polar metabolites in glycerolic extractions, but has little impact on the extraction of polar metabolites in aqueous extractions. Extracts prepared with neat water contained significantly higher concentration of polar metabolites than extracts prepared with neat glycerol (approximately three times higher in water than in glycerol). Given the significantly higher viscosity of glycerol compared with water (1.005 and 1412 centipoises for neat water and glycerol respectively [239]), metabolites will more readily diffuse into aqueous solvents than glycerolic solvents. Additionally, water is significantly more polar than glycerol and as such would more favourably extract polar metabolites.

Figure 6-5 and Figure 6-6 show the peak heights for peak B at 1-20‰ mol/mol PS/glycerol and PS/water mixtures respectively. It can be seen that there are significant increases in the measured peak heights

for 8-20‰ mol/mol PS20/glycerol and 8-20‰ mol/mol PS80/glycerol, with maximum increases observed at 12‰ mol/mol PS20/glycerol (1696.0 ± 114.28 mAU.g(flower)$^{-1}$) and 20‰ mol/mol PS80/glycerol (1268.1 ± 114.29 mAU.g(flower)$^{-1}$) when compared with extracts prepared from neat glycerol (997.19 ± 65.722 mAU.g(flower)$^{-1}$). Significant increases were observed in aqueous extracts prepared in 12‰ mol/mol PS20/water (5412.7 ± 162.73 mAU.g(flower)$^{-1}$) when compared with extracts prepared with neat water (4138.7 ± 519.92 mAU.g(flower)$^{-1}$). This result indicates that the addition of surfactants may increase the extraction of less polar metabolites in both glycerolic and aqueous extractions. Extracts prepared with neat water contained significantly higher concentrations of metabolites than extracts prepared with neat glycerol.

Significant increases in peak heights can be observed in less polar metabolites. Figure 6-7 shows the peak heights for Peak G at 1-20‰ mol/mol PS/glycerol, and Figure 6-8 shows the peak heights for Peak G at 1-20‰ mol/mol PS/water. Significant increases can be observed in all glycerolic extracts containing surfactants when compared with extracts prepared with neat glycerol. Similarly, increases can be observed in aqueous extracts containing 1-12‰ mol/mol PS/water; however, these were only significant for extracts prepared with 1‰ mol/mol PS20/water and 8‰ mol/mol PS80/water. This is due to the larger variability of results observed in aqueous extracts. Similarly, Figure 6-9 shows the peak heights for Peak I at 1-20‰ mol/mol PS/glycerol, and Figure 6-10 shows the peak heights for Peak I at 1-20‰ mol/mol PS/water, and it can be seen that significant increases can be observed for all glycerolic and aqueous extracts except for 1‰ mol/mol PS80/glycerol and 20‰ mol/mol PS20/water when compared to extracts prepared with neat glycerol or water respectively. Given that surfactants are typically used to solubilise nonpolar compounds, it was anticipated that the addition of surfactants would increase the extraction of nonpolar metabolites in *C. officinalis*. The concentrations used in the PS/glycerol and PS/water composites exceeded the Critical Micelle Concentration. Extracts prepared with neat water contained significantly higher concentrations of metabolites than extracts prepared with neat glycerol (approximately one and a half times higher in water than in glycerol for peaks G and I).

Similar increases in peak height were observed in glycerolic PS extracts for Peaks C-H, with significant increases in extracts prepared with PS/glycerol composites for Peaks C-H, typically at 8-12‰ mol/mol PS/glycerol. Increases were also observed for Peaks C-H in aqueous PS extracts, with significant increases observed for Peaks D, E, G, and H, typically at 8-12‰ mol/mol PS/water. Information for Peaks C-H are available in Appendix 5.

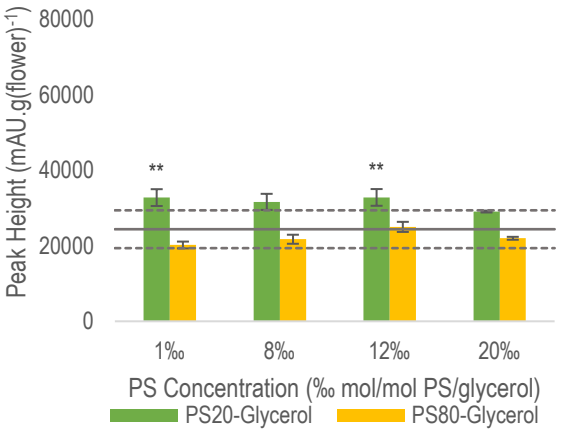


Figure 6-3: Peak heights for Peak A in C. officinalis *extracts prepared with PS/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the height of Peak A in glycerolic extracts, with dashed lines representing the error margin.*

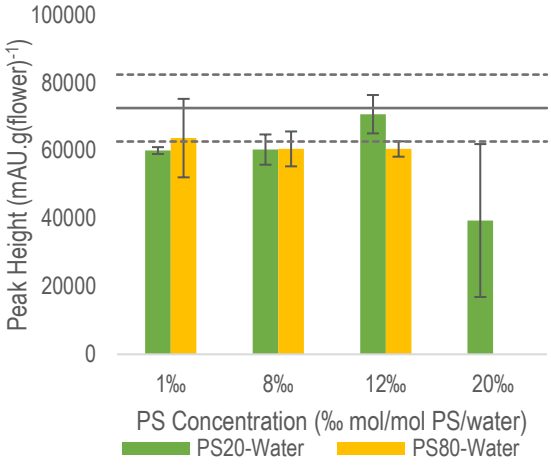


Figure 6-4: Peak heights for Peak A in C. officinalis *extracts prepared with PS/water composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat water. The line represents the height of Peak A in aqueous extracts, with dashed lines representing the error margin.*

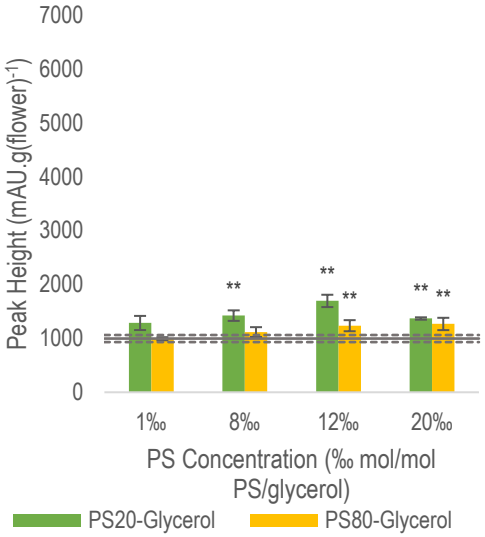


Figure 6-5: Peak heights for Peak B in C. officinalis *extracts prepared with PS/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the height of Peak B in glycerolic extracts, with dashed lines representing the error margin.*

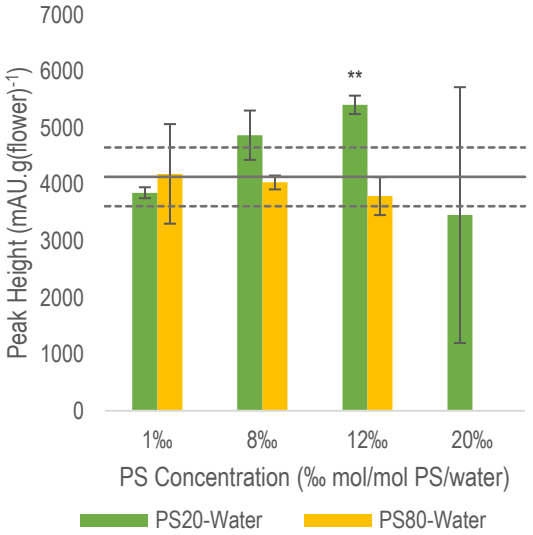


Figure 6-6: Peak heights for Peak B in C. officinalis *extracts prepared with PS/water composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat water. The line represents the height of Peak B in aqueous extracts, with dashed lines representing the error margin.*

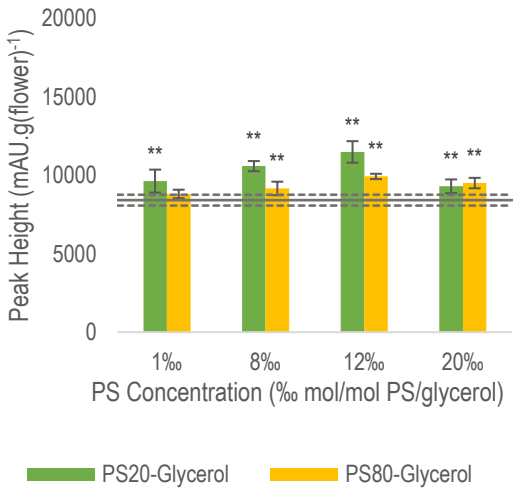


Figure 6-7: Peak heights for Peak G in C. officinalis *extracts prepared with PS/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the height of Peak G in glycerolic extracts, with dashed lines representing the error margin.*

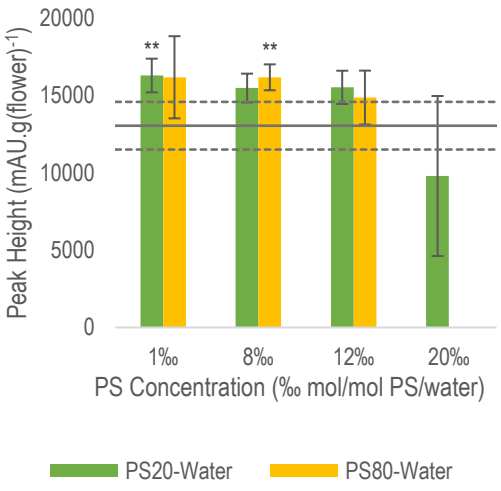


Figure 6-8: Peak heights for Peak G in C. officinalis *extracts prepared with PS/water composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat water. The line represents the height of Peak G in aqueous extracts, with dashed lines representing the error margin.*

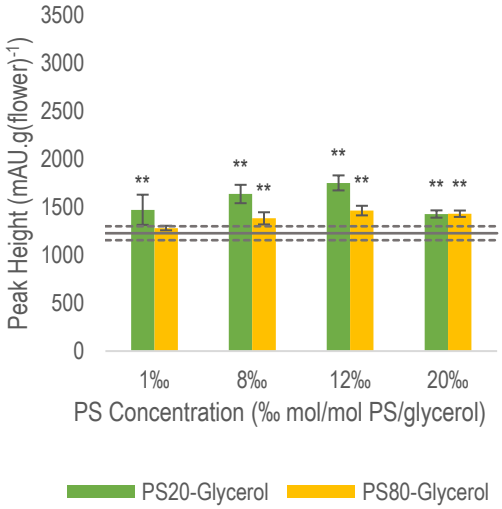


Figure 6-9: Peak heights for Peak I in C. officinalis extracts prepared with PS/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the height of Peak I in glycerolic extracts, with dashed lines representing the error margin.

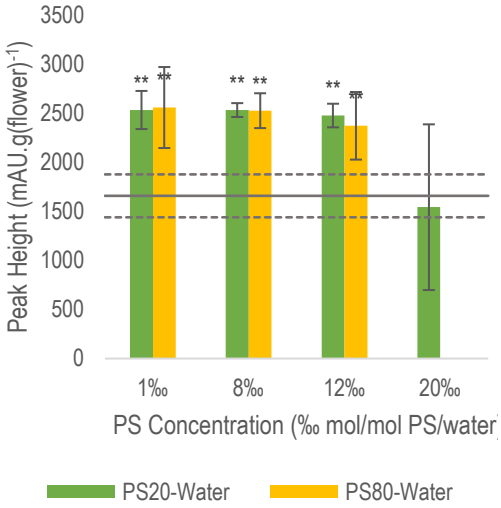


Figure 6-10: Peak heights for Peak I in C. officinalis extracts prepared with PS/water composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat water. The line represents the height of Peak I in aqueous extracts, with dashed lines representing the error margin.

UHPLC experiments were conducted with a view to determining the total peak area of *C. officinalis* extracts prepared with polysorbate/glycerol and polysorbate/water composites. Figure 6-11 shows the total peak areas of *C. officinalis* extracts prepared with 1-20‰ mol/mol PS/glycerol composites. It can be seen from Figure 6-11 that significant increases are observed in prepared with 1-20‰ PS20/glycerol, with maximum increases observable in extracts prepared with 12‰ PS20/glycerol (10798 ± 534.37 mAU.s.g(flower)$^{-1}$). Significant increases are also observed in extracts prepared with 8-20‰ mol/mol PS80/glycerol, with maximum increases observable in extracts prepared with 12‰ mol/mol PS80/glycerol (9231.3 ± 41.863 mAU.s.g(flower)$^{-1}$ when compared with an extract prepared with neat glycerol (7888.7 ± 267.81 mAU.s.g(flower)$^{-1}$). Figure 6-12 shows the total peak areas of *C. officinalis* extracts prepared with 1-20‰ mol PS20/water and 1-12‰ mol PS80/water composites. It can be seen that extracts prepared with 12‰ mol/mol PS20/water exhibited a significant increase in total peak area (18952 ± 675.09 mAU.s.g(flower)$^{-1}$) when compared with extracts prepared with neat water (15848 ± 1379.4 mAU.s.g(flower)$^{-1}$). Extracts prepared with PS80/water composites exhibited apparent increases in total peak area; however, these were determined to not be significant. Increases in total peak area exhibited by aqueous and glycerolic PS composites suggests that the use of surfactants in phytochemical extraction increases the yield of metabolites extracted.

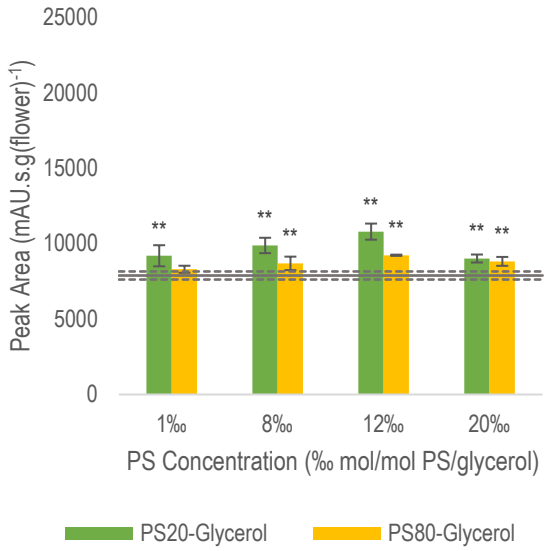


Figure 6-11: Total Peak Area of C. officinalis *extracts prepared with PS/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol. The line represents the total peak area of glycerolic extracts, with dashed lines representing the error margin.*

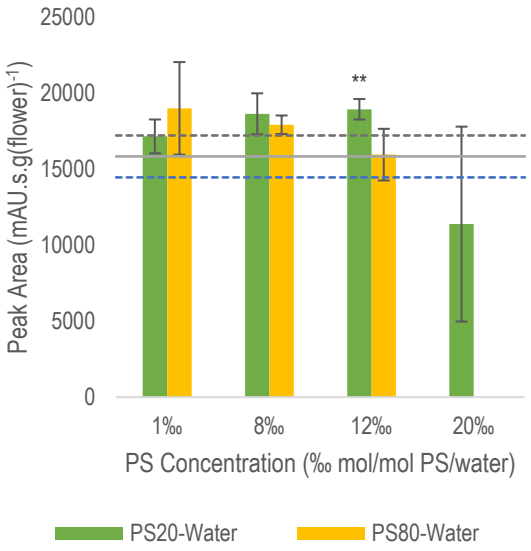


Figure 6-12: Total Peak Area of C. officinalis *extracts prepared with PS/water composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with extracts prepared with neat water. The line represents the total peak area of aqueous extracts, with dashed lines representing the error margin.*

In summary, the addition of surfactants to glycerolic extracts of *C. officinalis* significantly increased the extraction of polar and nonpolar metabolites, whereas the addition of surfactants to aqueous *C. officinalis* extracts significantly improved the extraction of nonpolar compounds but exhibited no significant effect on the extraction of polar metabolites. Extracts prepared with PS/water composites presented increased peak heights when compared to extracts prepared with PS/glycerol at equivalent PS concentrations. Extracts prepared with neat water presented increased peak heights when compared to extracts prepared with neat glycerol. This increase was greater for metabolites with low retention times (approximately 3x and 4x for peaks A and B respectively) compared with strongly retained metabolites (1.2x and 1.3x for peaks H and I respectively), indicating that while water is significantly better at extracting polar compounds, both solvents extract equivalent amounts of less-polar metabolites. Total peak areas for extracts prepared with glycerolic PS composites were observed to increase significantly when compared with extracts prepared from neat glycerol, and total peak areas for extracts prepared with aqueous PS composites were observed to significantly increase in aqueous PS20 extracts. Although the increases in peak heights and area were greater in glycerolic extracts when compared with extracts prepared with neat glycerol, extracts prepared with aqueous PS composites exhibited overall greater peak heights and

total peak area. These increases indicate that aqueous and glycerolic PS composites can be used to increase the extraction of phytochemicals from *C. officinalis*.

Chemiluminescence response. Experiments were conducted with a view to determining the chemiluminescence response of *C. officinalis* extracts prepared using polysorbate/glycerol composites. Table 9-51 demonstrates that significant increases in chemiluminescence response can be observed in all *C. officinalis* extracts prepared with polysorbate/glycerol composites when compared with extracts prepared with neat glycerol. Maximum increases were observable in extracts prepared with 20‰ mol/mol PS20/glycerol (16224 ± 9055.5mV.s^{-1}.g(flower)$^{-1}$) and 12‰ mol/mol PS80/glycerol (16736 ± 299.8 mV.s^{-1}.g(flower)$^{-1}$) when compared to extracts prepared with glycerol (8924.5 ± 1700 mV.s^{-1}.g(flower)$^{-1}$). Increases in chemiluminescence response were also observed in *C. officinalis* extracts prepared with PS/water composites, with maximum increases occurring at 1‰ mol/mol PS20/water (33268 ± 3285.9 mV.s^{-1}.g(flower)$^{-1}$) and 8‰ PS80/water (31900 ± 2144.4 mV.s^{-1}.g(flower)$^{-1}$) when compared with an extract prepared with water (23265 ± 2972.8 mV.s^{-1}.g(flower)$^{-1}$). Additionally, *C. officinalis* extracts prepared with water exhibited significantly higher chemiluminescence response than an extract prepared with glycerol. The lower number of significant results observed in PS/water composites can be attributed to the increased variability in observed chemiluminescence assays which occurred as a result of difficulties in extract workability that resulted from the substantial increase in viscosity observed in extracts.

Although it appears that *C. officinalis* extracts prepared with PS/glycerol and PS/water composites exhibit significantly higher chemiluminescence activities, it is likely that the chemiluminescence response is being enhanced by PS micelles within both PS/glycerol and PS/water extracts. Micelles were expected to be present in all extracts as all samples were diluted with a 30% ethanol/water solvent prior to analysis (critical micelle concentrations for aqueous PS20 and PS80 are 60 mg.L^{-1} [240] and 13-15 mg.L^{-1} respectively). The presence of micelles in chemiluminescence reactions is well-known to result in an increase in chemiluminescence response [241]. Kato *et al.* [242] demonstrated that Tween®20 and Tween®85 both enhanced the chemiluminescence response of sulfur dioxide in aqueous $KMnO_4$, with Tween®85 exhibiting a larger enhancement than Tween®20. This was hypothesised by to be the result of Tween®85 forming bilayer aggregates (with either vesicular or lamellar structures), which reduced quenching effects and intramolecular interactions that may be competing with the light emissions [242].

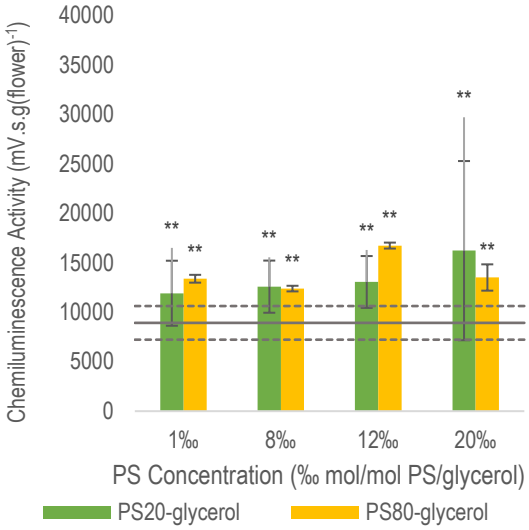


Figure 6-13: Chemiluminescence response of C. officinalis extracts prepared with PS/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=9). Asterisks indicate statistical significance (p<0.05) when compared with extracts prepared with neat water. The line represents the chemiluminescence response in glycerolic extracts, with dashed lines representing the error margin.

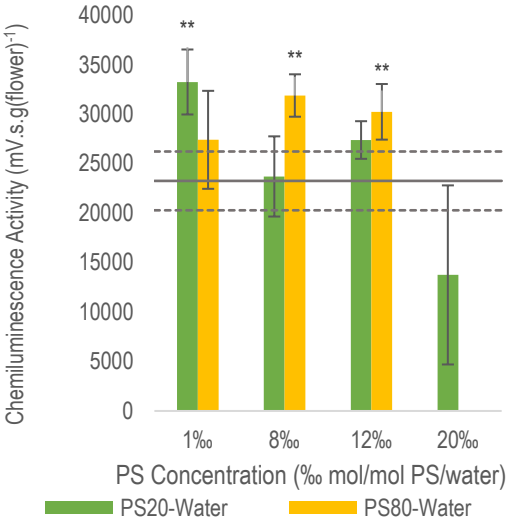


Figure 6-14: Chemiluminescence response of C. officinalis extracts prepared with PS/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=9). Asterisks indicate statistical significance (p<0.05) when compared with extracts prepared with neat water. The line represents the chemiluminescence response in aqueous extracts, with dashed lines representing the error margin.

Radical Scavenging Activity. Experiments were conducted with a view to determining the radical scavenging activity of *C. officinalis* extracts prepared using PS/glycerol composites. Table 9-52 demonstrates that apparent increases in radical scavenging activity can be observed in *C. officinalis* extracts prepared with 12‰ mol/mol PS20/glycerol and 1-20‰ mol/mol PS80/glycerol composites when compared with extracts prepared with neat glycerol (0.5907 ± 0.10998 mg(quercetin).g(flower)$^{-1}$). These increases were significant for extracts prepared with 1-20‰ mol/mol PS80/glycerol composites (0.74574 ± 0.032 mg(quercetin).g(flower)$^{-1}$, 0.76561 ± 0.066113 mg(quercetin).g(flower)$^{-1}$, 0.7219 ± 0.055455 mg(quercetin).g(flower)$^{-1}$, and 0.75206 ± 0.090451 mg(quercetin).g(flower)$^{-1}$ for 1‰, 8‰, 12‰, and 20‰ mol/mol PS80/glycerol respectively). Significant decreases were observed in extracts prepared with 1‰ and 20‰ mol/mol PS20/glycerol composites. Similar increases were observed in *C. officinalis* extracts prepared with PS20/water and PS80/water composites, with significant increases observed for 8-12‰ mol/mol PS20/water (5212.9 ± 459.73 mg(quercetin).g(flower)$^{-1}$ and 5454.8 ± 429.98 mg(quercetin).g(flower)$^{-1}$ for 8‰ and 12‰ respectively) and 8-12‰ mol/mol PS80/water (5280.7 ± 280.69 mg(quercetin).g(flower)$^{-1}$ and 5537.1 ± 275.2 mg(quercetin).g(flower)$^{-1}$ for 8‰ and 12‰ respectively) composites when compared with an extract prepared from neat water (4662.4 ± 874.87 mg(quercetin).g(flower)$^{-1}$). Maximum increases were observed at 12‰ mol/mol

PS20/water and 12‰ mol/mol PS80/water. An apparent decrease in radical scavenging activity was observed in *C. officinalis* extracts prepared with 20‰ mol/mol PS20/water composites; however, this was not significant. *C. officinalis* extracts prepared with neat glycerol appeared higher than extracts prepared with water; however, this was not found to be significant.

Yao *et al.* [243] indicate that PSs degrade in the presence of radical initiators such as 2,2'-azobis-2-methyl-propanimidamide dihydrochloride by autoxidation with the oxyethylene moieties or by oxidation of the fatty acid functionality (in the case of mono-, di-, and tri-unsaturated fatty acids). The latter oxidation mechanism is observed more strongly in extracts containing PS80 due to the higher proportions of unsaturated fatty acids (Table 6-1) which is reflected in Table 9-52 where extracts prepared with PS80 composites demonstrate higher apparent radical scavenging activity when compared with extracts prepared with PS20 composites. It is therefore possible that the use of a stable free radical such as DPPH will increase the rate of oxidation and autoxidation in *C. officinalis* extracts that contain PSs. In particular, extracts that contain PSs with longer polyoxyethylene moieties and unsaturated fatty acids will exhibit increased radical consumption and subsequently increase the apparent inhibition of the DPPH radical by the extract.

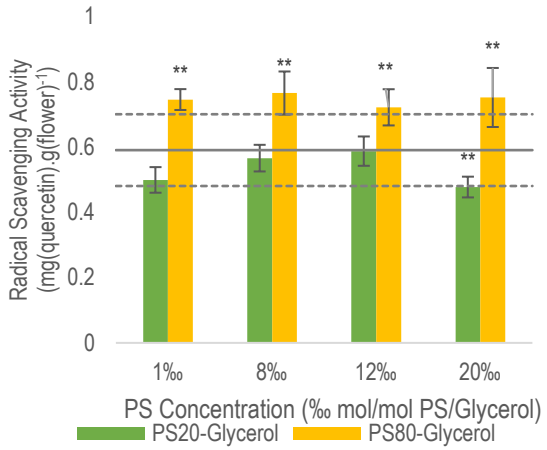


Figure 6-15: Radical scavenging activity of C. officinalis *extracts prepared with PS/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol. The line represents the radical scavenging activity in glycerolic extracts, with dashed lines representing the error margin.*

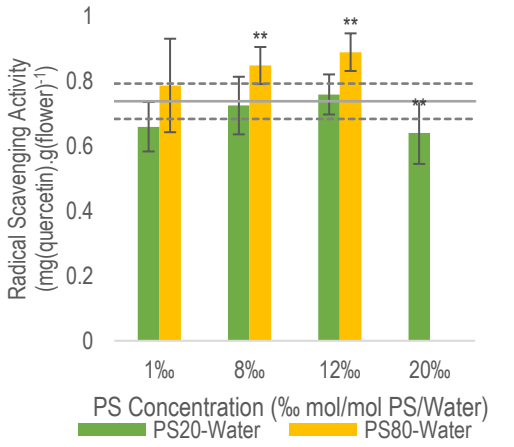


Figure 6-16: Radical scavenging activity of C. officinalis *extracts prepared with PS/water composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with extracts prepared with neat water. The line represents the radical scavenging activity in aqueous extracts, with dashed lines representing the error margin.*

Total Phenolic Content. Experiments were conducted with a view to determine the total phenolic content of *C. officinalis* extracts prepared with PS/glycerol and PS/water composites. Figure 6-17 shows the Gallic Acid Equivalent (GAE) content of *C. officinalis* extracts prepared with 1-20‰ mol/mol PS/glycerol composites. It can be seen that significant increases were observed in all extracts prepared with PS/glycerol composites, with maximum increases in phenolic content occurring at 12‰ mol/mol PS20/glycerol (1.9249 ± 0.12359 mg(GAE).g(flower)$^{-1}$) and 12‰ mol/mol PS80/glycerol (2.0087 ± 0.065121 mg(GAE).g(flower)$^{-1}$) when compared with an extract prepared with neat glycerol (1.3442 ± 0.063658 mg(GAE).g(flower)$^{-1}$). Figure 6-18 shows the total phenolic content of *C. officinalis* extracts prepared with 1-20‰ mol/mol PS20/water and 1-12‰ mol/mol PS80/water composites. It can be seen that significant increases in absorbance are observed in extracts prepared with PS/water composites, with a maximum increase in phenolic content observed at 12‰ mol/mol PS20/water (4.3063 ± 0.27725 mg(GAE).g(flower)$^{-1}$) and 8‰ PS80/water (3.9349 ± 0.17851 mg(GAE).g(flower)$^{-1}$) when compared with an extract prepared with neat water (2.5364 ± 0.18121 mg(GAE).g(flower)$^{-1}$). *C. officinalis* extracts prepared with water were also observed to demonstrate significantly higher total phenolic content than extracts prepared with glycerol.

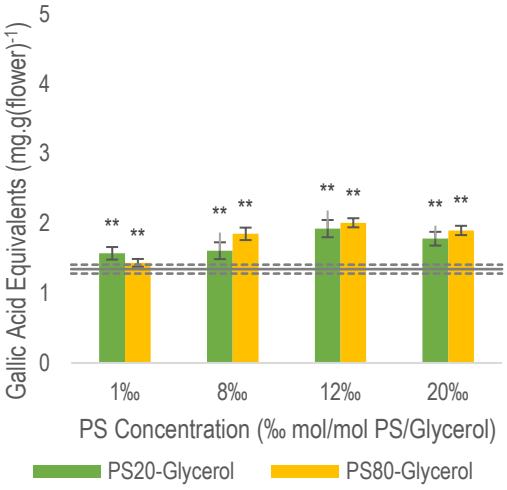


Figure 6-17: Total phenolic content of C. officinalis *extracts prepared with PS/glycerol composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat glycerol. The line represents the total phenolic content of glycerolic extracts, with dashed lines representing the error margin.*

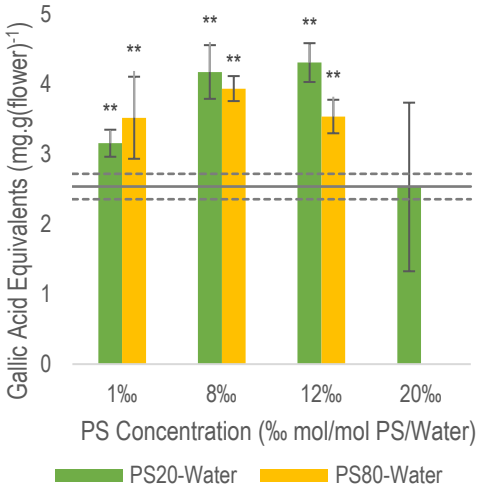


Figure 6-18: Total phenolic content of C. officinalis *extracts prepared with PS/water composites. Data is expressed as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance (p<0.05) when compared with an extract prepared with neat water. The line represents the total phenolic content of aqueous extracts, with dashed lines representing the error margin.*

6.5 SUMMARY

The use of polysorbate/glycerol composites in the preparation of *C. officinalis* extracts demonstrate significant increases in the extraction of individual metabolites, total peak area, chemiluminescence, radical scavenging activity, and phenolic content when compared with extracts prepared with neat glycerol. Results obtained from chemiluminescence analysis and radical scavenging analysis were disregarded due to complications arising from interactions between the matrix and the assays. Similar increases were observed in *C. officinalis* extracts prepared with polysorbate/water composites, with significant increases demonstrated in extracts prepared with PS20/water composites. Similar matrix effects were observed in chemiluminescence and radical scavenging assays.

7 THE OPTIMAL SOLVENT? AQUEOUS ACIDIC POLYSORBATES AS POTENTIAL EXTRACTION SOLVENTS

7.1 CHAPTER SYNOPSIS

This chapter explores the use of an aqueous organic acid/polysorbate solvent mixture for extraction of plant metabolites from *C. officinalis*. Dried *C. officinalis* flowers were used as a model plant material due to their high content of antioxidant and polyphenolic phytochemicals. Total phytochemical content and individual phytochemical concentrations were determined by UHPLC-DAD analysis. Phenolic content was determined with Folin-Ciocalteu assays. Results from HPLC and FC assays show that *C. officinalis* extracts prepared with acidified aqueous polysorbate composites exhibit almost identical phytochemical composition and equivalent concentration, and significantly increased phenolic content to traditional hydroethanolic solvents when used in maceration. Similarly, extracts prepared with acidified aqueous polysorbate composites in ultrasonic extraction processes exhibit almost identical phytochemical composition, and significant increases in phytochemical content and phenolic content when compared with traditional hydroethanolic solvents. These results show that acidified aqueous polysorbates show potential as alternative 'green' solvents in the extraction of plant metabolites.

7.2 INTRODUCTION

In Chapter 6, the use of polysorbate composites in the extraction of phytochemicals from *C. officinalis* demonstrated significant increases in the extraction of individual metabolites, total peak area, chemiluminescence response, radical scavenging activity, and phenolic content when compared with extracts prepared with neat water. It was hypothesised that aqueous polysorbate composites in particular could be used as 'green' alternatives to traditional hydroethanolic solvents in the extraction of metabolites from *C. officinalis*. PS20 was selected due to the greater increases and visually lower viscosities observed. Previous work by Hosseinzadeh *et al.* [238] showed that the addition of acid to pH 3 was optimal for the extraction of polyphenolics from apple pulp. This agreed with results in Chapter 5, which indicated that the presence of organic acid increases the glycerolic extraction of metabolites from *C. officinalis*. Given the presence of nonpolar surfactant micelles, it was hypothesised that the use of ultrasonic extraction processes may further increase the extraction of less polar metabolites through the formation of nonpolar microcavitations [26]. However, room-temperature maceration extraction processes were also tested as lower-energy alternative extraction methods. Thus, aqueous acidified polysorbate composites were selected for final testing as alternative 'green' solvents to replace traditional hydroethanolic solvents in the extraction of phytochemicals from *C. officinalis*.

7.3 EXPERIMENTAL

Calendula officinalis samples. Dried *C. officinalis* flower heads were used as supplied. Flowers were grown in the Adelaide Hills, South Australia on a certified biodynamic farm (Jurlique International, certified under the National Association for Sustainable Agriculture, Australia), hand-picked and air-dried in sheds. Dried *C. officinalis* was ground using a commercial grinder (Sunbeam Coffee Grinder, purchased commercially) and stored in sealed plastic containers in darkness at ambient conditions prior to use.

Chemicals and Reagents. Ethanol (99.96%) and glycerol (99.5%) was purchased from Chem-Supply (Gillman, SA, AUS). Tartaric acid (Food grade, McKenzies Foods) was purchased from local supermarkets. Centrifuge vials (10 mL, polypropylene) and storage vials (3 mL, polypropylene) were purchased from Sarstedt (Nümbrecht, DE). Plastic syringes (1 mL, 5 mL, polypropylene) and cotton wool were purchased from Livingstone (Rosebery, NSW, AUS). Polysorbate 20 (as Eumulgin® SML 20) was supplied by Jurlique International (Mount Barker, SA, AUS). Deionised water was purified to 18 MΩ using a Thermo Fisher Scientific (Waltham, MA, USA) Barnstead™ E-Pure™ water system.

AAP composites. PS20/water composites were prepared by titrating PS20 with water to 10‰ mol/mol PS/water with stirring for 3 hours and then acidifying to pH 3 with saturated aqueous tartaric acid.

Macerated extracts. Extracts were prepared by combining *C. officinalis* (0.1 g) with hydroethanolic or AAP composite solvents (5 g) with stirring (120 minutes) and subsequent centrifugation (Clements Orbital 325, 3000 rpm, 30 min). Extracts were then filtered through a syringe packed with clean cotton wool. Process blanks were prepared and analysed concurrently using identical techniques. Samples and blanks were diluted with hydroethanolic solvent (30% v/v) prior to analysis.

Ultrasonic extracts. Extracts were prepared by combining *C. officinalis* (0.1 g) with hydroethanolic solvent (30% v/v) (5 g) or AAP composite (3 g) with ultrasonication (Elmasonic S30, 120 min) and subsequent centrifugation (Clements Orbital 325, 3000 rpm, 30 min). Extracts were then filtered through a syringe packed with clean cotton wool. Process blanks were prepared and analysed concurrently using identical techniques. Samples and blanks were diluted with hydroethanolic solvents prior to analysis.

Chemiluminescence response. SIA-CL assays were not conducted due to the matrix effects observed in Chapter 6 from the presence of surfactants.

Radical Scavenging Activity. DPPH assays were not conducted due to the matrix effects observed in Chapter 5 from the presence of tartaric acid.

Phenolic Content. Folin-Ciocalteu assays were conducted using the method detailed in Chapter 2.5. Samples were centrifuged (Clements Orbital 325, 3000 rpm, 5 min) prior to UV/Vis analysis. Reference standards of aqueous gallic acid (10, 20, 30, 40, and 50 mg/mL) were run concurrently.

Statistical Testing. F-testing and T-testing to determine statistical significance was conducted using the method outlined in Chapter 2.6.

7.4 Results & Discussion

UHPLC Analysis. UHPLC analyses were conducted with a view to determining the phytochemical composition of ultrasonic and macerated *C. officinalis* extracts prepared with aqueous ethanol and with acidified aqueous polysorbate (AAP) composites. Figure 7-2 shows the phytochemical composition of *C. officinalis* extracts prepared with hydroethanolic solvent (30% v/v) and with AAP. Figure 7-3, Figure 7-4, Figure 7-5, and Figure 7-6 show selected peak heights of metabolites in *C. officinalis* extracts prepared with aqueous ethanol and with AAP. It can be seen that extracts prepared with AAP have similar phytochemical composition to extracts prepared with aqueous ethanol. An additional compound was observed at 15.6 minutes. Peaks were selected at 0.8, 8.5, 11.1, 11.2, 11.5, 11.9, 12.8, 13.3, and 15.6 for further analysis due to clear peak resolution and antioxidant activity determined in Chapter 3.4.1 and labelled peaks A-I respectively (Figure 7-1).

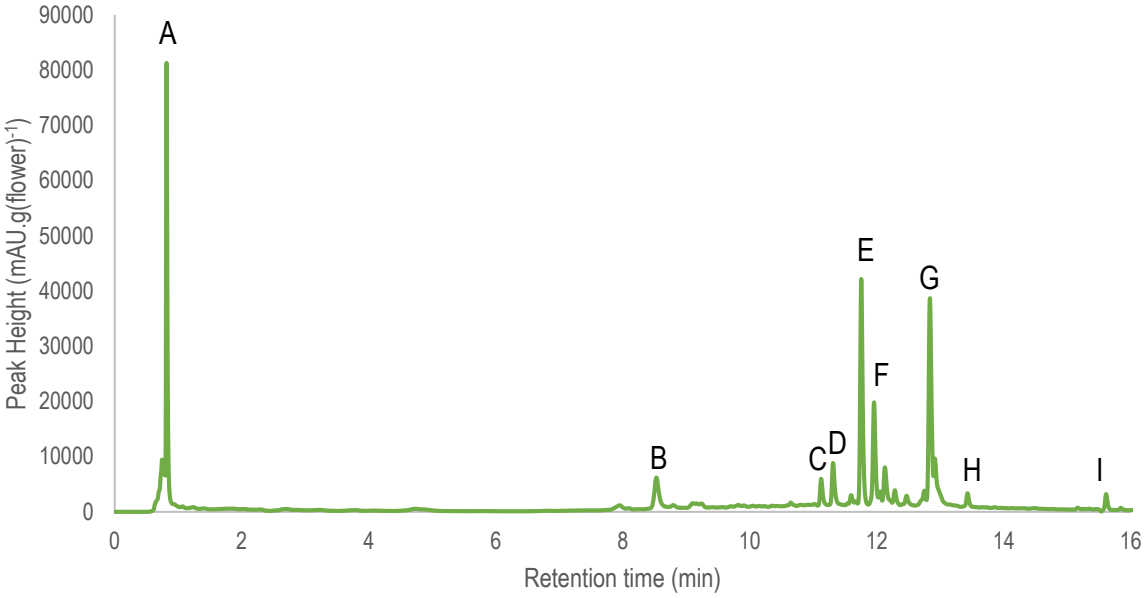


Figure 7-1: UHPLC analysis of an ultrasonic C. officinalis *extract prepared with AAP solvent. Peaks at 0.8, 8.5, 11.1, 11.2, 11.5, 11.9, 12.8, 13.3, and 15.6 min were selected and labelled Peaks A-I respectively.*

Maceration extracts prepared with AAP exhibit an apparent decrease in unretained compounds (Peak A) when compared with extracts prepared with aqueous ethanol; however, this result was not significant. Significant increases in Peak A height were observed in ultrasonic extracts prepared with AAP (6773.1 ± 174.35 mAU.g(flower)$^{-1}$) when compared with extracts prepared with aqueous ethanol (4406.6 ± 391.41 mAU.g(flower)$^{-1}$). This trend was observed in compounds B-I. Significant increases in Peak I height were observed in extracts prepared with AAP when compared with hydroethanolic extracts

for both maceration (609.48 ± 61.135 mAU.g(flower)$^{-1}$ and 52.7379 ± 4.4233 mAU.g(flower)$^{-1}$ for AAP and hydroethanolic solvent respectively) and ultrasonic (2656.4 ± 239.84 mAU.g(flower)$^{-1}$ and 66.536 ± 3.7316 mAU.g(flower)$^{-1}$ for AAP and hydroethanolic solvent respectively) extraction methods. Increases in peak heights were observed to be greater in polar compounds (Peak A) than in less polar compounds (Peak F). However, an additional nonpolar compound was extracted, indicating that the use of PS20 is solubilising additional components. Additionally, the height of Peak I in ultrasonic extracts is significantly higher than in maceration extracts, indicating that ultrasonic extraction is increasing the solubility of this compound. An additional peak was observed at 12.9 minutes as a shoulder on Peak G in ultrasonic extracts that is not present in maceration extracts. However, this peak was not investigated due to poor separation.

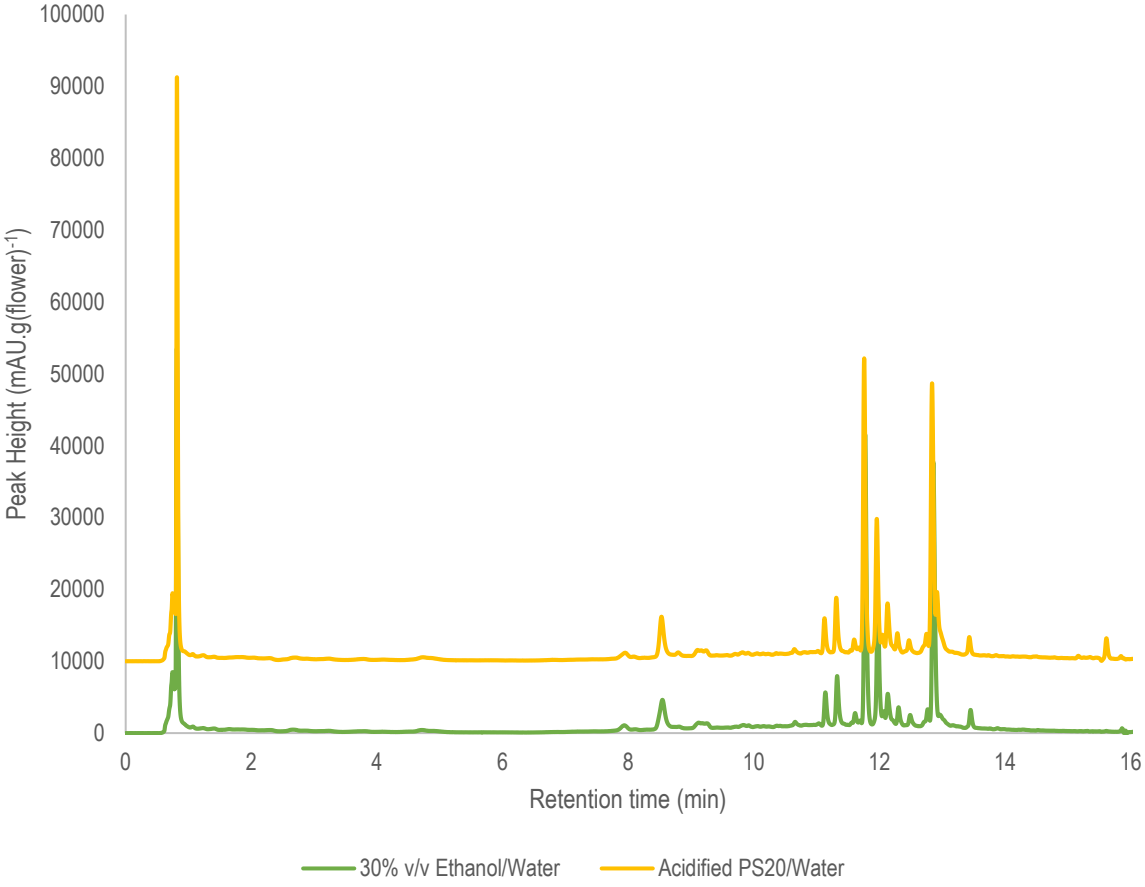


Figure 7-2: UHPLC chromatograms of ultrasonic C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Sequential chromatograms are offset vertically by 10000 mAU.g(flower)$^{-1}$.*

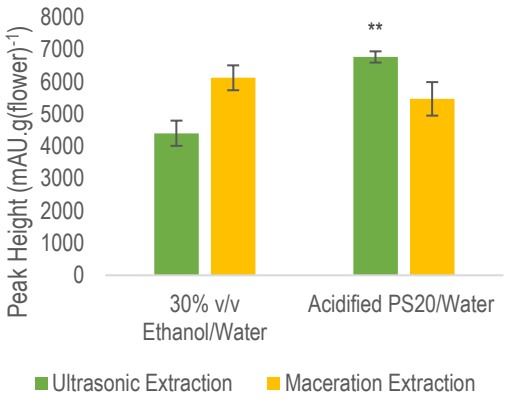


Figure 7-3: Heights of Peak A in macerated and ultrasonic C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is presented as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance when compared with hydroethanolic extracts prepared under similar conditions.*

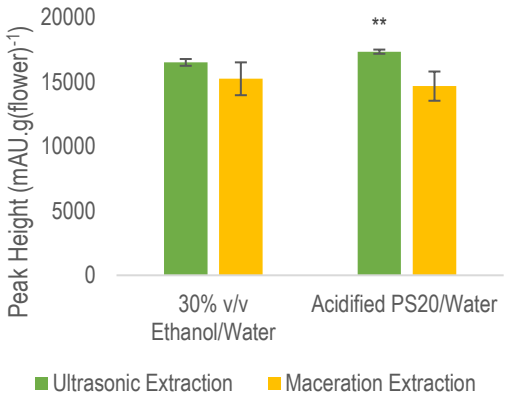


Figure 7-4: Heights of Peak F in macerated and ultrasonic C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is presented as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance when compared with hydroethanolic extracts prepared under similar conditions.*

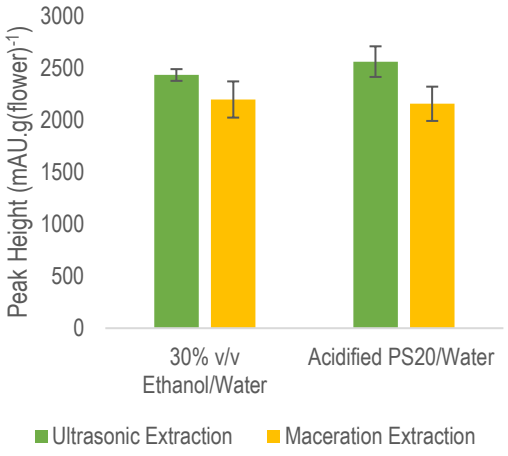


Figure 7-5: Heights of Peak H in macerated and ultrasonic C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is presented as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance when compared with hydroethanolic extracts prepared under similar conditions.*

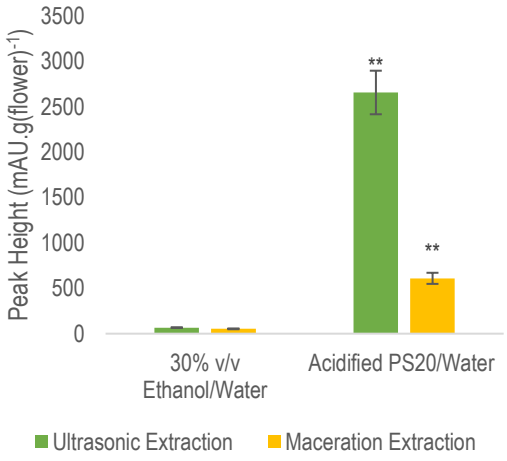


Figure 7-6: Heights of Peak I in macerated and ultrasonic C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is presented as means ± standard deviation of replicates (n=3). Asterisks indicate statistical significance when compared with hydroethanolic extracts prepared under similar conditions.*

The total peak areas of ultrasonic extracts prepared with AAP were observed to be significantly higher than hydroethanolic extracts prepared under identical conditions. Maceration extracts prepared with acidified PS20/water were observed to exhibit and apparent increase in total peak area when compared with hydroethanolic extracts prepared under identical conditions; however, this difference was not significant. This result indicates that the use of acidified PS/water composites in maceration extraction offers an extract of equivalent phytochemical content to hydroethanolic solvents. The use of acidified

PS/water composites in ultrasonic extraction show significantly increased phytochemical content (20793 ± 714.99 mAU.s.g(flower)$^{-1}$) when compared to hydroethanolic solvents (17258 ± 1774.1 mAU.s.g(flower)$^{-1}$).

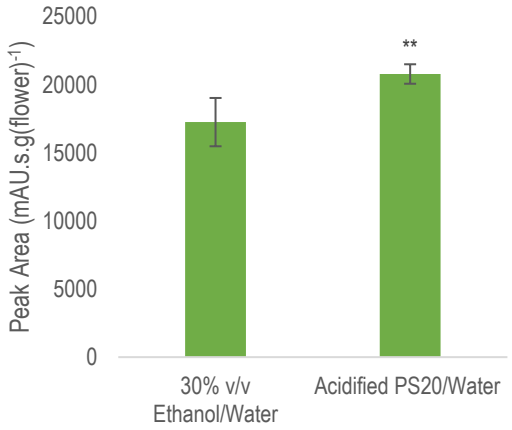


Figure 7-7: Total UHPLC peak areas of macerated and ultrasonic C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is presented as means ± standard deviation of replicates (n=9). Asterisks indicate statistical significance when compared with hydroethanolic extracts prepared under similar conditions.*

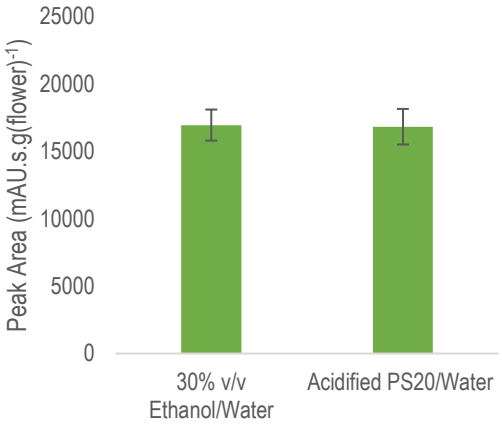


Figure 7-8: Total UHPLC peak areas of macerated and ultrasonic C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is presented as means ± standard deviation of replicates (n=9). Asterisks indicate statistical significance when compared with hydroethanolic extracts prepared under similar conditions.*

Phenolic content. Folin-Ciocalteu assays were conducted with a view to determining the total phenolic content of *C. officinalis* extracts prepared using ultrasonic and maceration extraction methods with aqueous ethanol and AAP solvents. Figure 7-9 and Figure 7-10 show the Gallic Acid Equivalent (GAE) content of *C. officinalis* extracts prepared with aqueous ethanol and AAP using ultrasonic and maceration extraction methods respectively. It can be seen that the use of AAP solvents in ultrasonic extraction offers significant increases in phenolic content (3.8824 ± 0.17148 mg(GAE).g(flower)$^{-1}$) when compared with an ultrasonic extract prepared with aqueous ethanol (3.0494 ± 0.12394 mg(GAE).g(flower)$^{-1}$). Similarly, the use of AAP solvents in macerated extraction methods offers increased phenolic content (3.1104 ± 0.17313 mg(GAE).g(flower)$^{-1}$) when compared with a macerated extract prepared with aqueous ethanol (2.6312 ± 0.14458 mg(GAE).g(flower)$^{-1}$).

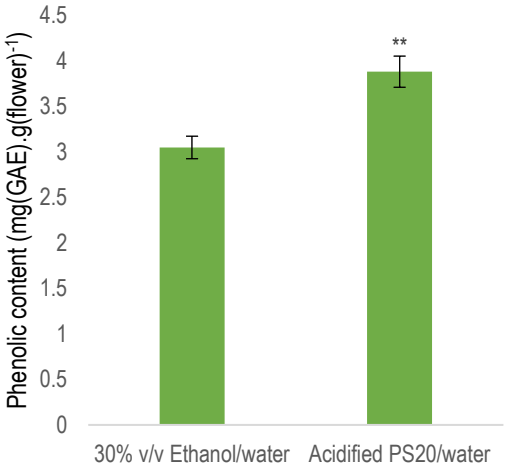


Figure 7-9: Total phenolic content of ultrasonic C. officinalis extracts prepared with hydroethanolic and AAP solvents. Data is presented as means ± standard deviations of replicates (n=9). Asterisks represent statistical significance when compared with C. officinalis *extracts prepared with aqueous ethanol under similar conditions.*

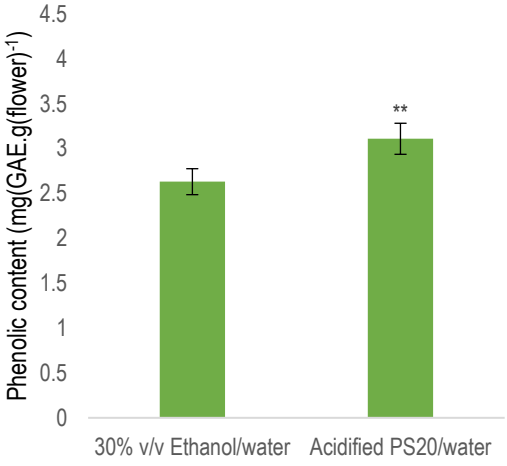


Figure 7-10: Total phenolic content of macerated C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is presented as means ± standard deviations of replicates (n=9). Asterisks represent statistical significance when compared with* C. officinalis *extracts prepared with aqueous ethanol under similar conditions.*

7.5 SUMMARY

The use of AAP solvents in the preparation of macerated *C. officinalis* extracts demonstrate equivalent extraction of individual metabolites, total peak area, and phenolic content when compared with extracts prepared with aqueous ethanol. Similarly, the use of AAP solvents in the preparation of ultrasonic *C. officinalis* extracts demonstrate significant increases in the ultrasonic extraction of individual metabolites, total peak area, and phenolic content when compared with extracts prepared with aqueous ethanol. Results were not obtained from chemiluminescence analysis and radical scavenging analysis due to complications observed in Chapters 5 and 6. Thus, acidified polysorbate/water composites offer a novel 'green' alternative to hydroethanolic solvents in the extraction of metabolites from *C. officinalis*.

8 CONCLUSIONS AND FUTURE WORK

8.1 CONCLUSIONS

The aim of this research was to develop a novel 'green' solvent as an alternative to more hazardous solvents (such as ethanol) in the extraction of metabolites from *C. officinalis* flowers. Chapter 3 describes the adaptation of an industrial extraction method to a laboratory-scale extraction method. It was found that a number of phytochemical compounds in hydroethanolic *C. officinalis* extracts exhibited chemiluminescence response. Grinding flowers to maintain sample homogeneity was found to not affect the phytochemical composition, antioxidant activity, or radical scavenging activity of hydroethanolic extracts. Similarly, the use of ultrasonic extraction methods were determined to produce hydroethanolic extracts of similar phytochemical composition, antioxidant activity, and radical scavenging activity to macerated extracts prepared under similar conditions with identical solvents.

Three 'green' alternative solvents were initially tested as alternative solvents to traditional hydroethanolic solvents for *C. officinalis* metabolite extraction. Chapter 4 describes investigations into the use of aqueous acids and bases, supercritical fluids, and natural deep eutectic solvents to produce *C. officinalis* extracts of similar phytochemical composition, antioxidant activity, and radical scavenging activity to hydroethanolic extracts. It was found that extracts prepared with pH 0-13 solvents exhibited decreased phytochemical content, antioxidant activity, and radical scavenging activity when compared with hydroethanolic extracts prepared under similar conditions and were therefore eliminated as alternative solvents. The large increase in total phytochemical content observed in pH 14 extracts was hypothesised to be the result of degradation of *C. officinalis* plant material. Supercritical fluid extracts were found only be soluble in 'non-green' ethanol/hexane solvents. UHPLC analysis of secondary liquid/liquid extracts with aqueous and acetonitrile solvents demonstrated that negligible metabolites could be recovered from the SCF residue and were therefore eliminated as alternative solvents. NaDES exhibited equivalent or greater antioxidant activity, radical scavenging activity, and phenolic content to hydroethanolic extracts. It was concluded that while aqueous acids and bases, and supercritical fluids are not suitable replacements for aqueous ethanol solvents, NaDES, particularly those prepared with glycerol, exhibited potential as alternative 'green' solvents and were investigated in Chapter 5.

Chapter 5 describes investigations into the effect of different concentrations of salts, sugars, and acids on the glycerolic solubility of *C. officinalis* metabolites. The use of acid/glycerol and sugar/glycerol composites demonstrated an increase in phytochemical concentration, antioxidant activity, radical scavenging activity, and phenolic content when compared with extracts prepared with neat glycerol. A

strong correlation was observed between the dielectric constant of the solvent and the extraction of polar metabolites from *C. officinalis*. Strong correlations were also observed between the chaotropicity of the solvent and phytochemical concentration, antioxidant activity, radical scavenging activity, and phenolic content in sugar/glycerol composites, which led to an investigation into the use of highly chaotropic substances in Chapter 6.

Chapter 6 explored the addition of polysorbates in glycerolic and aqueous extraction processes, and showed increases in phytochemical content, radical scavenging activity, and phenolic content when compared with neat glycerolic and aqueous *C. officinalis* extracts. Large increases in antioxidant activity were observed but these were determined to be due to matrix interactions. In conjunction with results obtained in Chapter 5, these observed increases led to an investigation into the use of acidified aqueous polysorbates as alternative 'green' solvents in Chapter 7.

Chapter 7 utilised aqueous acidic polysorbate composites as alternative solvents for the extraction of *C. officinalis* metabolites, and it was found that AAP extracts presented significant increases in phytochemical content with ultrasonic extraction methods and equivalent phytochemical content with maceration extraction methods when compared with hydroethanolic extracts. Similarly, AAP extracts presented significant increases in total phenolic content for both maceration and ultrasonic extraction methods. Thus, acidified aqueous polysorbate solvents were shown to be a novel 'green' alternative solvent to traditional hydroethanolic solvents.

8.2 FUTURE WORK

8.2.1 Identification of phytochemicals in *C. officinalis* extracts

Peaks in hydroethanolic *C. officinalis* extracts were found to exhibit chemiluminescence response in Chapter 3. However, structural elucidation was unachievable due to the lack of access to a LC-MS system. Further work to elucidate the structure of the phytochemicals present in *C. officinalis* using LC-MS to identify molecular ions and fragments thereof, particularly if complemented with NMR of purified hydroethanolic extract fractions would allow for further understanding into the effect of solvent chaotropicity upon phytochemical extraction.

8.2.2 Determining chaotropicity for tartaric and citric acids

Chaotropicity values used for the Unscrambler® correlation analyses in Chapter 5 were determined by Cray *et al.* [218] by determination of the change in melting point of agar gel doped with analyte when compared with plain agar gel. Chaotropicities were calculated for glycerol composites prepared with LiCl, NaCl, KCl, sucrose, glucose, and fructose; however, the chaotropicity of glycerol composites prepared with tartaric and citric acids were not calculated as the chaotropicity of these compounds was not elucidated by Cray *et al.*

Since tartaric acid/glycerol and citric acid/glycerol composites exhibited increases in phytochemical content, chemiluminescence response, and phenolic content similar to those observed in extracts prepared with sugar/glycerol composites, correlations between acid/glycerol composite chaotropicities and the aforementioned extract properties would provide further insight into the effect of solvent chaotropicity on the extraction process.

8.2.3 Determining surfactant/metabolite interactions

Correlations were observed between solvent parameters such as chaotropicity and increased phytochemical extraction, leading to the development of surfactant-ased solvent systems (Chapter 6) which demonstrated significant increases in phytochemical content. However, the exact mechanism(s) by which this increase occurred were not explored. Future experiments using NMR peak shifts with model systems such as D_2O/PS and D_2O/PS/rutin would allow for further understanding of solvent-metabolite interactions, with a view to controlling and increasing phytochemical extraction. If this were to be

investigated, however, purification of the PS would be necessary to ensure that the chemical shifts were not due to differences in polyethoxylene chain lengths or fatty acid ester moieties.

8.2.4 Antioxidant and radical scavenging assays for AAP composite extracts

The use of AAP composites in the extraction of *C. officinalis* metabolites allows for increased phytochemical extraction when compared with hydroethanolic solvents. This solvent demonstrates a positive step in the development of alternative 'green' solvents that extract similar or greater phytochemical quantities when compared with organic solvents. However, the future studies discussed below are necessary to advance the use of AAP composites as alternative 'green' solvents for phytochemical extraction.

C. officinalis extracts prepared with AAP composites were analysed with UHPLC and FC assays. SIA-CL assays could not be conducted, however, due to the matrix interactions observed between polysorbates and permanganate chemiluminescence due to the presence of micelles. Alternative colourimetric approaches to measuring antioxidant activity such as the Ferric Reducing Antioxidant Potential (FRAP) assay or the Ferric Thiocyanate (FTC) assay used by Zheleva-Dimitrova *et al.* [244] may be used in order to directly measure the antioxidant activity without micellar interference.

Similarly, DPPH radical scavenging activity was observed to be inhibited by the presence of organic acids in Chapter 5. Approaches to measuring radical scavenging activity such as Oxygen Radical Absorbance Capacity (ORAC) assay can be developed for analysis of radical scavenging activity. Alternatively, since radical scavenging activity strongly correlates to antioxidant activity, radical scavenging assays can simply be replaced by alternative antioxidant assays as described earlier.

8.2.5 Determining micellar structures in AAP composites

Final experiments into the suitability of AAP solvents as alternative 'green' replacements for hydroethanolic solvents were conducted using PS20 and tartaric acid as these both exhibited increases in phytochemical content, antioxidant activity, DPPH radical scavenging activity, and phenolic content in Chapters 5 (in glycerolic composites) and 6 (in aqueous and glycerolic composites). A concentration of 20‰ mol/mol PS20/water was selected as optimal from results in Chapter 5. However, the phase behaviour at this and other concentrations was not investigated. Future experiments conducted with a view to identifying the effect of micellar phase structure upon the phytochemical composition of AAP

extracts would allow for determination of the optimal micellar phase for phytochemical extraction. Pseudobinary phase diagrams of AAP solvents can be constructed through observation of the change in 'cloud point' of various AAP solvents prepared with different water/surfactant ratios [245].

8.2.6 Continuing investigations into AAP parameters

The final AAP solvent tested was acidified to pH 3 following research by Hosseinzadeh *et al.* [238]. Tartaric acid was used following results in Chapter 5. However, different pH values and alternative acids containing additional chemical moieties such as quinic acid (for additional hydroxyl functionalities) or caffeic acid (for aromatic functionalities) may be used to further manipulate the properties of the solvents. Similarly, different nonhazardous non-ionic surfactants such as lecithin and sodium lauryl sulfate were not investigated. Further research in this area would be beneficial as micellar structure is highly dependent on the surfactant used; as such, different surfactants may further increase the phytochemical extraction from *C. officinalis*. Additionally, surfactants such as lecithin are naturally occurring, renewable, and nonhazardous and therefore show potential as 'green' solvents. Future experiments should therefore be conducted with a view to determining the effect of pH and choice of acid and surfactant upon the phytochemical composition of *C. officinalis* extracts prepared using AAP solvents.

APPENDIX A

Table 9-1: Selected peak heights for extracts prepared with aqueous acid and hydroethanolic solvents

Peak	*pH 0*	*pH 1*	*pH 2*	*pH 3*	*pH 4*	*pH 5*	*pH 6*	*pH 7*	*ethanol/water*
A	125.54	124.15	211.01	259.39	246.82	262.29	258.8	268.99	99.951
B	13.041	46.475	53.303	57.632	50.208	53.635	51.566	62.423	44.63
C	48.752	72.299	68.514	70.499	75.5	69.878	66.935	67.141	51.383
D	81.151	88.943	76.556	73.402	77.713	75.872	70.017	74.633	73.361
E	9.6511	26.884	33.184	31.222	34.726	30.773	30.353	29.141	30.628
F	457.78	615.87	626.16	605.84	692.96	601.17	593.28	553.58	455.6
G	154.3	205.68	196.07	184.43	210.32	188.23	185.67	181.01	169.82
H	350.13	482.86	497.42	419.08	531.51	409.98	468.6	383.11	437.81
I	3.1855	19.237	53.998	47.146	59.467	50.394	53.12	45.739	46.781

Table 9-2: Selected peak heights for extracts prepared with aqueous base and hydroethanolic solvents

Peak	*pH 7*	*pH 8*	*pH 9*	*pH 10*	*pH 11*	*pH 12*	*pH 13*	*pH 14*	*ethanol/water*
A	268.99	243.59	258.25	257.49	263.19	238.4	220.46	399.96	99.951
B	62.423	42.258	58.731	52.101	52.332	35.139	7.5876	9.6569	44.63
C	67.141	68.879	72.329	69.922	68.331	60.536	16.246	24.036	51.383
D	74.633	72.716	78.076	73.116	72.016	60.177	13.407	18.287	73.361
E	29.141	33.369	30.418	32.286	30.413	27.402	17.315	15.779	30.628
F	553.58	660.58	646.3	630.28	602.99	575.36	310	329.94	455.6
G	181.01	200.22	204.16	196.47	194.14	169.77	127.84	160.72	169.82
H	383.11	517.76	499.19	452.01	429.2	427.5	158.82	224.41	437.81
I	45.739	57.546	30.987	50.691	49.183	42.988	9.7032	30.886	46.781

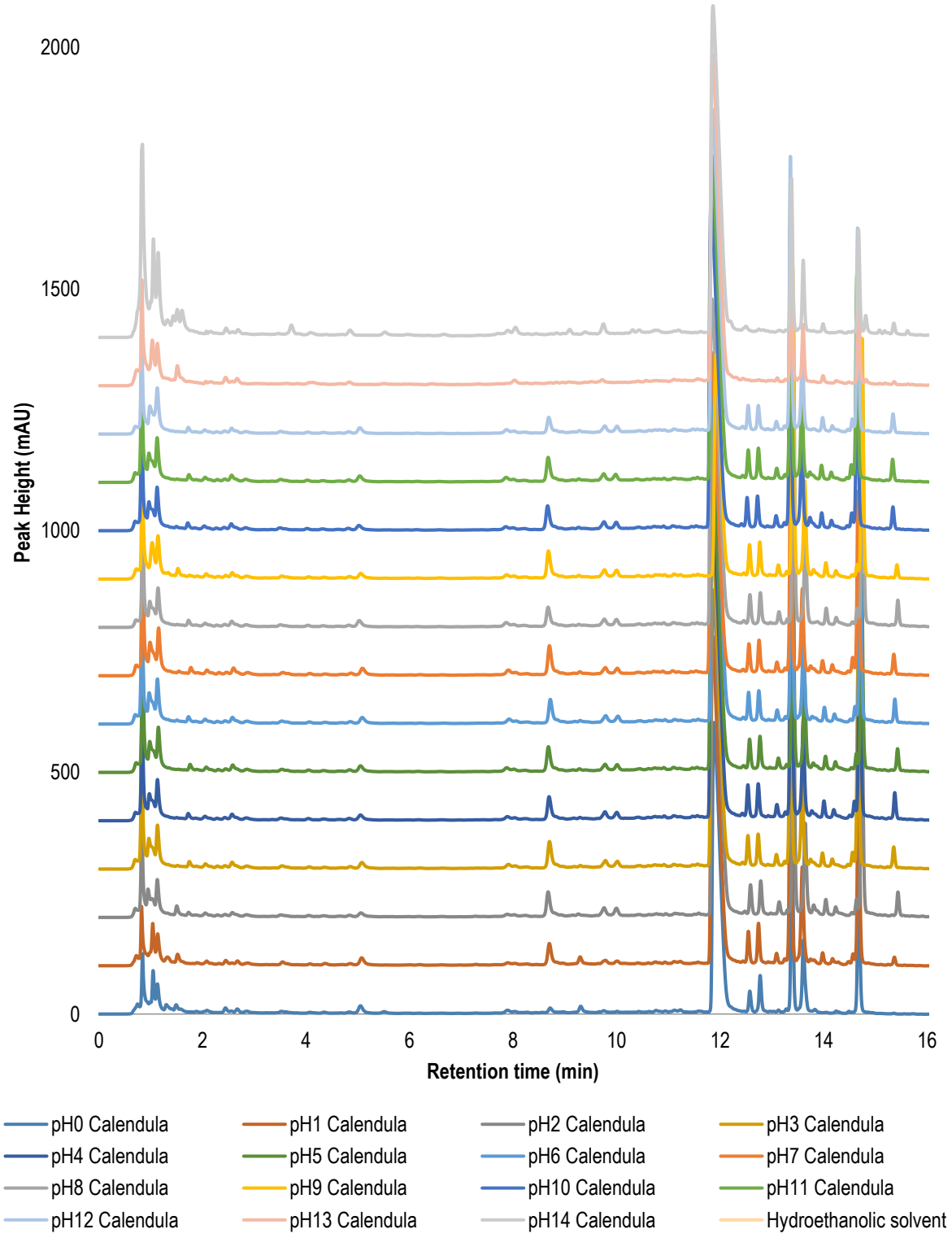


Figure 9-1: UHPLC analysis of C. officinalis *extracts prepared with hydroethanolic and aqueous acid and base solvents. Sequential chromatograms are offset vertically by 100 mAU.*

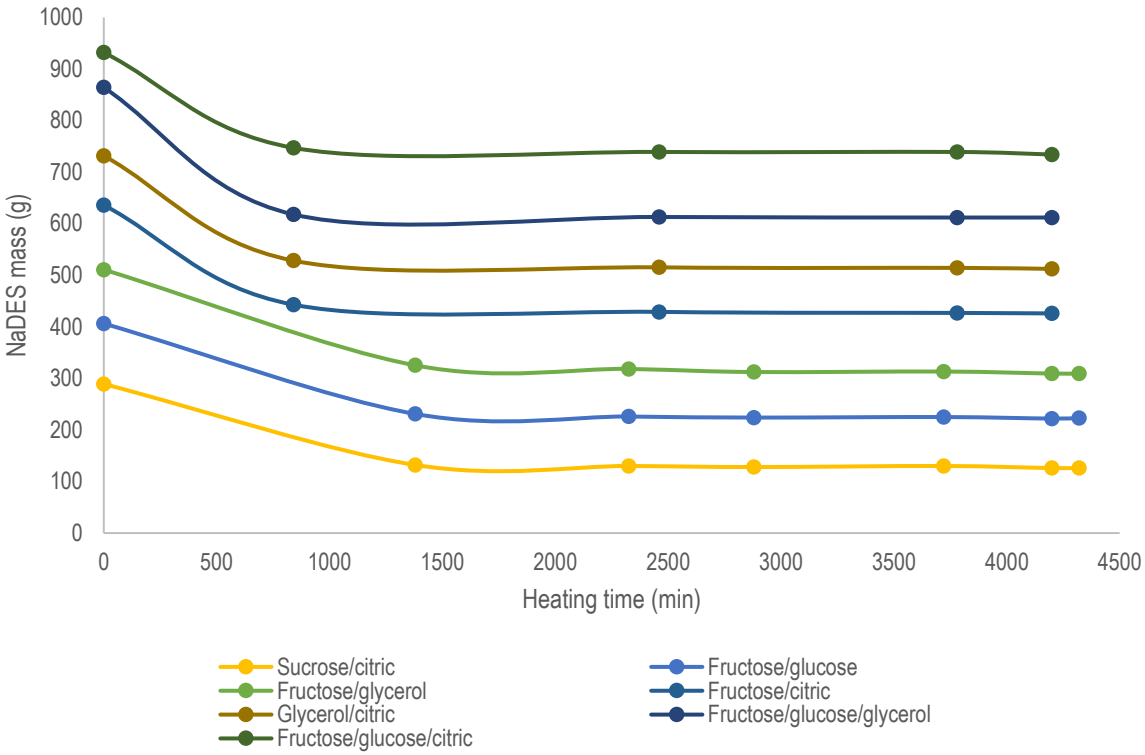


Figure 9-2: Total weight of NaDES during solvent preparation. Sequential weights are offset vertically by 100 g.

Table 9-3: Water weights in NaDES after heating for 72 hours

NaDES combination	*water weight per 100 g NaDES materials (g)*
Sucrose/citric	27.287
Fructose/glucose	22.992
Fructose/glycerol	10.445
Fructose/citric	26.433
Glycerol/citric	13.232
Fructose/glucose/glycerol	11.738
Fructose/glucose/citric	36.529

APPENDIX B

Salt/Glycerol Composites

Table 9-4: Height of Peak A in C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared with neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Salt Concentration (mol/mol)	*LiCl/glycerol composite*	*NaCl/glycerol composite*	*KCl/glycerol composite*	*$MgCl_2$/glycerol composite*
0‰	4738.6 ± 1734.6	4738.6 ± 1734.6	4738.6 ± 1734.6	4738.6 ± 1734.6
20‰	3572.9 ± 60.666	2907.8 ± 742.95	**1512.6 ± 523.45**	4873.6 ± 641.38
40‰	**3123.3 ± 84.452**	**2226.4 ± 454.95**	2712.5 ± 1180.3	5684.5 ± 2487.6
60‰	**1930.5 ± 112.3**	2501.1 ± 651.03	3353.2 ± 803.88	3075.8 ± 1698.2
80‰	3260 ± 667.96	**2201.4 ± 496.47**	3499.3 ± 454.6	4364.7 ± 714.17
100‰	2405.2 ± 781.22	**1945.9 ± 532.09**	**2664.3 ± 168.79**	**2088.3 ± 526.03**

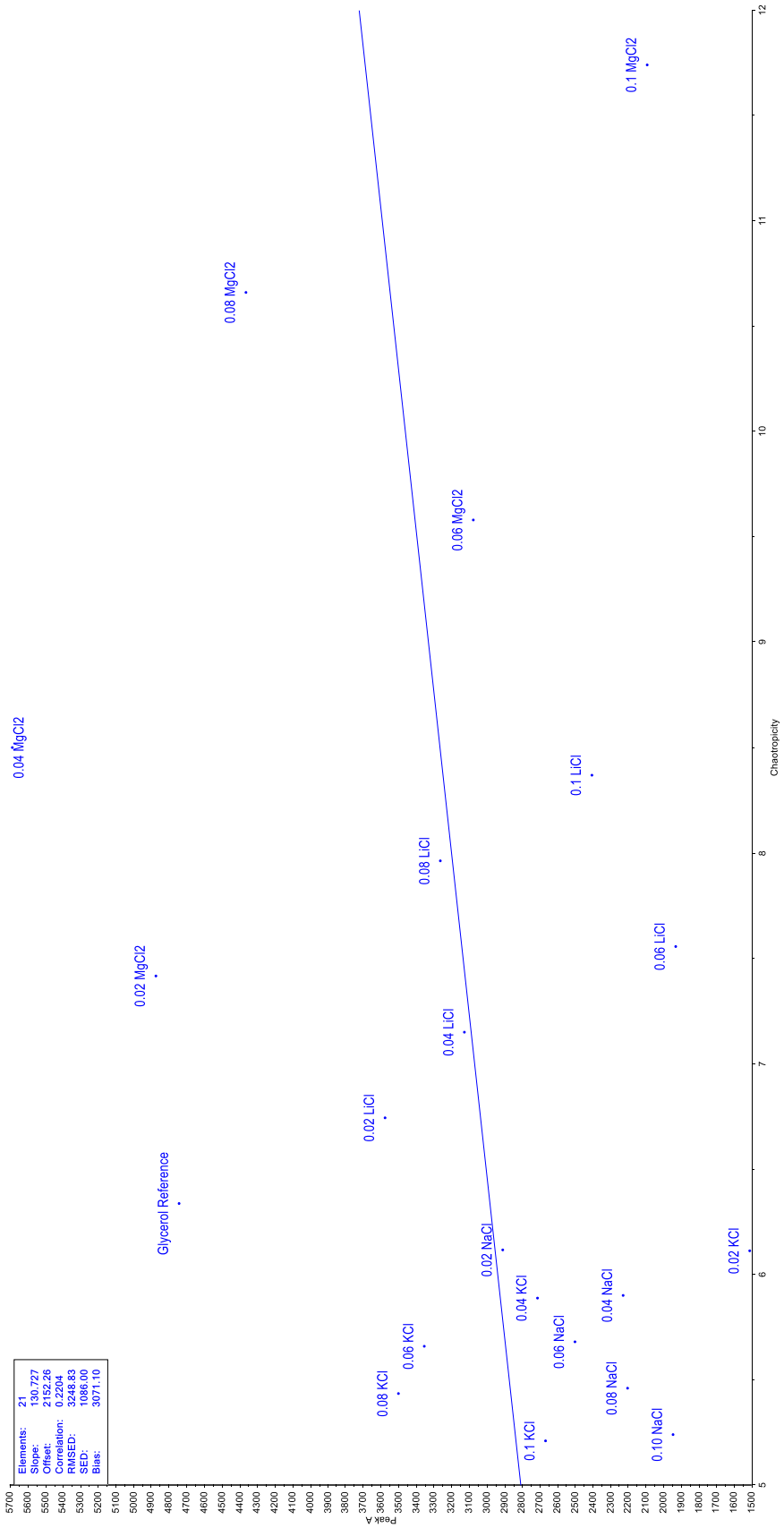


Figure 9-3: Unscrambler® correlation between solvent chaotropicity and Peak A height in C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means of replicates (n=3).*

Table 9-5: Height of Peak B in C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared with neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Salt Concentration (mol/mol)	*LiCl/glycerol composite*	*NaCl/glycerol composite*	*KCl/glycerol composite*	*$MgCl_2$/glycerol composite*
0‰	1150.1 ± 279.55	1150.1 ± 279.55	1150.1 ± 279.55	1150.1 ± 279.55
20‰	925.24 ± 147.87	**766.96 ± 195.84**	914.17 ± 205.11	1223.2 ± 315.45
40‰	**802.57 ± 5.1548**	**704.77 ± 19.522**	867.49 ± 222.99	1246.3 ± 498.85
60‰	**764.5 ± 3.7061**	**712.25 ± 145.28**	1038 ± 96.124	855.94 ± 309.91
80‰	**677.08 ± 92.613**	**674.66 ± 104.3**	1045.3 ± 66.002	1004.3 ± 171.11
100‰	**666.48 ± 189.93**	**664.53 ± 172.54**	891.48 ± 89.965	**718.72 ± 200.57**

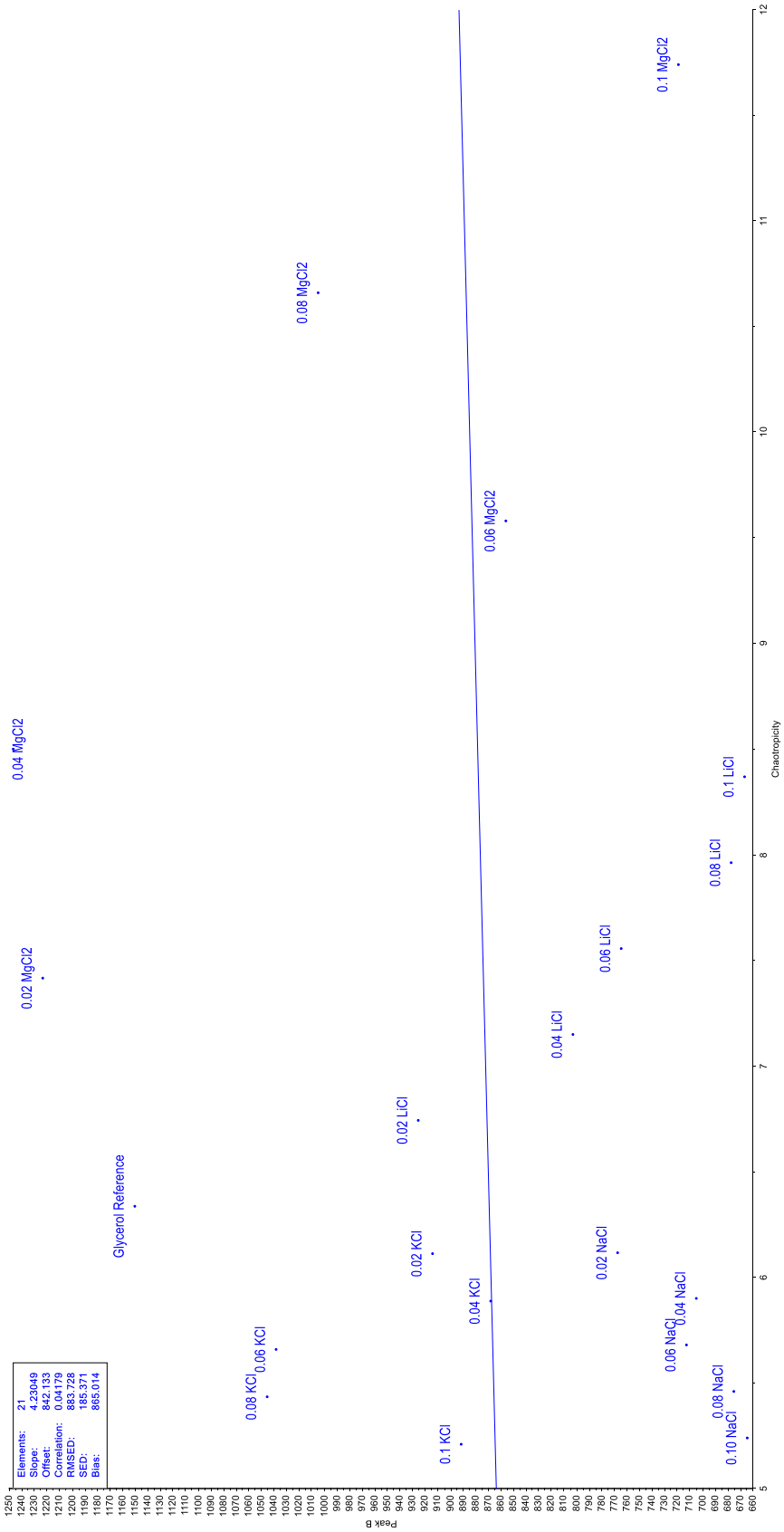


Figure 9-4: Unscrambler® correlation between solvent chaotropicity and Peak B height in C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means of replicates (n=3).*

Table 9-6: Height of Peak C in C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Salt Concentration (mol/mol)	*LiCl/glycerol composite*	*NaCl/glycerol composite*	*KCl/glycerol composite*	*MgCl2/glycerol composite*
0‰	1664.4 ± 277.25	1664.4 ± 277.25	1664.4 ± 277.25	1664.4 ± 277.25
20‰	1253.8 ± 170.83	**951.08 ± 604.55**	1348.2 ± 199.79	1205.8 ± 230.89
40‰	**1172 ± 57.293**	**692.38 ± 450.35**	1339.2 ± 262.89	1736.2 ± 665.38
60‰	1657.2 ± 71.297	**768.82 ± 484.55**	1540.5 ± 196.37	1258.3 ± 462.94
80‰	1721.3 ± 250.4	**839.26 ± 436.73**	899.25 ± 696.56	1311.4 ± 229.2
100‰	1722.5 ± 268.59	**1051.6 ± 188.82**	1388.8 ± 66.303	**1133.7 ± 195.47**

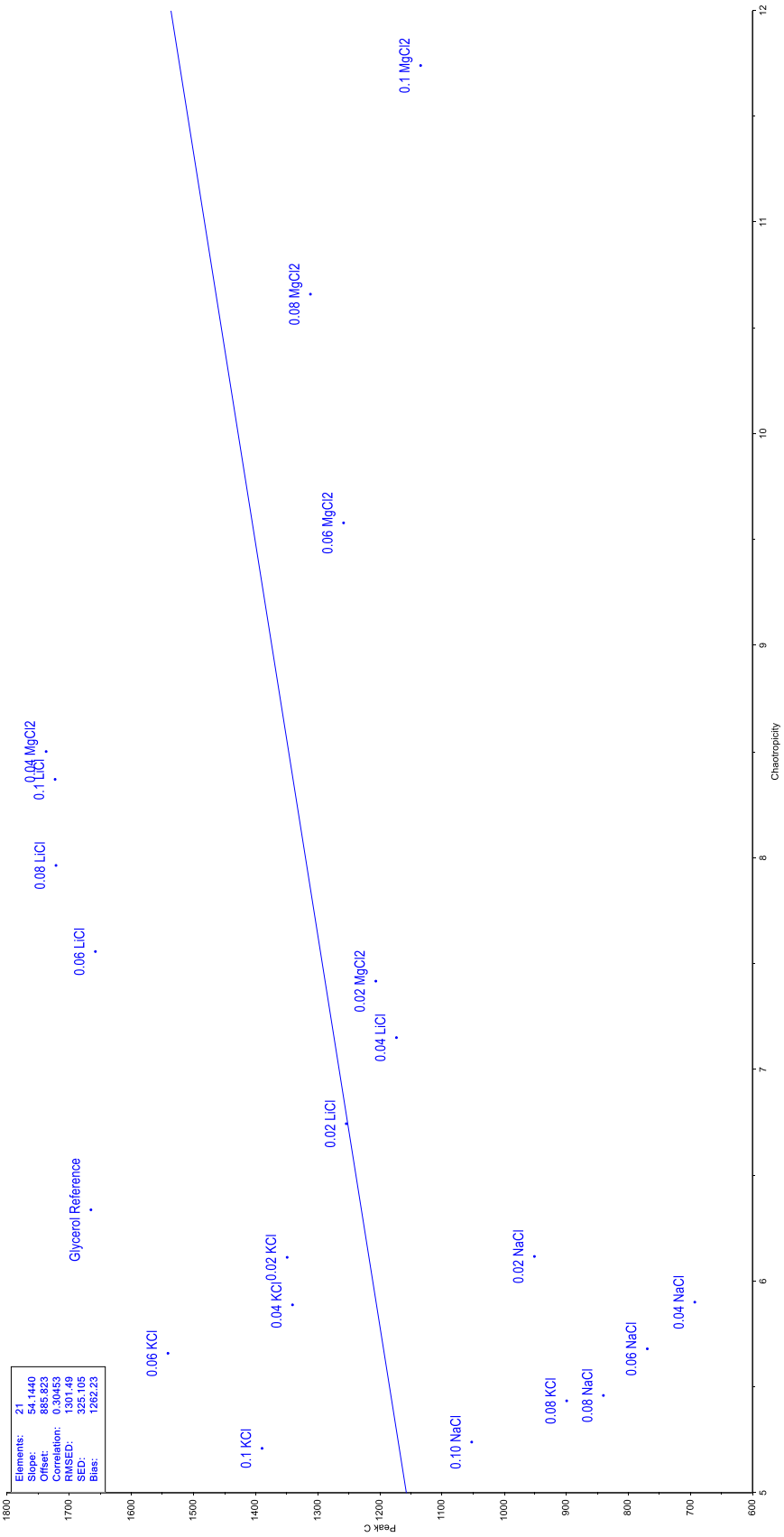


Figure 9-5: Unscrambler® correlation between solvent chaotropicity and Peak C height in C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means of replicates (n=3).*

Table 9-7: Height of Peak D in C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Salt Concentration (mol/mol)	*LiCl/glycerol composite*	*NaCl/glycerol composite*	*KCl/glycerol composite*	*MgCl2/glycerol composite*
0‰	2475.2 ± 442.53	2475.2 ± 442.53	2475.2 ± 442.53	2475.2 ± 442.53
20‰	**1878.7 ± 195.12**	**1814.2 ± 218.83**	2022.5 ± 255.16	2540.5 ± 506.74
40‰	**1667.4 ± 43.749**	**1676 ± 253.79**	1966.1 ± 362.99	2661.1 ± 958.9
60‰	**1595.2 ± 95.919**	**1681.1 ± 194.9**	2302.3 ± 222.59	1898.8 ± 620.41
80‰	**1595.7 ± 202.17**	**1691.1 ± 243.22**	2207.2 ± 4.9618	2230.3 ± 331.16
100‰	**1659.2 ± 224.12**	**1602.6 ± 338.06**	**2136 ± 16.06**	**1724 ± 331.96**

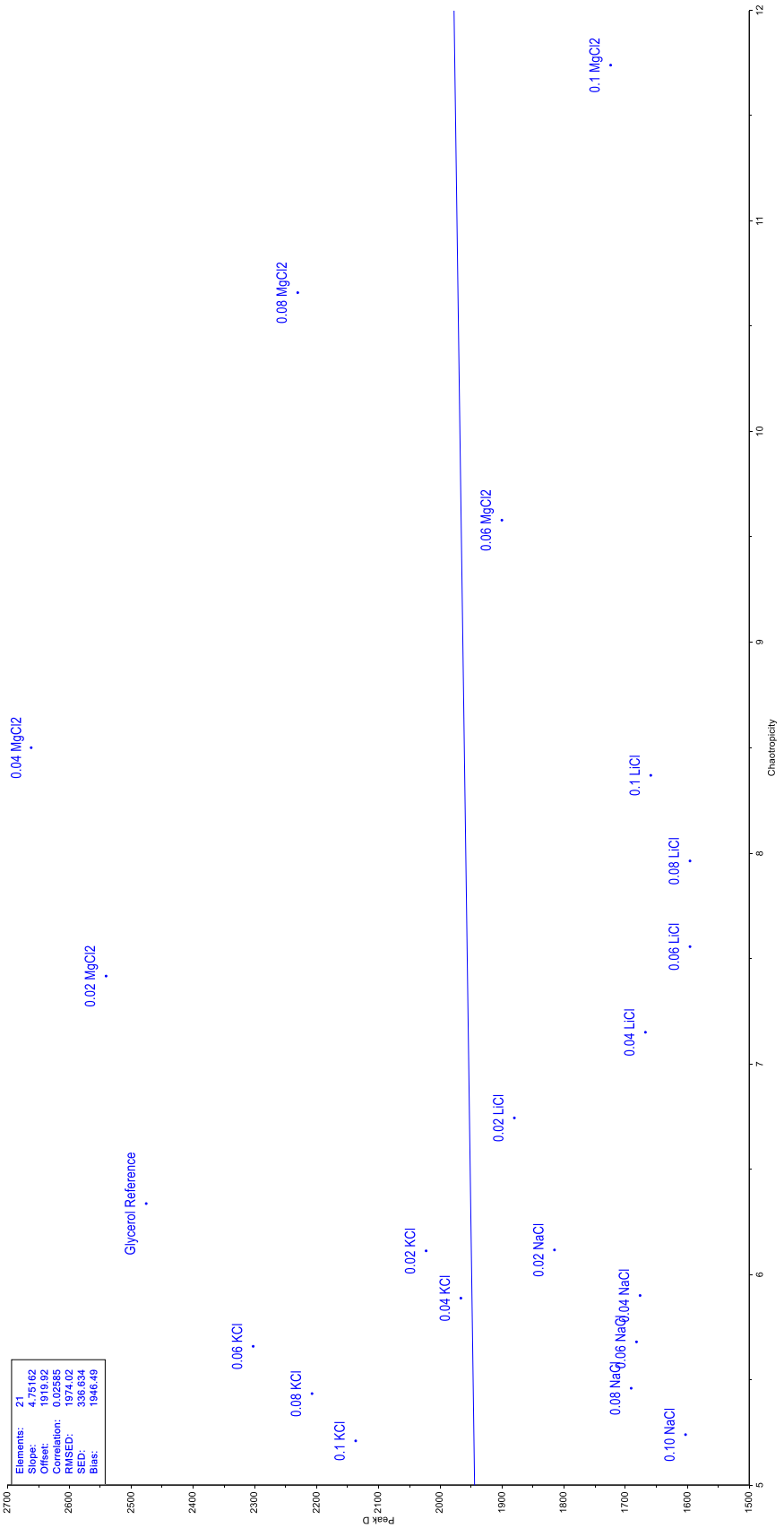


Figure 9-6: Unscrambler® correlation between solvent chaotropicity and Peak D height in C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means of replicates (n=3).*

Table 9-8: Height of Peak E in C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared with neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Salt Concentration (mol/mol)	*LiCl/glycerol composite*	*NaCl/glycerol composite*	*KCl/glycerol composite*	*MgCl2/glycerol composite*
0‰	13279 ± 2214.2	13279 ± 2214.2	13279 ± 2214.2	13279 ± 2214.2
20‰	10529 ± 914.7	10427 ± 1151.4	10623 ± 1306.2	12151 ± 2570.2
40‰	**9629.9 ± 138.5**	**9293.5 ± 1239.9**	10619 ± 2140.5	13738 ± 5136.9
60‰	**9020.3 ± 347.73**	**9187.1 ± 1099.6**	12361 ± 1121	**9906.7 ± 2918.1**
80‰	**9183.8 ± 1270**	**8864.2 ± 1279.7**	11657 ± 299.68	11075 ± 1734.2
100‰	**8500.2 ± 1117.1**	**8400.9 ± 1723.5**	**11109 ± 302.03**	**9032.8 ± 1670.9**

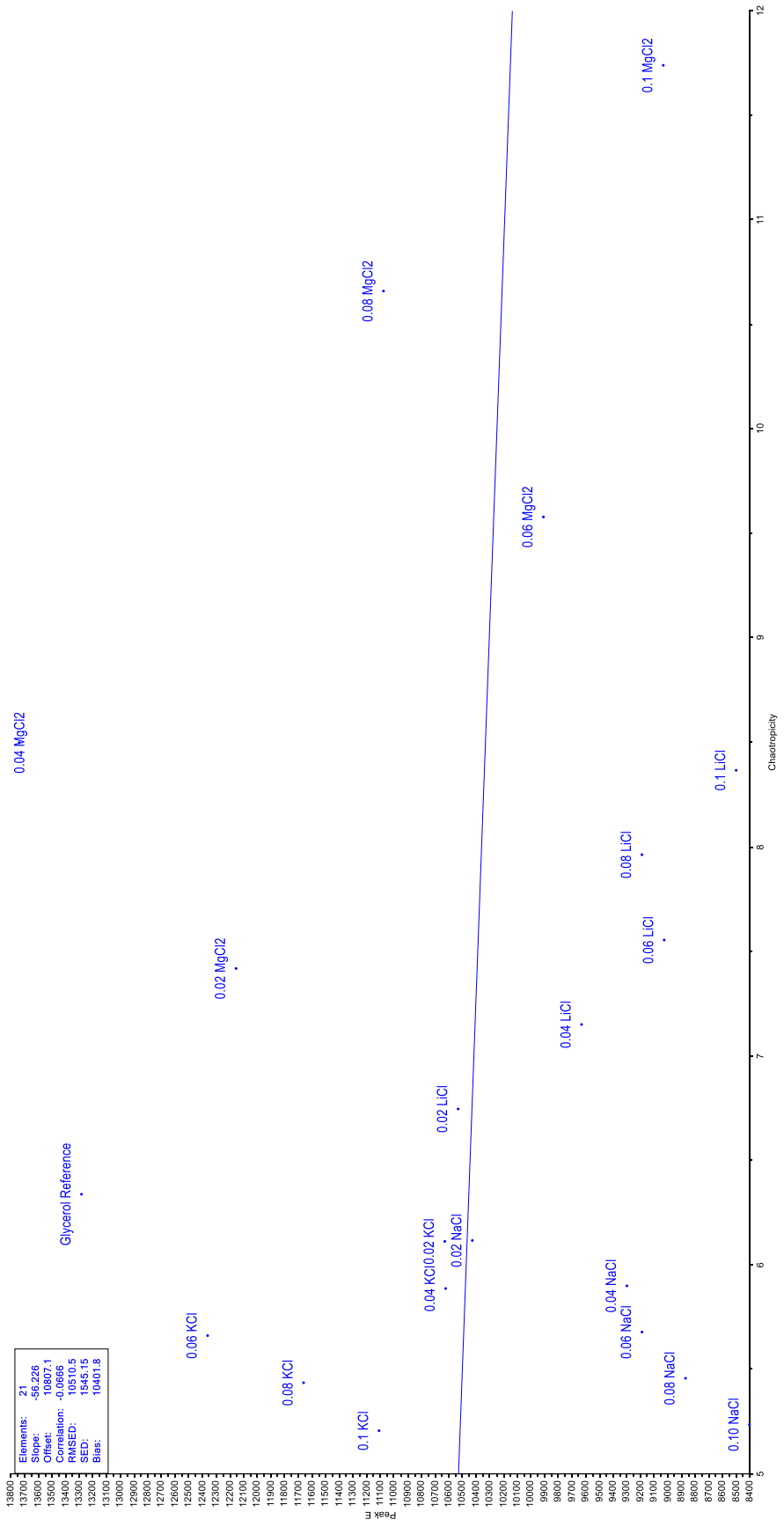


Figure 9-7: Unscrambler® correlation between solvent chaotropicity and Peak E height in C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means of replicates (n=3).*

Table 9-9: Height of Peak F in C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means ± standard deviation of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared with neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Salt Concentration (mol/mol)	*LiCl/glycerol composite*	*NaCl/glycerol composite*	*KCl/glycerol composite*	*MgCl2/glycerol composite*
0‰	4819.3 ± 922.06	4819.3 ± 922.06	4819.3 ± 922.06	4819.3 ± 922.06
20‰	4163.5 ± 420.33	4198.6 ± 354.91	4592.8 ± 569.87	3616.4 ± 1049.5
40‰	**3760.2 ± 8.6513**	4067.6 ± 3165.2	4519.7 ± 865.62	**3134.6 ± 994.56**
60‰	3636.3 ± 192.74	3799.3 ± 351.88	5274 ± 507.67	**3066.9 ± 1074.4**
80‰	3652.8 ± 536.9	**2855.8 ± 1181.4**	4882.4 ± 263.44	**2035.4 ± 161.03**
100‰	3511.7 ± 546.28	**2477.5 ± 1260.3**	4806.4 ± 102.99	**2625.6 ± 1170.1**

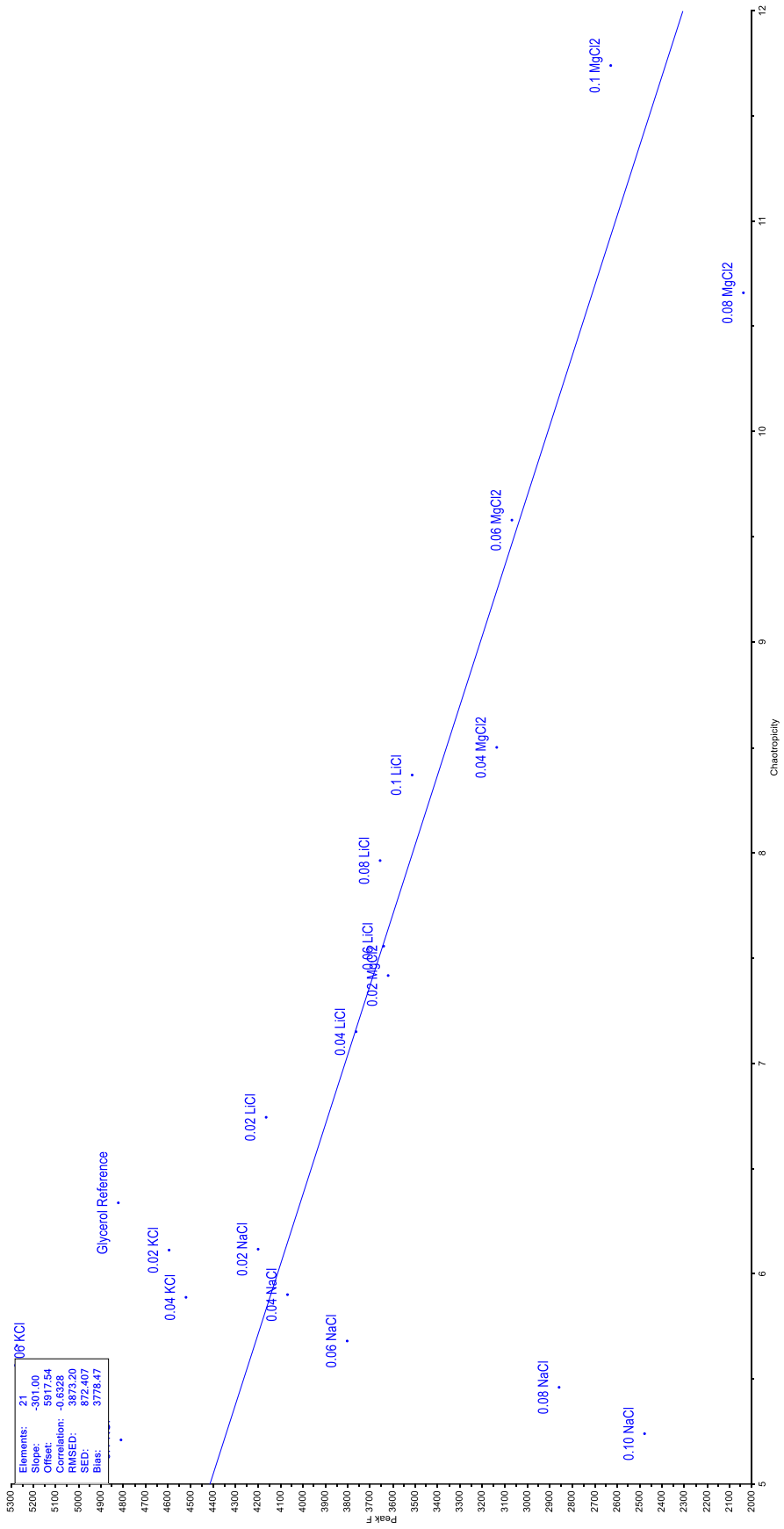


Figure 9-8:Unscrambler® correlation between solvent chaotropicity and Peak F height in C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means of replicates (n=3).*

Table 9-10: Height of Peak G in C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means ± standard deviation of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared with neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Salt Concentration (mol/mol)	*LiCl/glycerol composite*	*NaCl/glycerol composite*	*KCl/glycerol composite*	*$MgCl_2$/glycerol composite*
0‰	12342 ± 1510.6	12342 ± 1510.6	12342 ± 1510.6	12342 ± 1510.6
20‰	**10444 ± 977.88**	**8380.6 ± 826.93**	11049 ± 1296.6	11938 ± 2166.3
40‰	**8796.8 ± 138.42**	**8005.4 ± 1290**	11111 ± 2027.8	13382 ± 4999.8
60‰	**8269.3 ± 193.79**	**7912.1 ± 832.06**	12690 ± 552.88	9824.8 ± 3074.7
80‰	**8028.5 ± 1288**	**8165.3 ± 1274.5**	12121 ± 638.4	10528 ± 1682.7
100‰	**7488.1 ± 974.01**	**7304.8 ± 1662.6**	**11238 ± 251.32**	**7861.6 ± 1700.8**

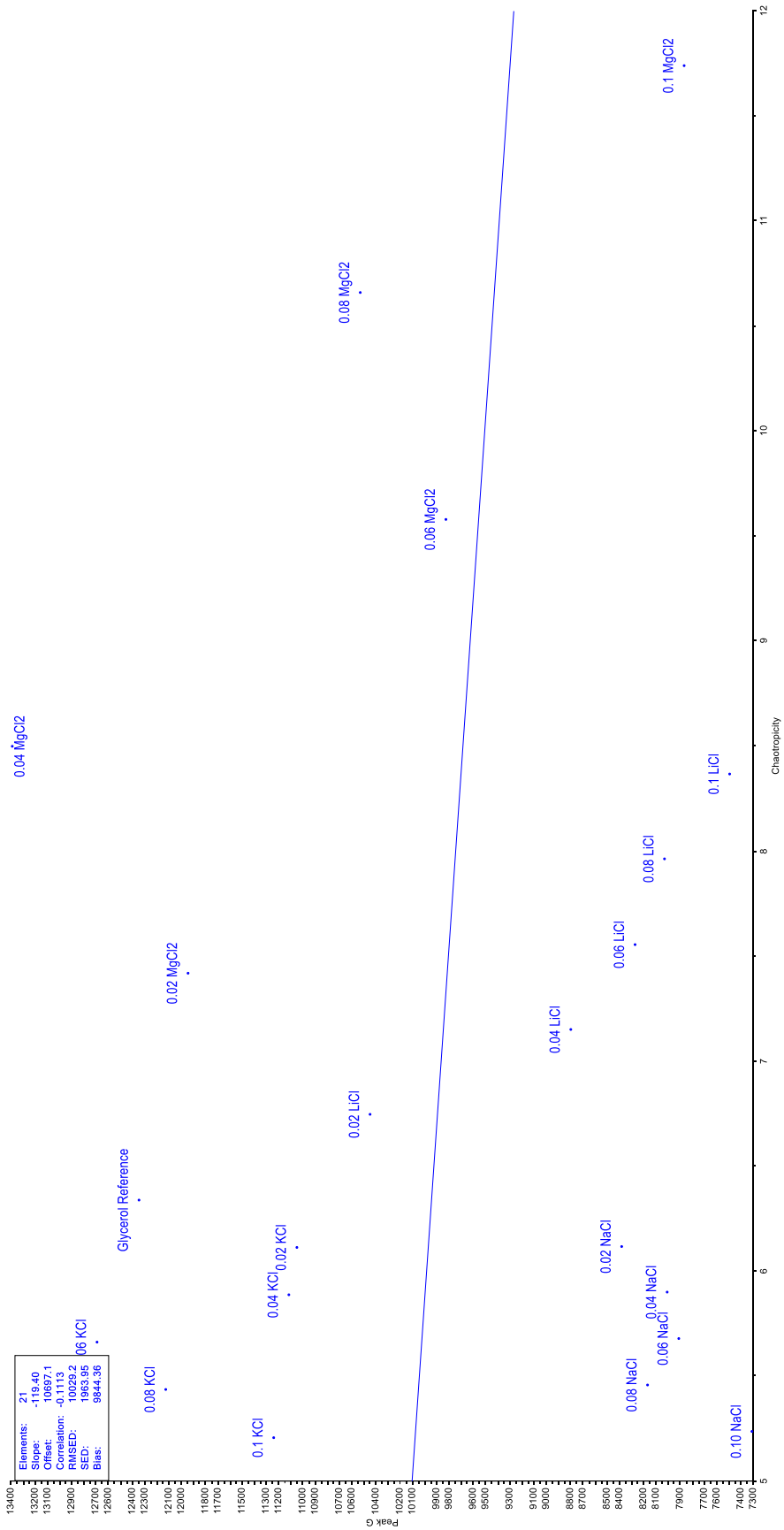


Figure 9-9: Unscrambler® correlation between solvent chaotropicity and Peak A height in C. officinalis *extracts prepared with salt/glycerol composites. Data is presented as means of replicates (n=3).*

Table 9-11: Total Peak Areas of C. officinalis *extracts prepared with salt/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=3). Bold values indicate statistically significant results ($p<0.05$) when compared with extracts prepared with neat glycerol. Units are mAU.s.g(flower)$^{-1}$.*

Salt Concentration (‰ mol/mol)	*LiCl/glycerol composite*	*NaCl/glycerol composite*	*KCl/glycerol composite*	*$MgCl_2$/glycerol composite*
0‰	4197.6 ± 658.62	4197.6 ± 658.62	4197.6 ± 658.62	4197.6 ± 658.62
20‰	4594.2 ± 125.79	**1890.2 ± 433.18**	3370.3 ± 430.98	5135.4 ± 1269.1
40‰	3531.8 ± 48.669	**1987.7 ± 871.28**	3767.3 ± 877.87	6032 ± 1969.4
60‰	3744.8 ± 156.81	**2545.3 ± 266.38**	4197.7 ± 480.49	4283.4 ± 1200
80‰	3150.3 ± 639.38	3158.5 ± 216.72	4639.5 ± 156.8	4521.2 ± 622.14
100‰	3575.1 ± 867.42	3305.7 ± 521.49	4167.7 ± 105.82	3826.7 ± 845.76

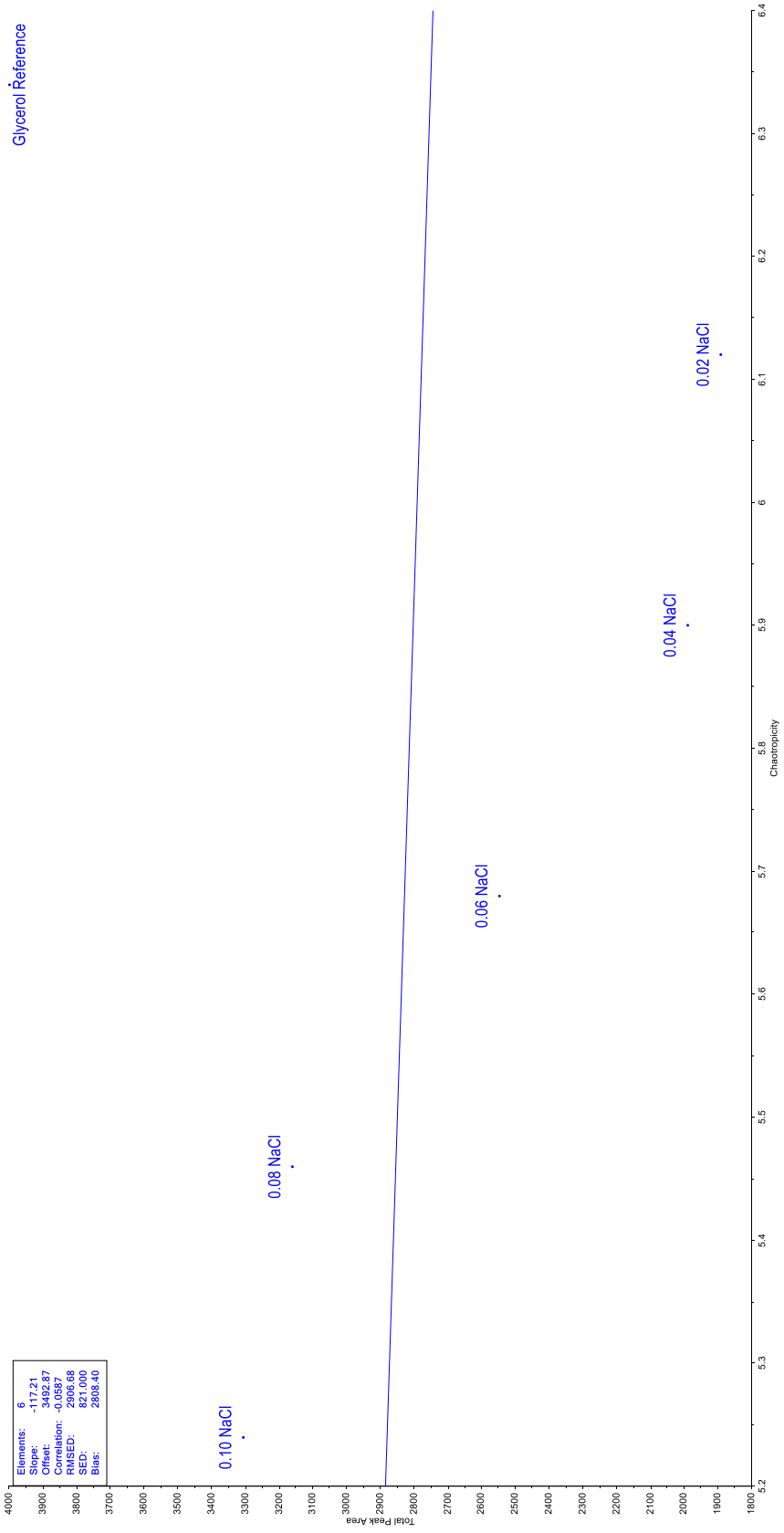


Figure 9-10: Unscrambler® correlation between total peak areas and solvent chaotropicity in ultrasonic C. officinalis *extracts prepared with NaCl/glycerol composites. Data is presented as means of replicates (n=3)*

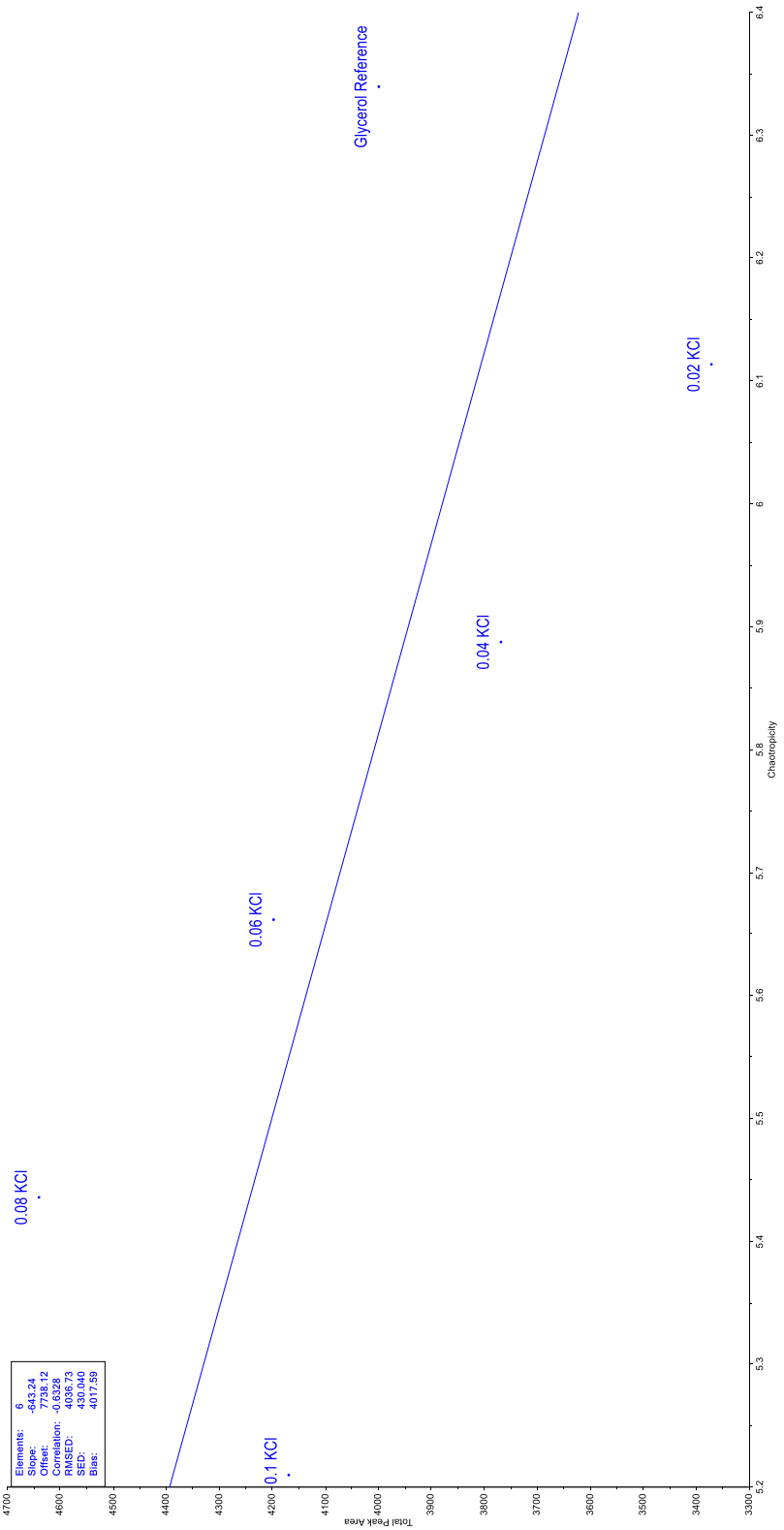


Figure 9-11: Unscrambler® correlation between total peak areas and solvent chaotropicity in ultrasonic C. officinalis *extracts prepared with KCl/glycerol composites. Data is presented as means of replicates (n=3)*

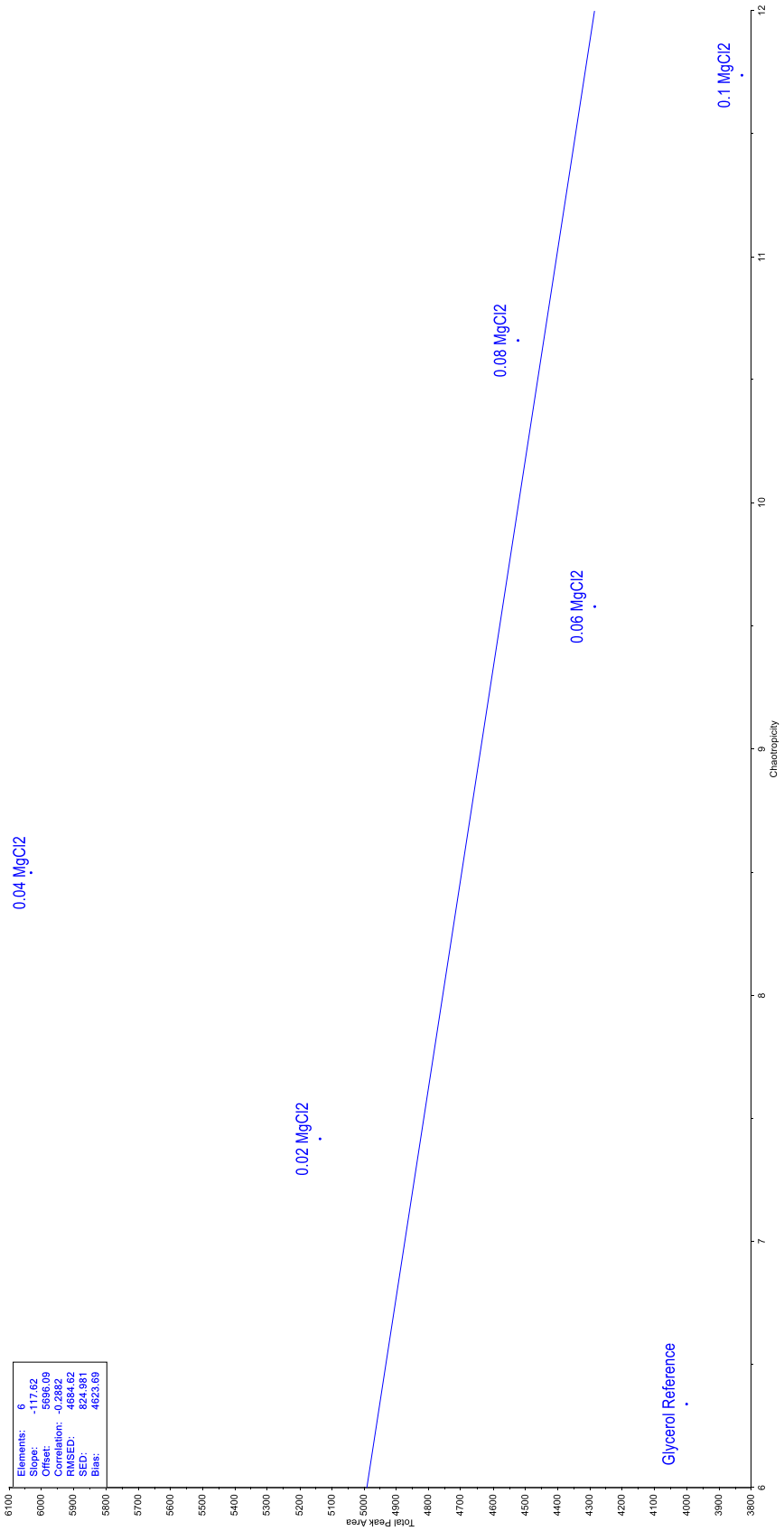


Figure 9-12: Unscrambler® correlation between total peak areas and solvent chaotropicity in ultrasonic C. officinalis *extracts prepared with $MgCl_2$/glycerol composites Data is presented as means of replicates (n=3)*

Table 9-12: Chemiluminescence response of C. officinalis *extracts prepared with salt/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=9). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared with neat glycerol. Units are mV.s.g(flower)$^{-1}$.*

Salt Concentration (‰ mol/mol)	*LiCl/glycerol composite*	*NaCl/glycerol composite*	*KCl/glycerol composite*	*$MgCl_2$/glycerol composite*
0‰	36251 ± 2464.4	36251 ± 2464.4	36251 ± 2464.4	-
20‰	**26258 ± 1217.5**	**23857 ± 3409.3**	**22983 ± 5521.6**	-
40‰	**24680 ± 4990.8**	28598 ± 1769	**26586 ± 4323.9**	-
60‰	**23021 ± 2989.9**	**26519 ± 1883.1**	27171 ± 3255.2	-
80‰	**24236 ± 5124.5**	**22950 ± 3341.1**	**22423 ± 2204.6**	-
100‰	**22423 ± 4960.9**	**25116 ± 1882.2**	**18276 ± 4666.5**	-

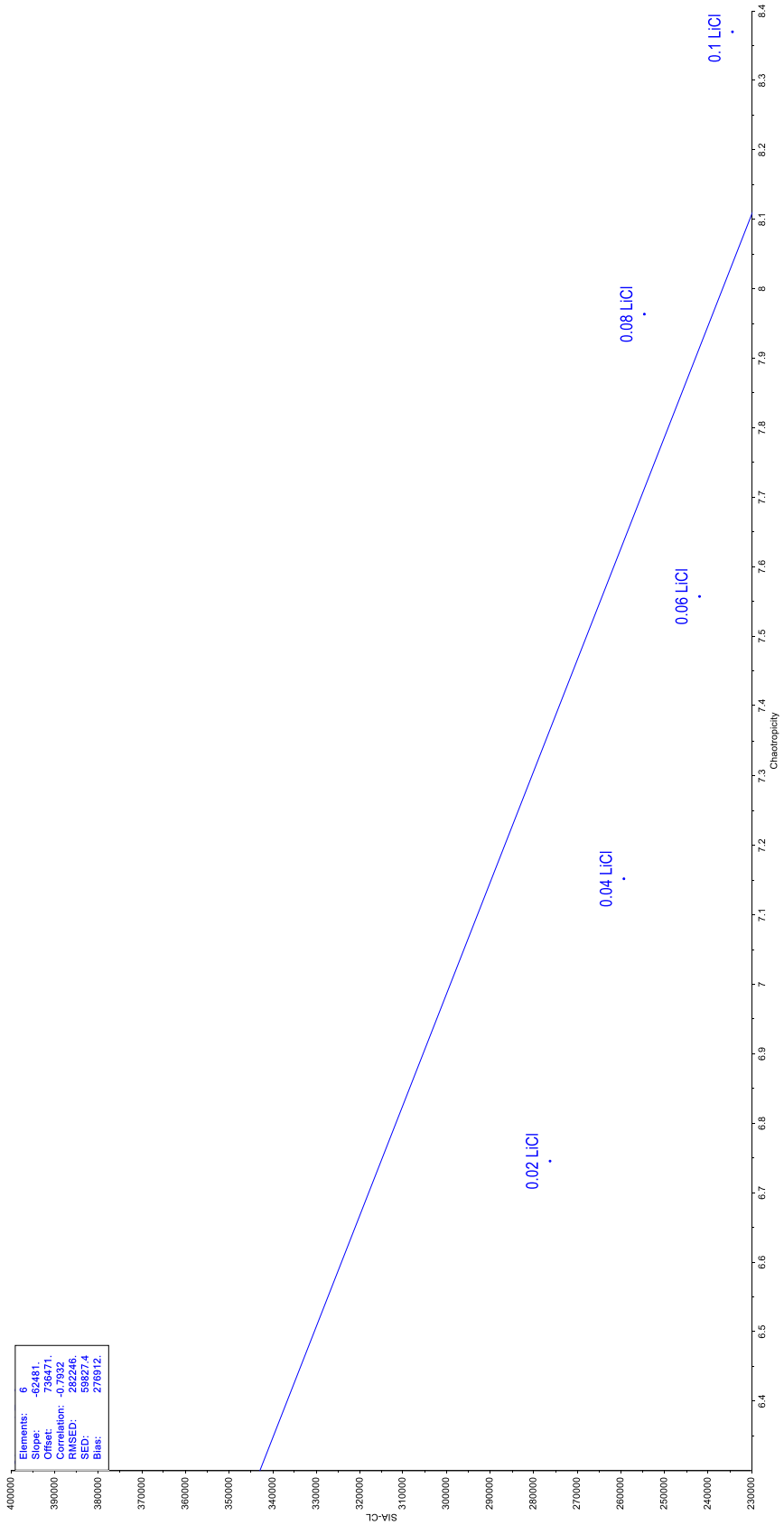


Figure 9-13: Unscrambler® correlation between chemiluminescence and solvent chaotropicity in ultrasonic C. officinalis *extracts prepared with LiCl/glycerol composites. Data is presented as means of replicates (n=3).*

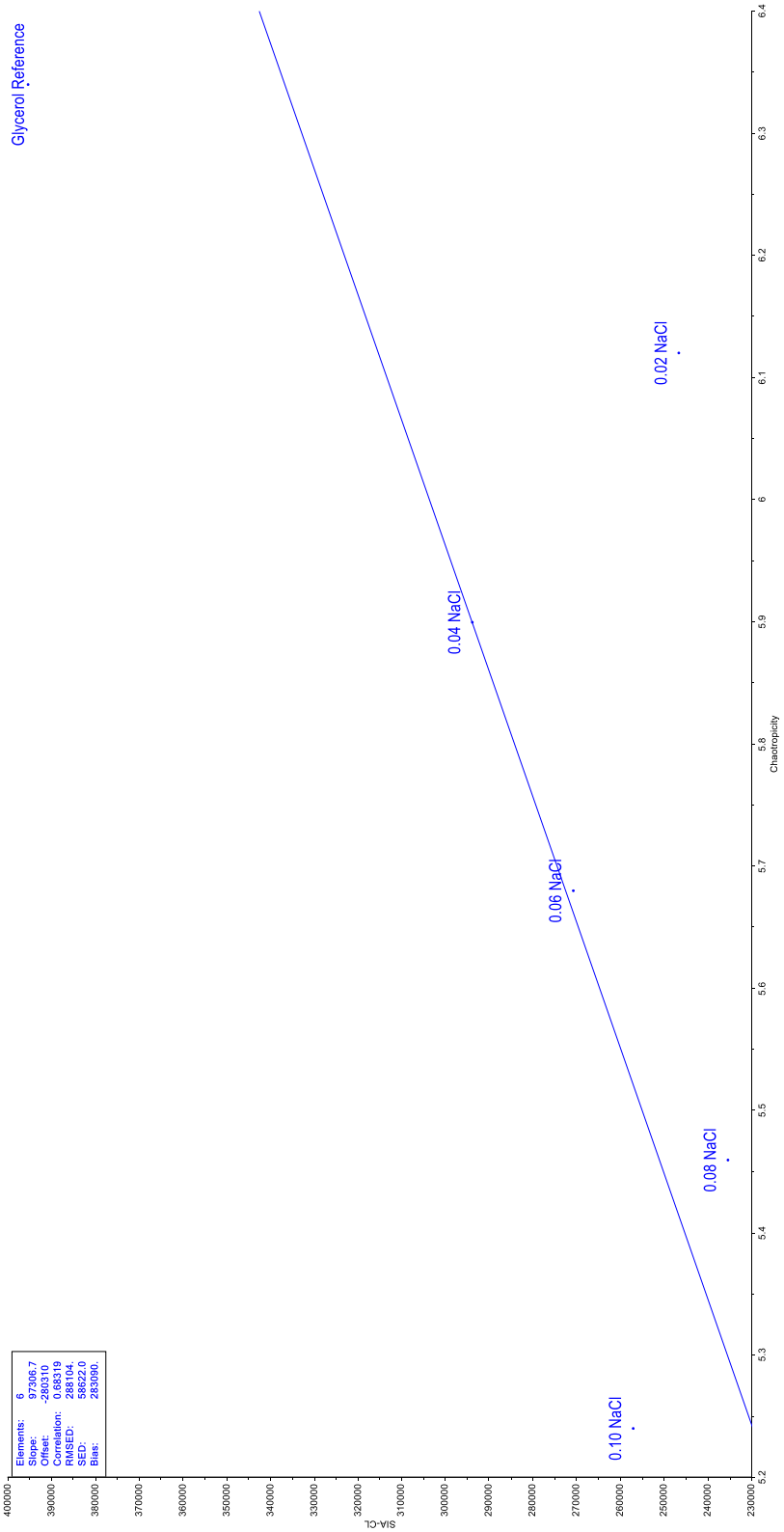


Figure 9-14: Unscrambler® correlation between chemiluminescence and solvent chaotropicity in ultrasonic C. officinalis *extracts prepared with NaCl/glycerol composites. Data is presented as means of replicates (n=3).*

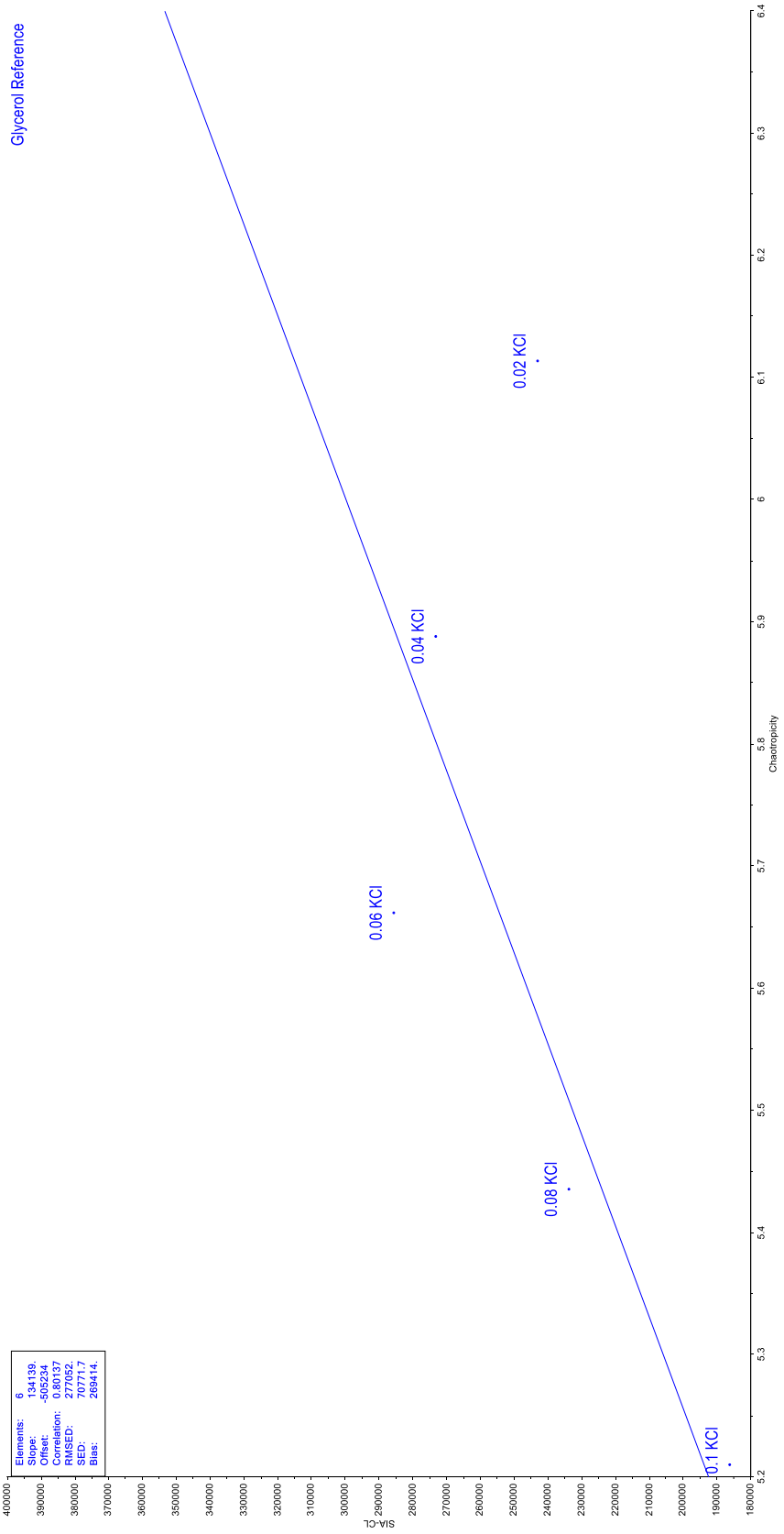


Figure 9-15: Unscrambler® correlation between chemiluminescence and solvent chaotropicity in ultrasonic C. officinalis *extracts prepared with KCl/glycerol composites. Data is presented as means of replicates (n=3).*

Table 9-13: Radical scavenging activity of C. officinalis *extracts prepared with salt/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=9). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol. Units are mV.s.g(flower)$^{-1}$.*

Salt Concentration (‰ mol/mol)	*LiCl/glycerol composite*	*NaCl/glycerol composite*	*KCl/glycerol composite*	*$MgCl_2$/glycerol composite*
0‰	1.787 ± 0.3065	1.787 ± 0.3065	1.787 ± 0.3065	-
20‰	1.928 ± 0.01028	1.547 ± 0.1188	1.583 ± 0.151	**-**
40‰	1.912 ± 0.06926	1.617 ± 0.1195	1.529 ± 0.1281	**-**
60‰	1.878 ± 0.02719	1.57 ± 0.1333	1.694 ± 0.1476	-
80‰	1.782 ± 0.01756	1.472 ± 0.09955	1.57 ± 0.1225	-
100‰	1.725 ± 0.1039	1.479 ± 0.1009	**1.439 ± 0.01241**	-

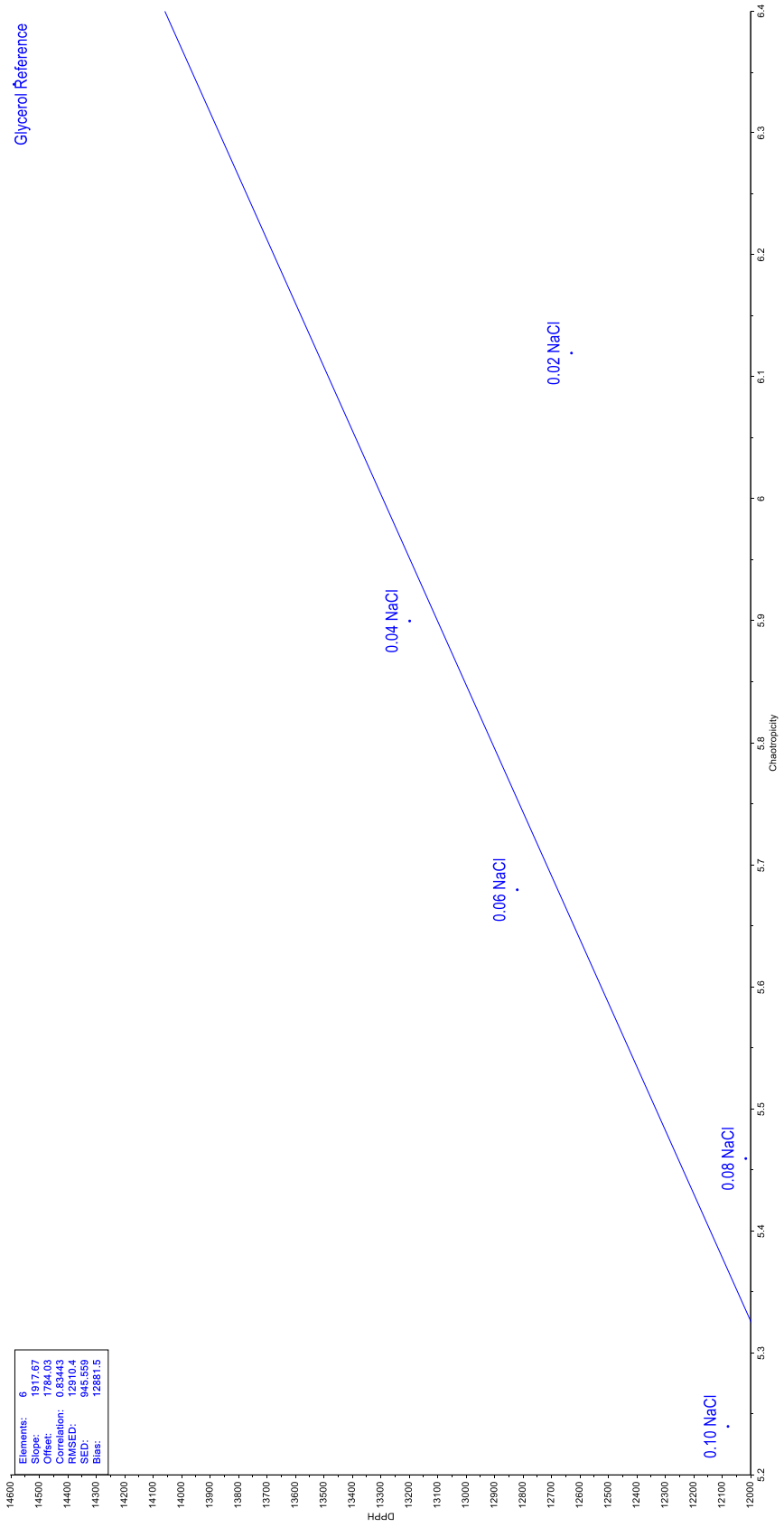


Figure 9-16: Unscrambler® correlation between radical scavenging activity and solvent chaotropicity in ultrasonic C. officinalis *extracts prepared with NaCl/glycerol composites. Data is presented as means of replicates (n=9).*

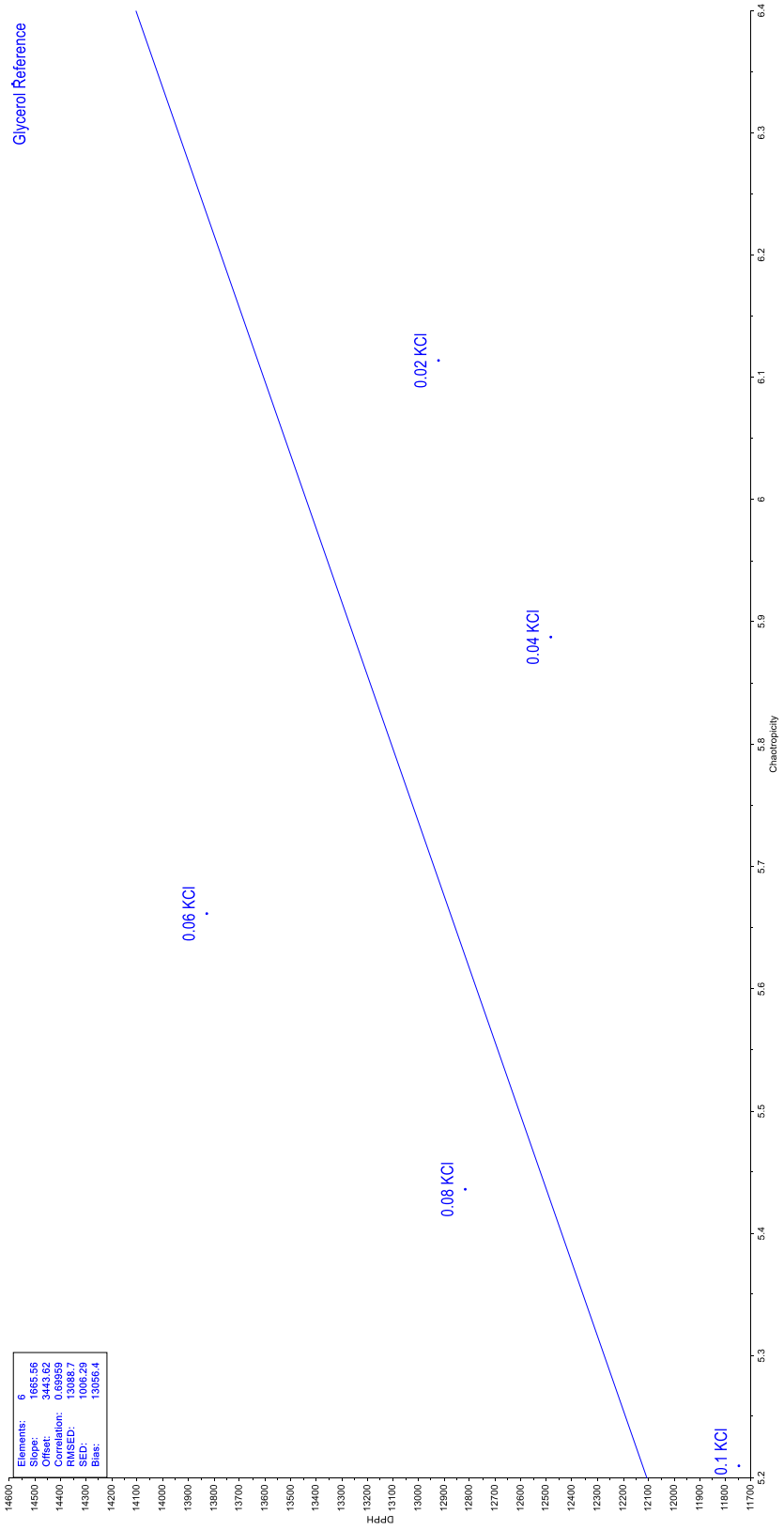


Figure 9-17: Unscrambler® correlation between radical scavenging activity and solvent chaotropicity in ultrasonic C. officinalis *extracts prepared with KCl/glycerol composites. Data is presented as means of replicates (n=9).*

Table 9-14: Total phenolic content of C. officinalis *extracts prepared with salt/glycerol composites. Data is expressed as means ± standard deviations of replicates (n=9). Units are mg(GAE).g(flower)$^{-1}$. Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol.*

Salt Concentration (‰ mol/mol)	*LiCl/glycerol composite*	*NaCl/glycerol composite*	*KCl/glycerol composite*	*$MgCl_2$/glycerol composite*
0‰	2.9113 ± 0.23174	2.9113 ± 0.23174	2.9113 ± 0.23174	2.9113 ± 0.23174
20‰	3.0509 ± 0.14106	**2.3568 ± 0.16828**	1.4317 ± 0.13033	2.5954 ± 0.34678
40‰	**2.6929 ± 0.23831**	2.5869 ± 0.12655	0.94357 ± 0.18222	**2.4914 ± 0.40897**
60‰	2.654 ± 0.17505	**2.3784 ± 0.12204**	-0.028423 ± 0.33863	2.5706 ± 0.25641
80‰	**2.3545 ± 0.1534**	**2.2449 ± 0.1016**	-0.42127 ± 0.21272	**2.302 ± 0.2996**
100‰	**2.4469 ± 0.22708**	**2.322 ± 0.13973**	0.34881 ± 0.33126	2.0631 ± 0.36197

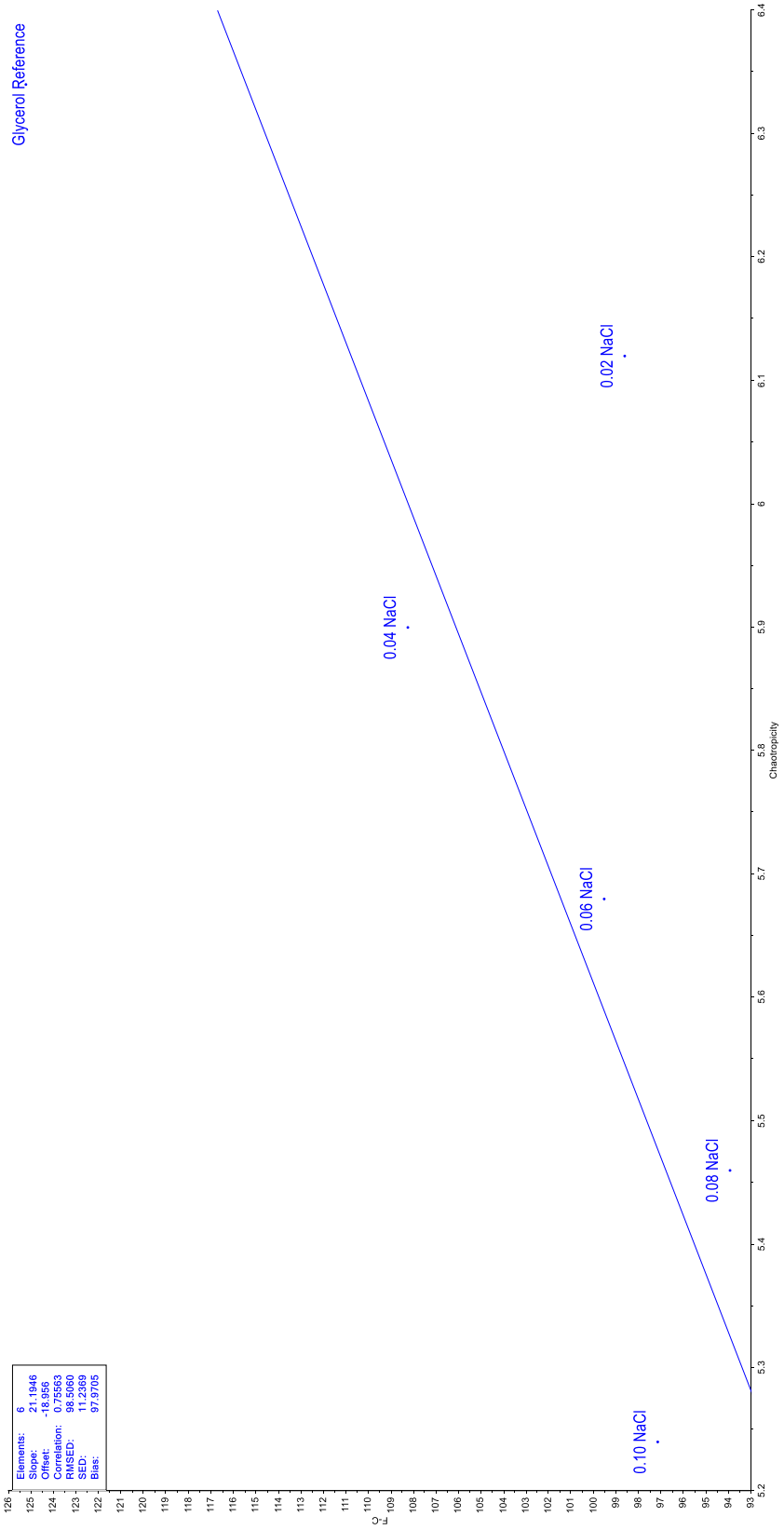


Figure 9-18: Unscrambler® correlation between total phenolic content and solvent chaotropicity in ultrasonic C. officinalis *extracts prepared with NaCl/glycerol composites. Data is presented as means of replicates (n=9).*

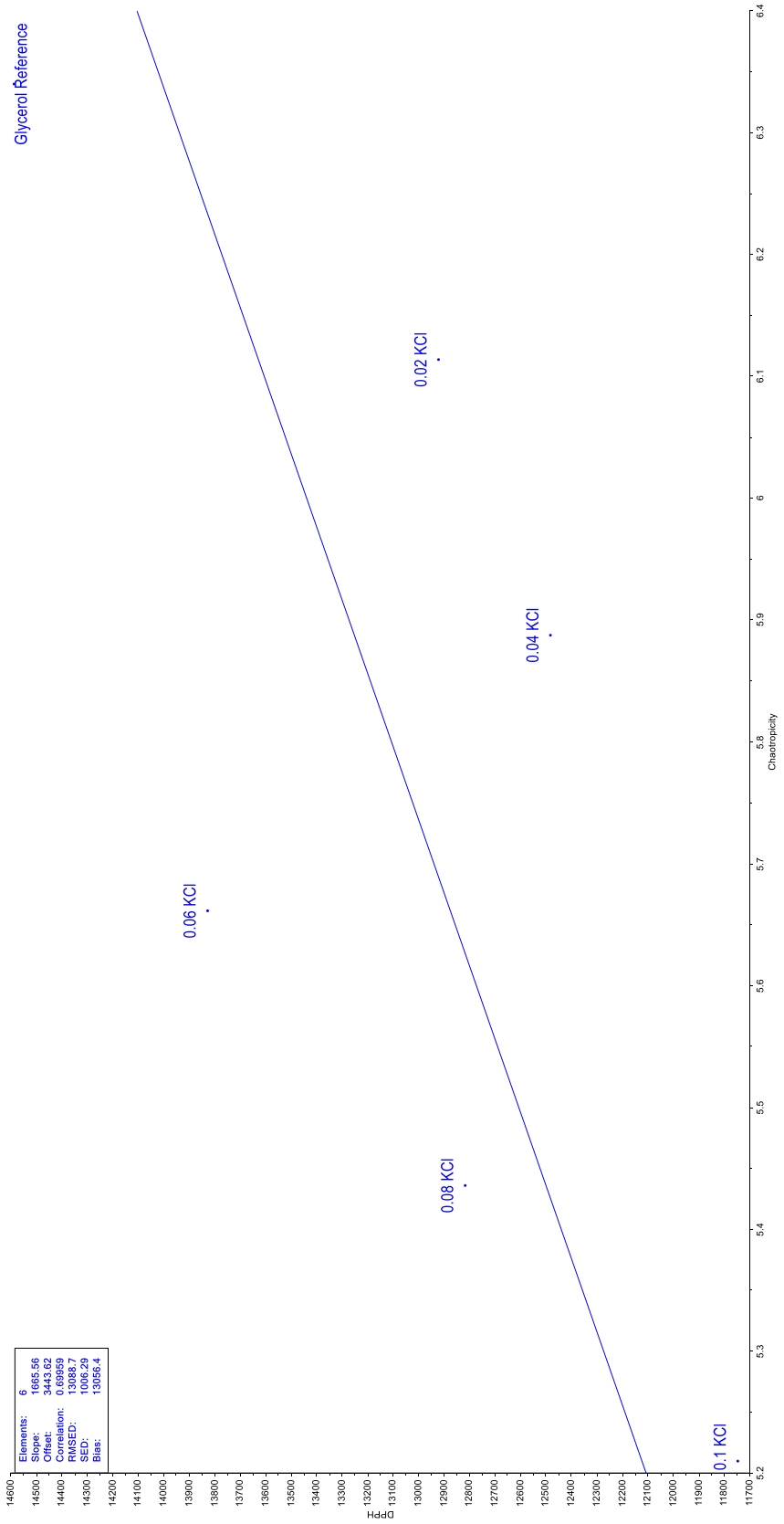


Figure 9-19: Figure 9-20: Unscrambler® correlation between total phenolic content and solvent chaotropicity in ultrasonic C. officinalis *extracts prepared with KCl/glycerol composites. Data is presented as means of replicates (n=9).*

Acid/Glycerol Composites

Table 9-15: Height of Peak A in C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared from neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Tartaric acid/glycerol composite*	*Citric acid/glycerol composite*
0‰	46853 ± 2934.2	46853 ± 2934.2
1‰	50561 ± 2535.8	47684 ± 2702.8
8‰	**56268 ± 3160.1**	47551 ± 3478.9
12‰	**51528 ± 4490.5**	44925 ± 3368.6
20‰	**55457 ± 2176.1**	44616 ± 1222.6
40‰	**51996 ± 4844**	**38193 ± 1748.1**
60‰	**38361 ± 2239.2**	**38388 ± 3964.7**

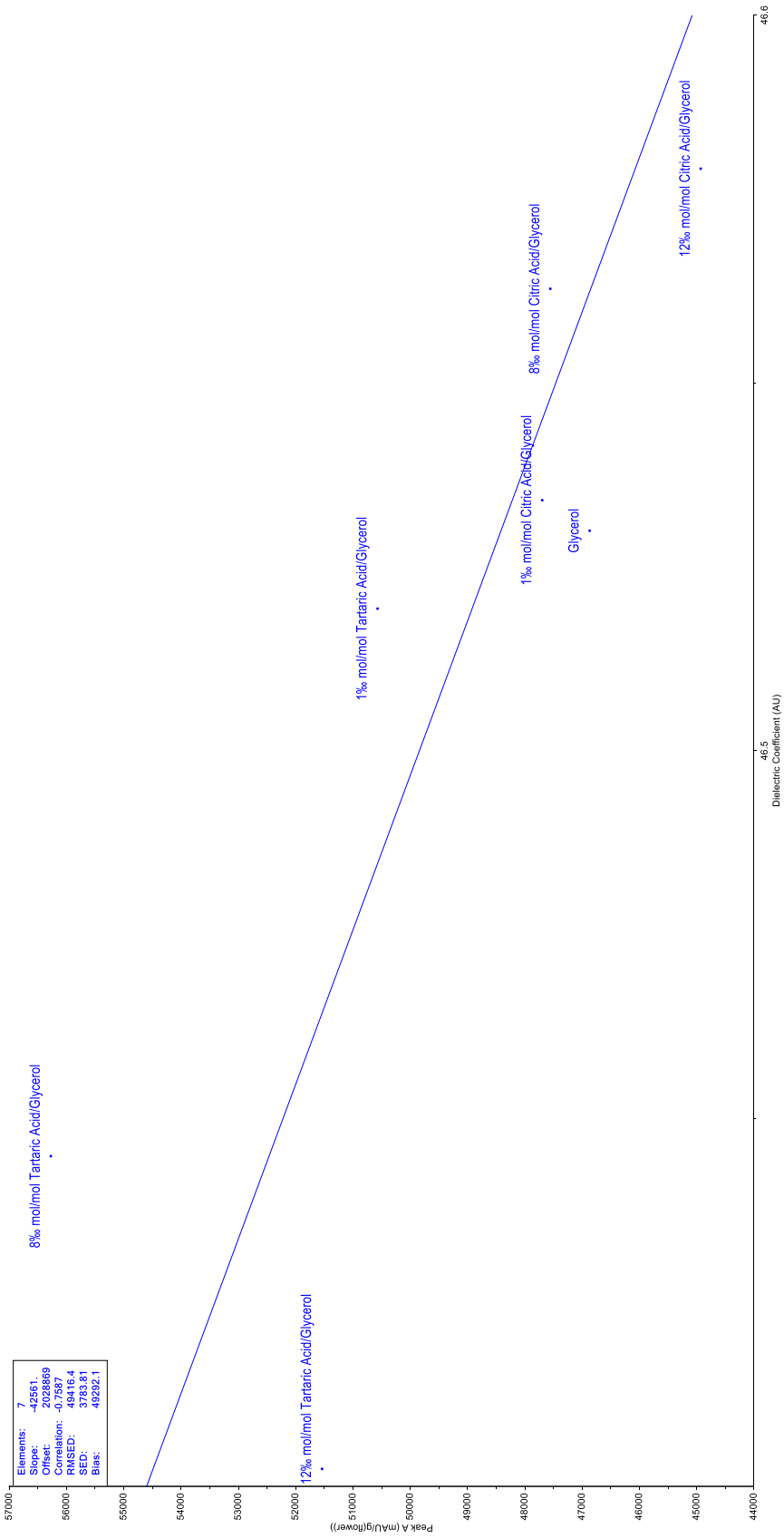


Figure 9-21: Unscrambler® correlation between Peak A height in C. officinalis extracts prepared with acid/glycerol solvents and dielectric constant of the solvent. Data is expressed as means of replicates (n=3).

Table 9-16: Height of Peak B in C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Tartaric acid/glycerol composite*	*Citric acid/glycerol composite*
0‰	1782.6 ± 183.44	1782.6 ± 183.44
1‰	2045.1 ± 38.064	1893.1 ± 148.36
8‰	**2383.2 ± 113.17**	1989.7 ± 147.53
12‰	**2116.6 ± 236.43**	2187.5 ± 392.65
20‰	**2384.1 ± 99.595**	1852.5 ± 68.257
40‰	**2146 ± 211.7**	1540.1 ± 45.658
60‰	**1472.2 ± 65.89**	**1523.6 ± 133.56**

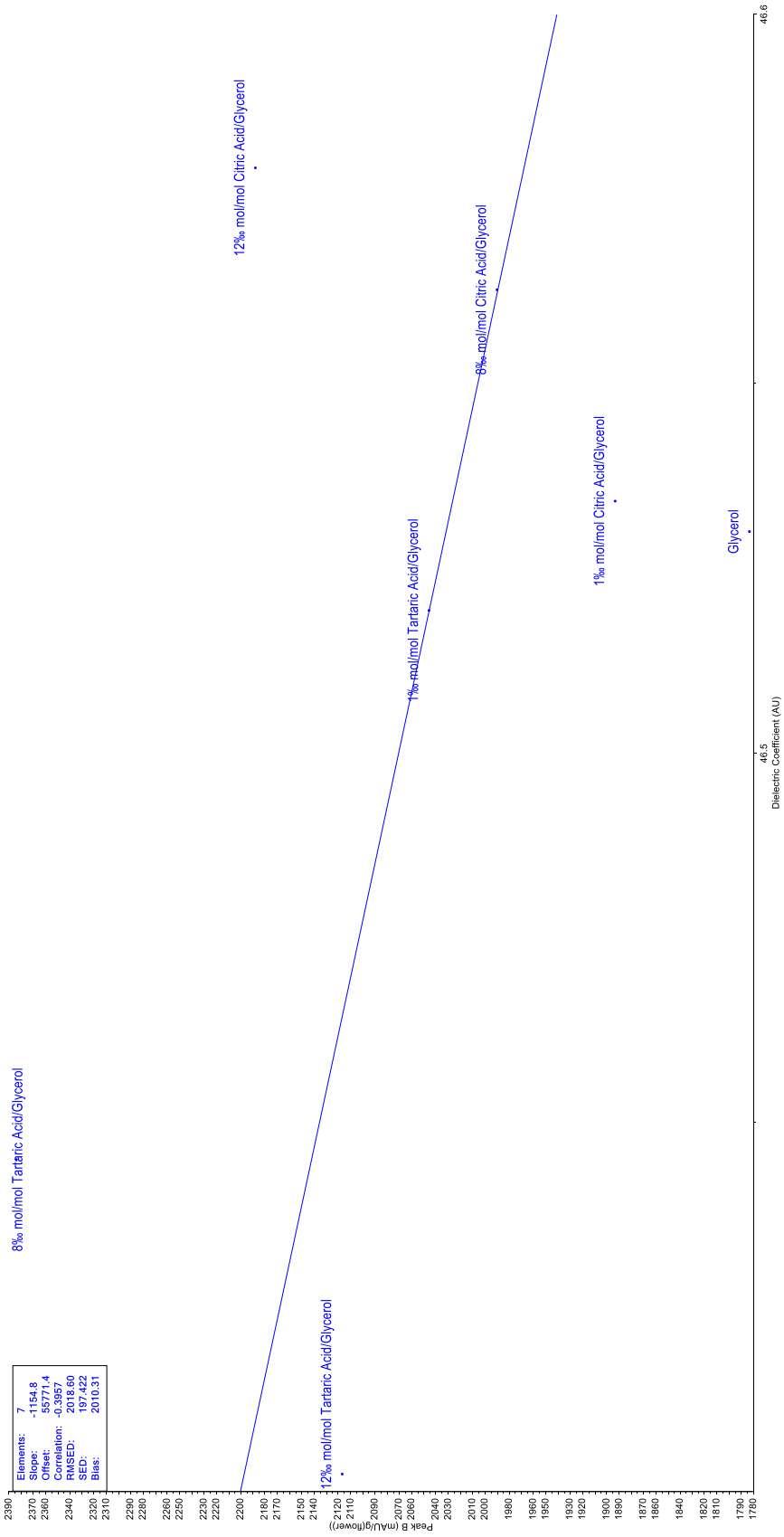


Figure 9-22: Unscrambler® correlation between Peak B height in C. officinalis extracts prepared with acid/glycerol solvents and dielectric constant of the solvent. Data is expressed as means of replicates (n=3).

Table 9-17: Height of Peak C in C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Tartaric acid/glycerol composite*	*Citric acid/glycerol composite*
0‰	4152.6 ± 309.47	4152.6 ± 309.47
1‰	4525.3 ± 117.62	4157.5 ± 208.23
8‰	**4683.2 ± 302.65**	4545.9 ± 442.9
12‰	4478.9 ± 421.52	4744.4 ± 819.91
20‰	**4862.4 ± 99.601**	4223.4 ± 69.309
40‰	4308.4 ± 334.7	3812.7 ± 173.56
60‰	**3279.6 ± 141.79**	**3622.9 ± 305.18**

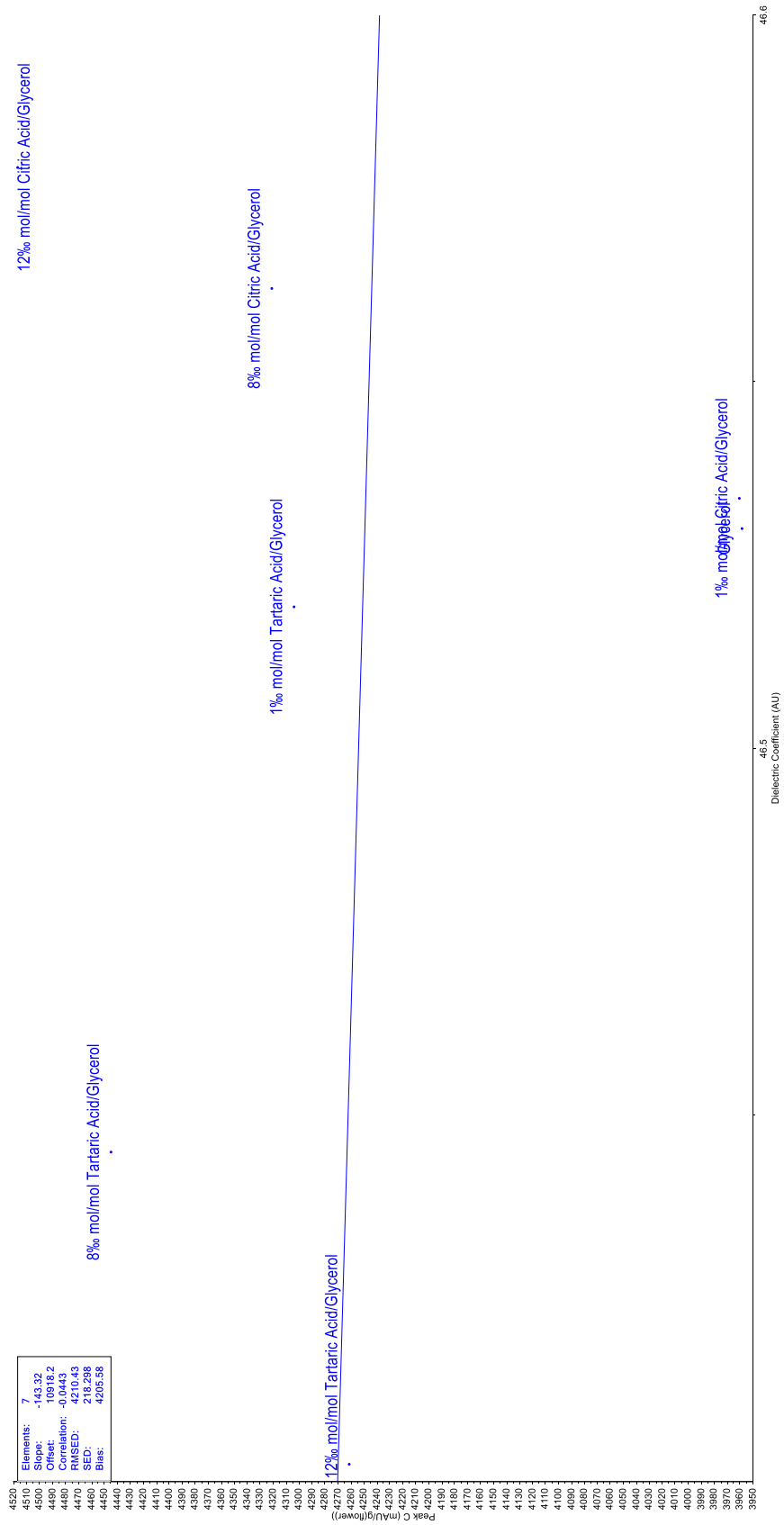


Figure 9-23: Unscrambler® correlation between Peak C height in C. officinalis *extracts prepared with acid/glycerol solvents and dielectric constant of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-18: Height of Peak D in C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as means ± standard deviation of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol.Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Tartaric acid/glycerol composite*	*Citric acid/glycerol composite*
0‰	5535.4 ± 441.57	5535.4 ± 441.57
1‰	**6140.4 ± 216.85**	5574.1 ± 241.57
8‰	**6430.3 ± 447.93**	6097.5 ± 553.52
12‰	6102.3 ± 605.97	6378.3 ± 1150.3
20‰	**6682.8 ± 156.88**	5726.8 ± 50.644
40‰	5944.1 ± 471.8	5145.1 ± 246.84
60‰	**4576.4 ± 206.07**	4957.5 ± 418.76

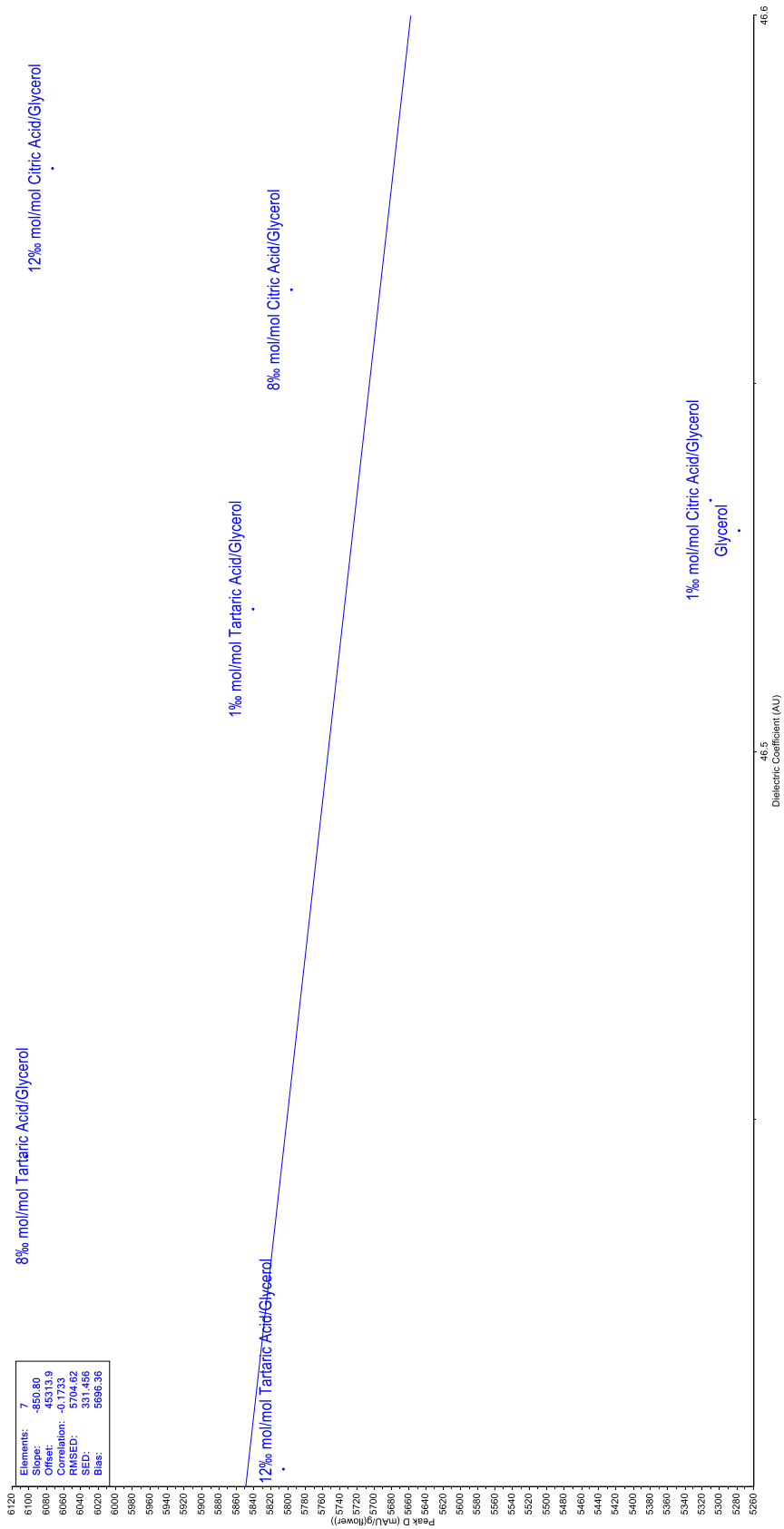


Figure 9-24: Figure 9-25: Unscrambler® correlation between Peak D height in C. officinalis *extracts prepared with acid/glycerol solvents and dielectric constant of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-19: Height of Peak E in C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Tartaric acid/glycerol composite*	*Citric acid/glycerol composite*
0‰	1274.7 ± 106.46	1274.7 ± 106.46
1‰	**1473.5 ± 51.993**	1260.8 ± 76.242
8‰	**1433.5 ± 94.815**	1351.9 ± 108.79
12‰	1333.2 ± 108.39	1412.8 ± 239.09
20‰	**1432.6 ± 37.846**	**1207.6 ± 22.085**
40‰	1163.9 ± 100.81	**1040.3 ± 36.635**
60‰	**798.63 ± 16.252**	**943.18 ± 80.555**

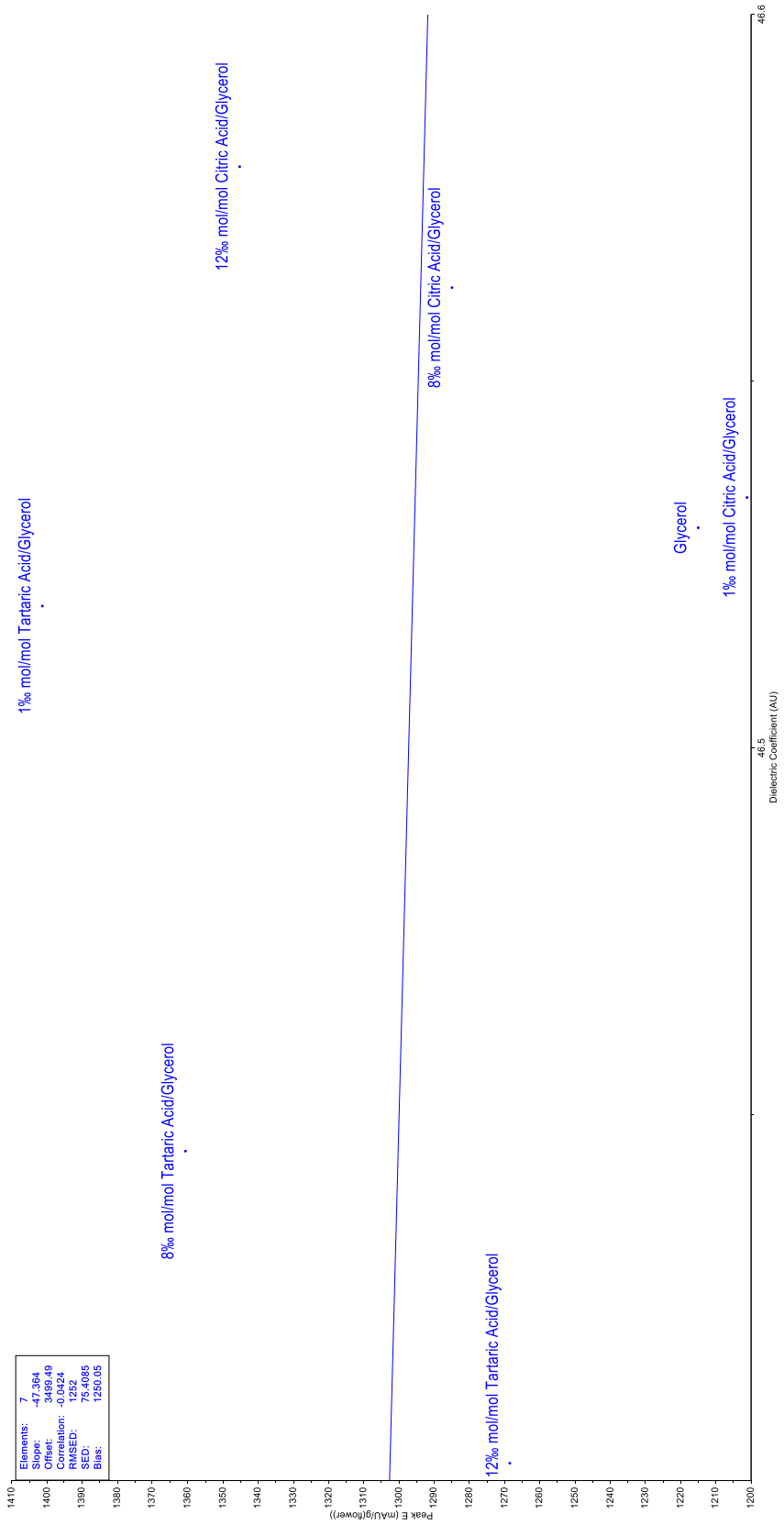


Figure 9-26: Unscrambler® correlation between Peak E height in C. officinalis extracts prepared with acid/glycerol solvents and dielectric constant of the solvent. Data is expressed as means of replicates (n=3).

Table 9-20: Height of Peak F in C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared from neat glycerol.Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Tartaric acid/glycerol composite*	*Citric acid/glycerol composite*
0‰	37435 ± 2969	37435 ± 2969
1‰	**41826 ± 1786.2**	36915 ± 2022.2
8‰	**43588 ± 3067.2**	39993 ± 3807.7
12‰	41505 ± 4069.4	41877 ± 6921.4
20‰	**44585 ± 1095.6**	37027 ± 1024.2
40‰	39640 ± 3377.2	**33226 ± 1381.9**
60‰	**29562 ± 1250.7**	**31236 ± 2610.6**

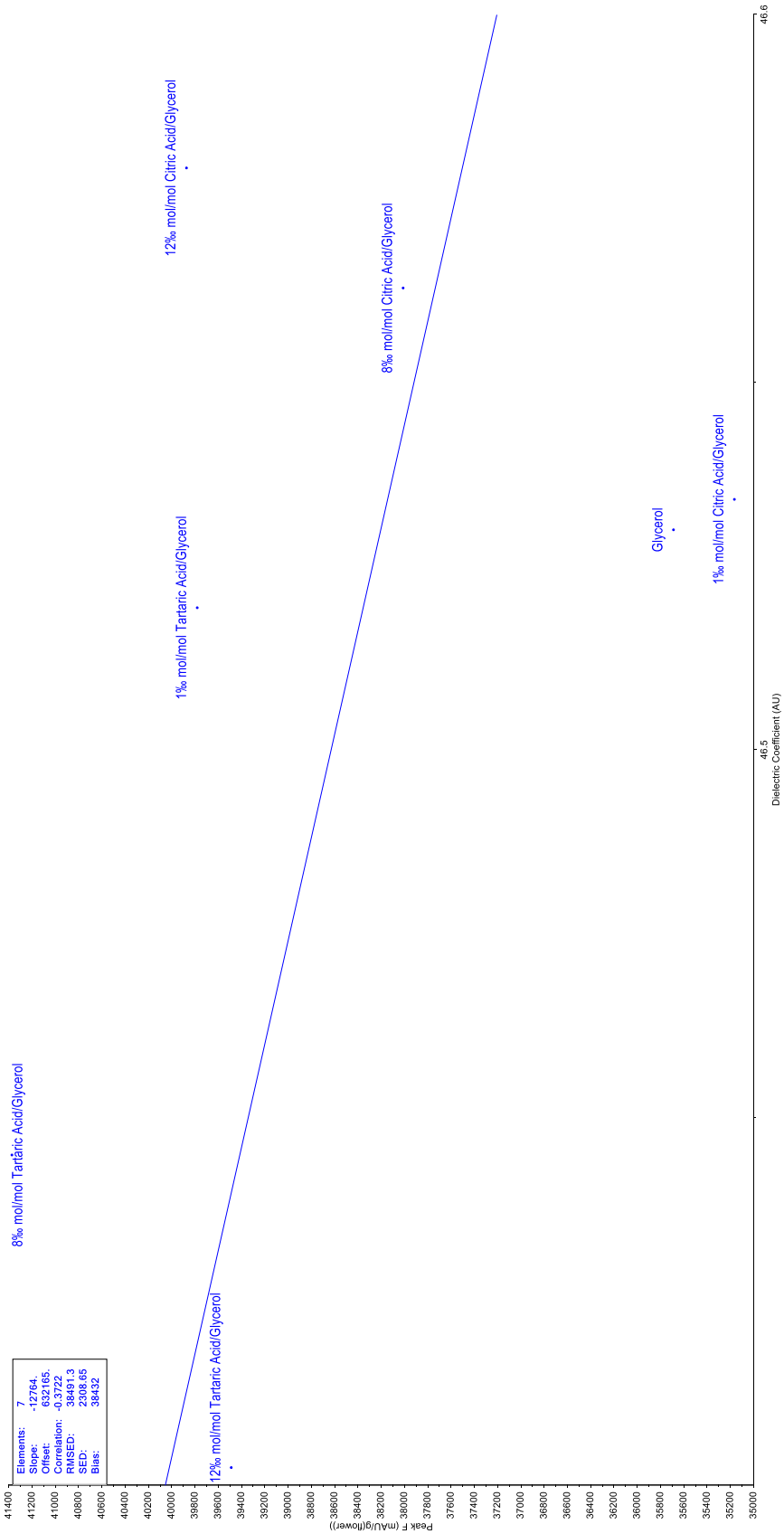


Figure 9-27: Unscrambler® correlation between Peak E height in C. officinalis extracts prepared with Facid/glycerol solvents and dielectric constant of the solvent. Data is expressed as means of replicates (n=3).

Table 9-21: Height of Peak G in C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Tartaric acid/glycerol composite*	*Citric acid/glycerol composite*
0‰	13260 ± 1108.7	13260 ± 1108.7
1‰	14787 ± 602.37	13092 ± 655.54
8‰	**15380 ± 1027.8**	14097 ± 1119.6
12‰	**14647 ± 1424.2**	14733 ± 2437.8
20‰	**15887 ± 297.59**	13267 ± 410.06
40‰	14024 ± 1148.8	11945 ± 613.13
60‰	**10536 ± 449.45**	**11325 ± 930.74**

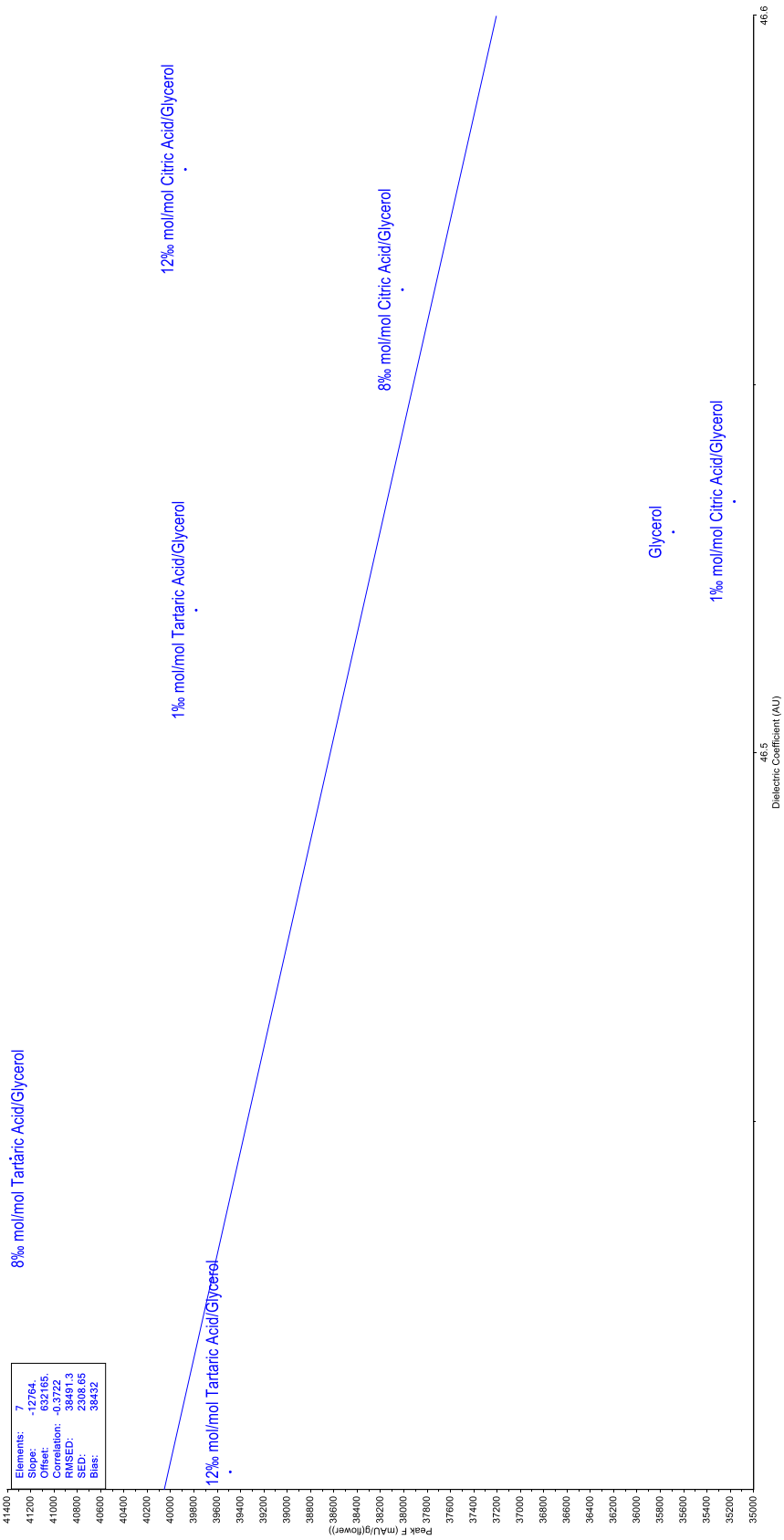


Figure 9-28: Unscrambler® correlation between Peak G height in C. officinalis extracts prepared with acid/glycerol solvents and dielectric constant of the solvent. Data is expressed as means of replicates (n=3).

Table 9-22: Height of Peak H in C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Tartaric acid/glycerol composite*	*Citric acid/glycerol composite*
0‰	34472 ± 3006	34472 ± 3006
1‰	**39956 ± 1985.7**	33647 ± 2207.9
8‰	**40232 ± 3019.3**	34924 ± 3754
12‰	37913 ± 3675.8	36350 ± 5830.2
20‰	**40698 ± 851.44**	**32635 ± 1263.6**
40‰	**36321 ± 3253.2**	**28852 ± 1352.9**
60‰	**26896 ± 1592.6**	**26759 ± 2319.9**

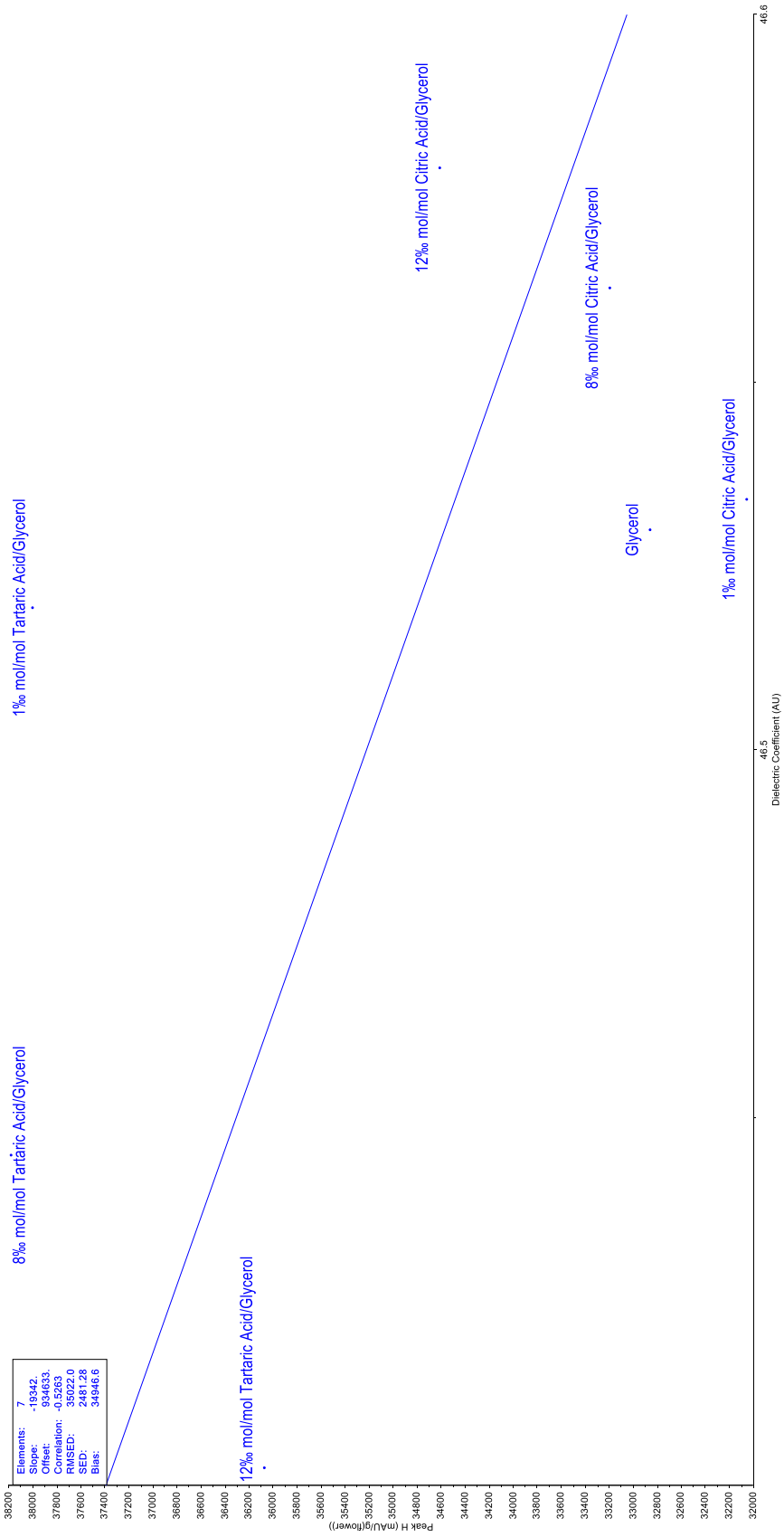


Figure 9-29: Unscrambler® correlation between Peak H height in C. officinalis extracts prepared with acid/glycerol solvents and dielectric constant of the solvent. Data is expressed as means of replicates (n=3).

Table 9-23: Height of Peak I in C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Tartaric acid/glycerol composite*	*Citric acid/glycerol composite*
0‰	1979 ± 159.19	1979 ± 159.19
1‰	2139.1 ± 116.15	1917.7 ± 119.81
8‰	**2216.7 ± 175.2**	1938.7 ± 132.85
12‰	2114.9 ± 191.13	2100.2 ± 378.42
20‰	**2315.3 ± 31.778**	1914.2 ± 49.499
40‰	2100.5 ± 183.26	**1702.8 ± 85.38**
60‰	**1570.2 ± 86.636**	**1637.8 ± 141.15**

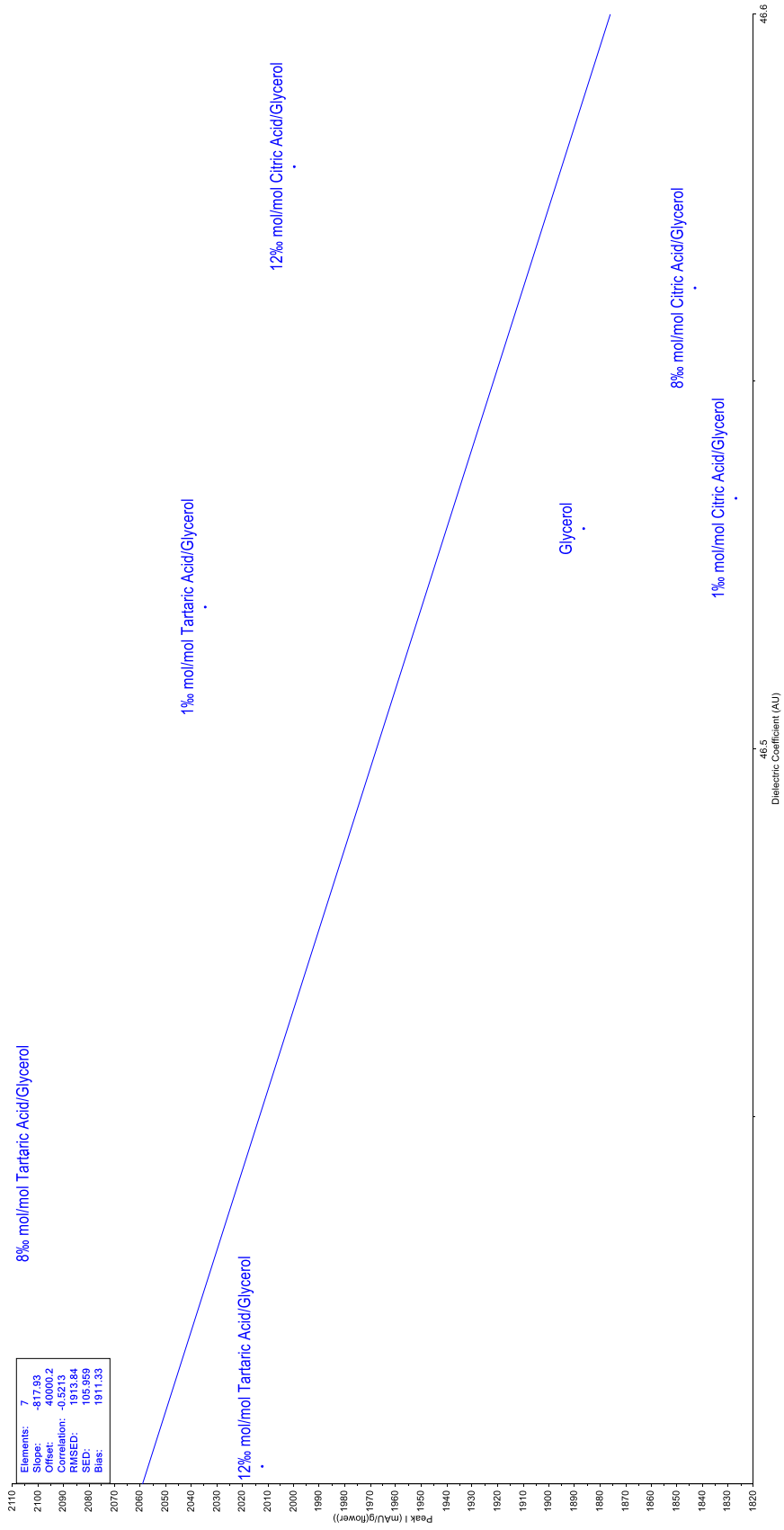


Figure 9-30: Unscrambler® correlation between Peak I height in C. officinalis extracts prepared with acid/glycerol solvents and dielectric constant of the solvent. Data is expressed as means of replicates (n=3).

Table 9-24: Total peak areas of C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Tartaric acid/glycerol composite*	*Citric acid/glycerol composite*
0‰	12156 ± 1065.2	12156 ± 1065.2
1‰	13426 ± 386.15	**10411 ± 1032.1**
8‰	**13920 ± 874.98**	11093 ± 2565.9
12‰	13002 ± 1256.2	13301 ± 2373.8
20‰	**14205 ± 311.53**	**10757 ± 570.23**
40‰	12237 ± 1072.6	**7960.1 ± 565.24**
60‰	**9618.3 ± 519.12**	**8221.7 ± 1337.2**

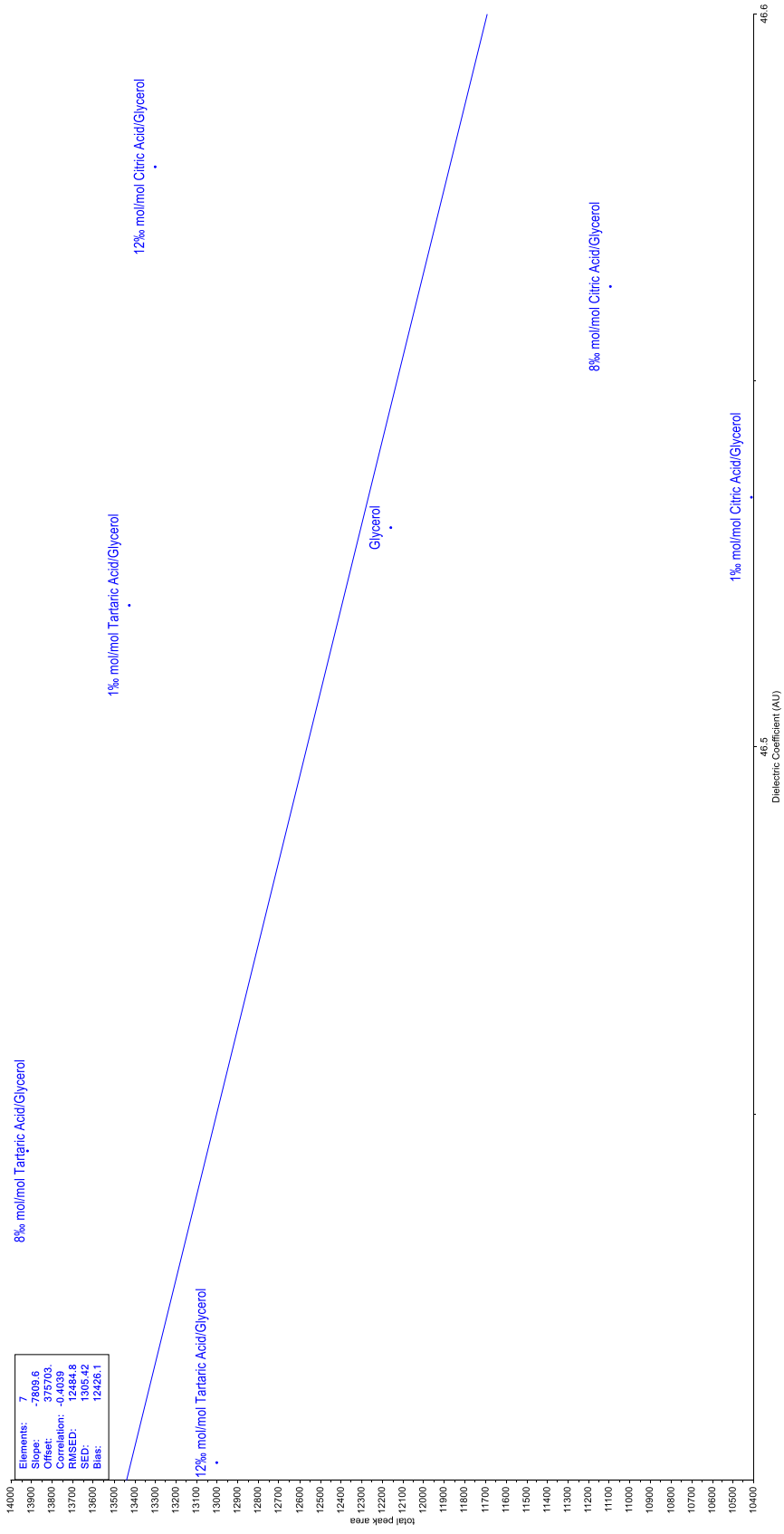


Figure 9-31: Unscrambler® correlation between total peak area in C. officinalis *extracts prepared with acid/glycerol solvents and dielectric constant of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-25: Chemiluminescence response of C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Tartaric acid/glycerol composite*	*Citric acid/glycerol composite*
0‰	29133 ± 4924.2	29133 ± 4924.2
1‰	33328 ± 1867.4	34483 ± 4669.1
8‰	**42278 ± 7690.5**	**34502 ± 642.29**
12‰	34735 ± 2282.1	34472 ± 5878.5
20‰	**36118 ± 2556.7**	29848 ± 2280.9
40‰	25737 ± 3188.5	27522 ± 3221.7
60‰	**16891 ± 3058.2**	**25196 ± 1050.4**

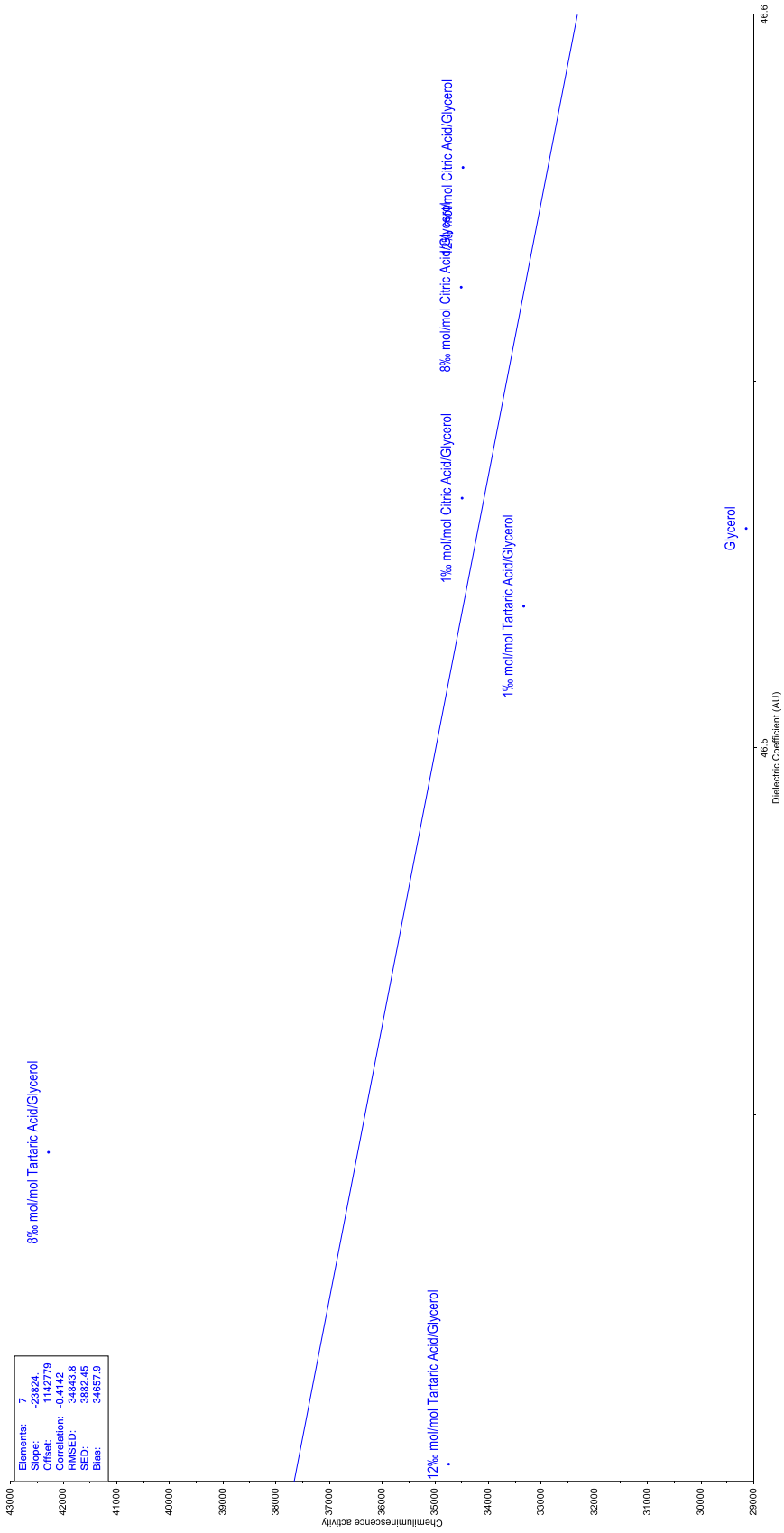


Figure 9-32 Unscrambler® correlation between chemiluminescence response in C. officinalis *extracts prepared with acid/glycerol solvents and dielectric constant of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-26: DPPH radical scavenging activity of C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Tartaric acid/glycerol composite*	*Citric acid/glycerol composite*
0‰,	0.7009 ± 0.092631	0.7009 ± 0.092259
1‰,	0.71369 ± 0.039481	0.70377 ± 0.038932
8‰,	0.66505 ± 0.034983	0.72371 ± 0.038069
12‰,	0.60324 ± 0.032164	**0.54087 ± 0.028838**
20‰,	**0.60545 ± 0.032459**	**0.59162 ± 0.031718**
40‰,	**0.48659 ± 0.023728**	**0.57016 ± 0.027803**
60‰,	**0.37332 ± 0.021329**	**0.37846 ± 0.021623**

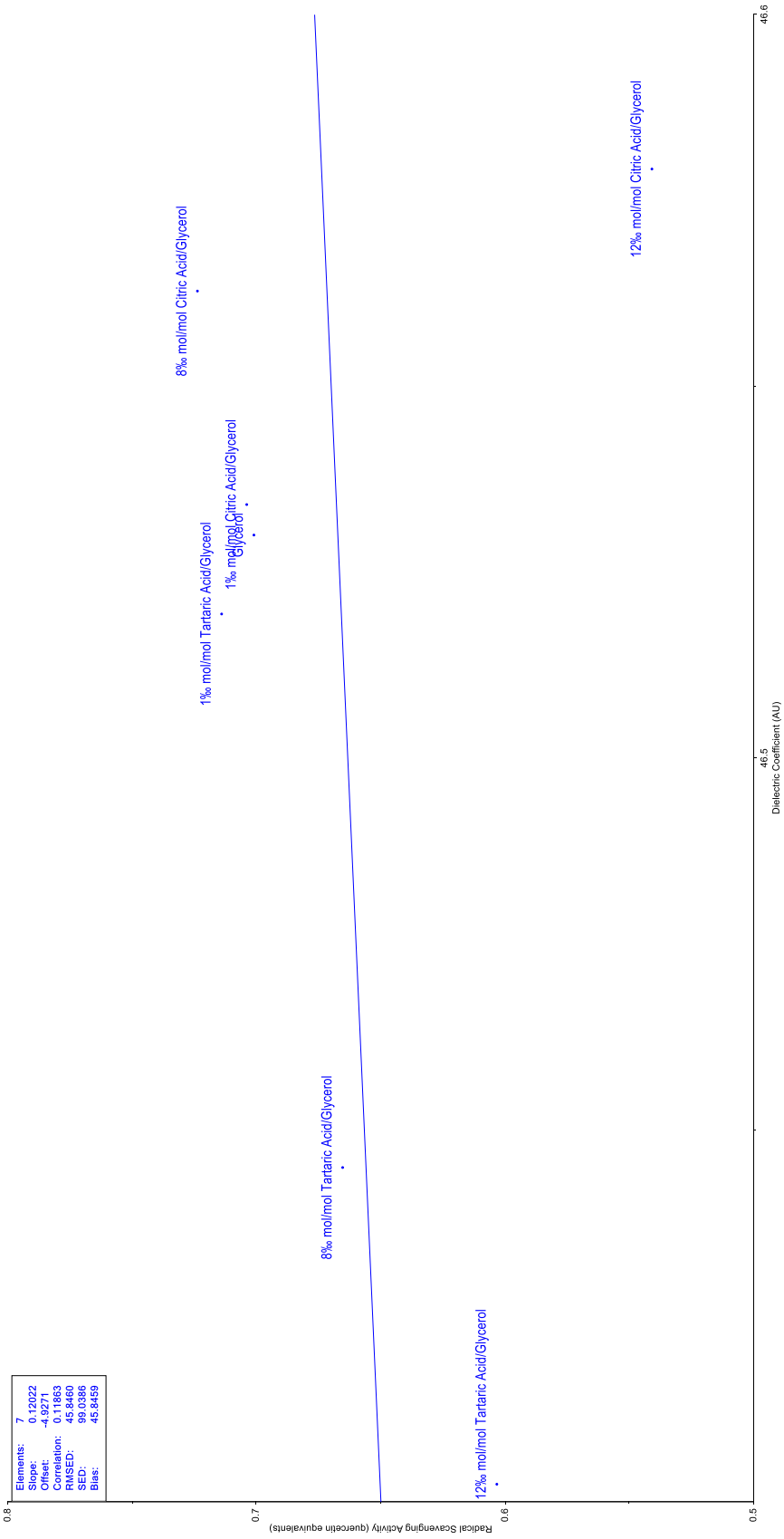


Figure 9-33: Unscrambler® correlation between DPPH radical scavenging activity in C. officinalis *extracts prepared with acid/glycerol solvents and dielectric constant of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-27: Phenolic content of C. officinalis *extracts prepared with acid/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. Units are mg(quercetin).g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Tartaric acid/glycerol composite*	*Citric acid/glycerol composite*
0‰	1.8289 ± 0.12883	1.8289 ± 0.12883
1‰	**2.0702 ± 0.087554**	1.8172 ± 0.12511
8‰	**2.1534 ± 0.17794**	1.88 ± 0.099959
12‰	**1.9346 ± 0.097247**	1.831 ± 0.14625
20‰	**2.1698 ± 0.17443**	1.8196 ± 0.0798
40‰	**2.0698 ± 0.14585**	**1.7014 ± 0.094653**
60‰	**1.6819 ± 0.14509**	**1.5441 ± 0.13452**

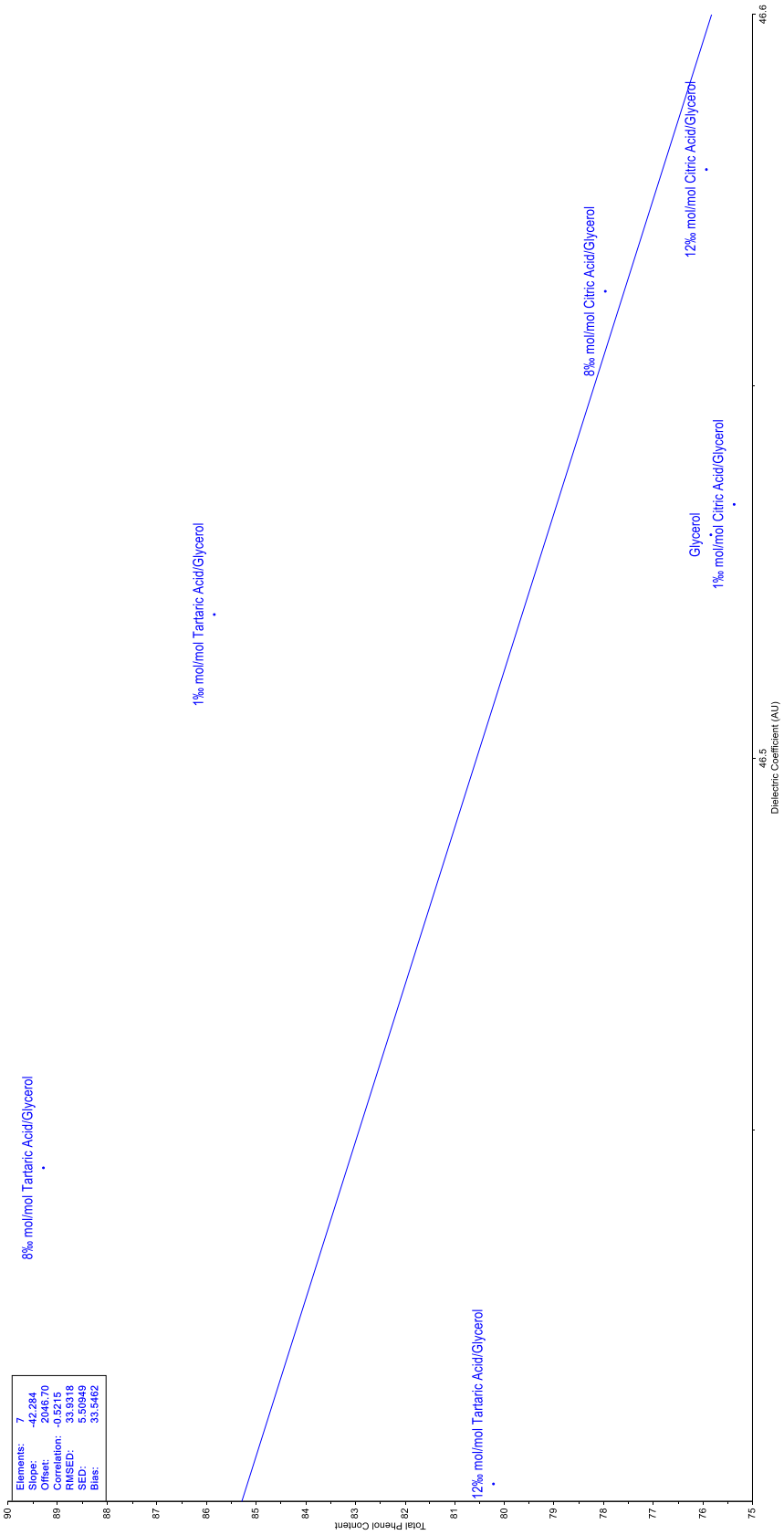


Figure 9-34: Unscrambler® correlation between phenolic content in C. officinalis *extracts prepared with acid/glycerol solvents and dielectric constant of the solvent. Data is expressed as means of replicates (n=3).*

Sugar/Glycerol Composites

Table 9-28: Peak A height of C. officinalis *extracts prepared with sugar/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Sucrose/glycerol composite*	*Fructose/glycerol composite*	*Glucose/glycerol composite*
0‰	46853 ± 2934.2	46853 ± 2934.2	46853 ± 2934.2
1‰	45458 ± 510.22	46755 ± 2207.5	50659 ± 2949.7
8‰	48188 ± 564.12	**53294 ± 2375.8**	**54365 ± 1298.5**
12‰	45424 ± 1383.8	**53583 ± 30.283**	**54861 ± 1841.5**
20‰	**38071 ± 1519.9**	47046 ± 1130.1	**51259 ± 1633.2**
40‰	**31673 ± 707.95**	44808 ± 1518.4	43086 ± 2369.6
60‰	**28485 ± 904.83**	**35530 ± 8340.8**	**40378 ± 739.08**

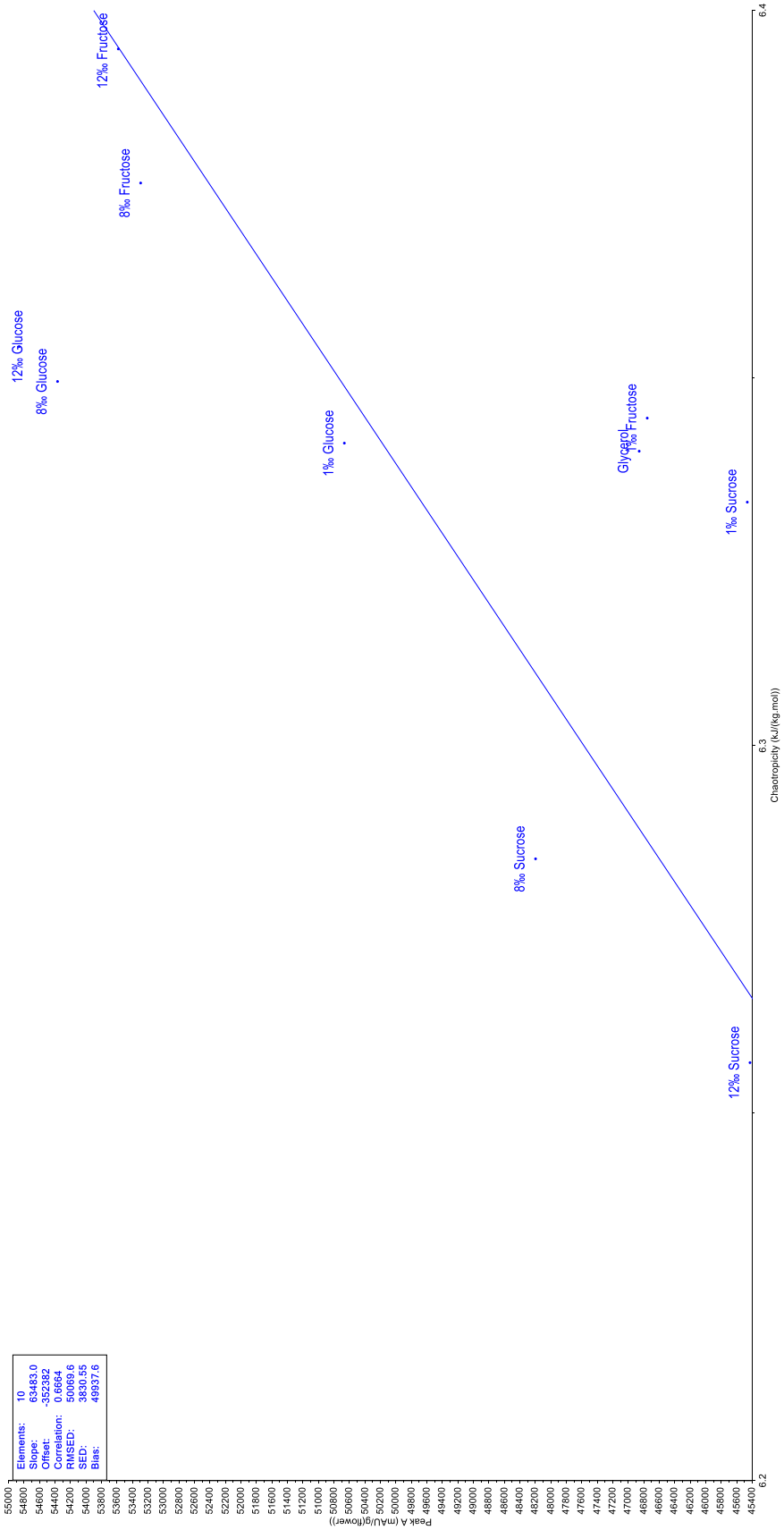


Figure 9-35: Unscrambler® correlation between Peak A height in C. officinalis *extracts prepared with sugar/glycerol solvents and chaotropicity of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-29: Peak B height of C. officinalis *extracts prepared with sugar/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared from neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Sucrose/glycerol composite*	*Fructose/glycerol composite*	*Glucose/glycerol composite*
0‰	1782.6 ± 183.44	1782.6 ± 183.44	1782.6 ± 183.44
1‰	1746.6 ± 83.381	1873.1 ± 116.17	**2181.2 ± 238.25**
8‰	1797.2 ± 88.336	2118.9 ± 121.75	**2265.8 ± 129.21**
12‰	1722.1 ± 105.54	2165.3 ± 75.809	**2360.5 ± 100.69**
20‰	**1386.6 ± 72.317**	1773.2 ± 168.58	**2271.3 ± 62.891**
40‰	**1050.6 ± 30.219**	1591 ± 112.35	1749 ± 79.656
60‰	**991.92 ± 96.974**	**1190.7 ± 284.75**	1617.1 ± 114.65

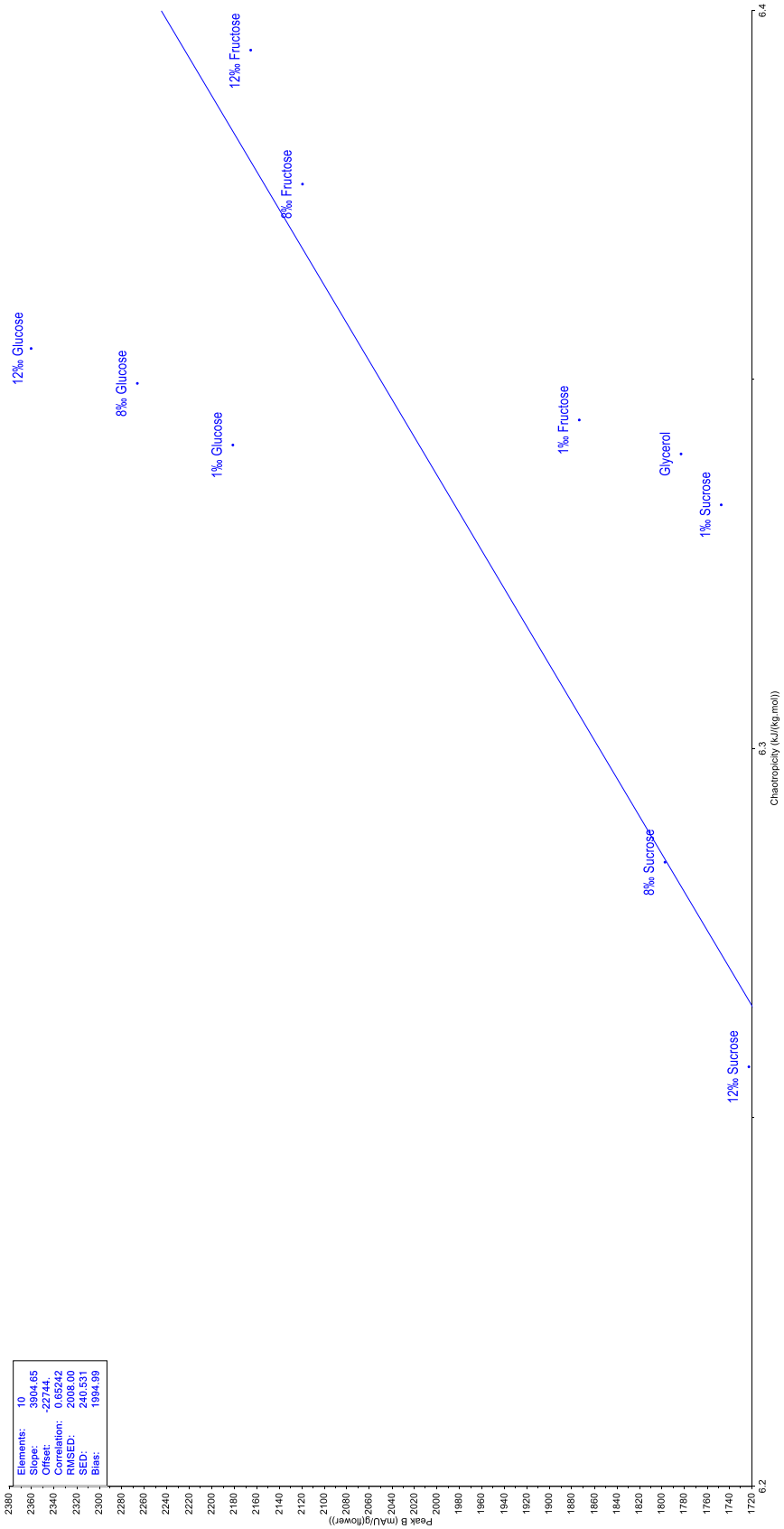


Figure 9-36: Unscrambler® correlation between Peak B height in C. officinalis *extracts prepared with sugar/glycerol solvents and chaotropicity of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-30: Peak C height of C. officinalis *extracts prepared with sugar/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared from neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Sucrose/glycerol composite*	*Fructose/glycerol composite*	*Glucose/glycerol composite*
0‰	3958.3 ± 296.23	3958.3 ± 296.23	3958.3 ± 296.23
1‰	3928.2 ± 214.69	3912.6 ± 221.43	3935 ± 125.23
8‰	3817.8 ± 227.98	4079.4 ± 206.27	3994.4 ± 208.97
12‰	3716.6 ± 270.37	4176.8 ± 248.84	4001.6 ± 128.37
20‰	**3092.7 ± 62.903**	3801.8 ± 143.22	4037.9 ± 139.88
40‰	**2536.7 ± 106.04**	**3564.5 ± 211.24**	**3444.5 ± 72.568**
60‰	**2284.1 ± 164.88**	2850.7 ± 659.66	**3332.4 ± 249.95**

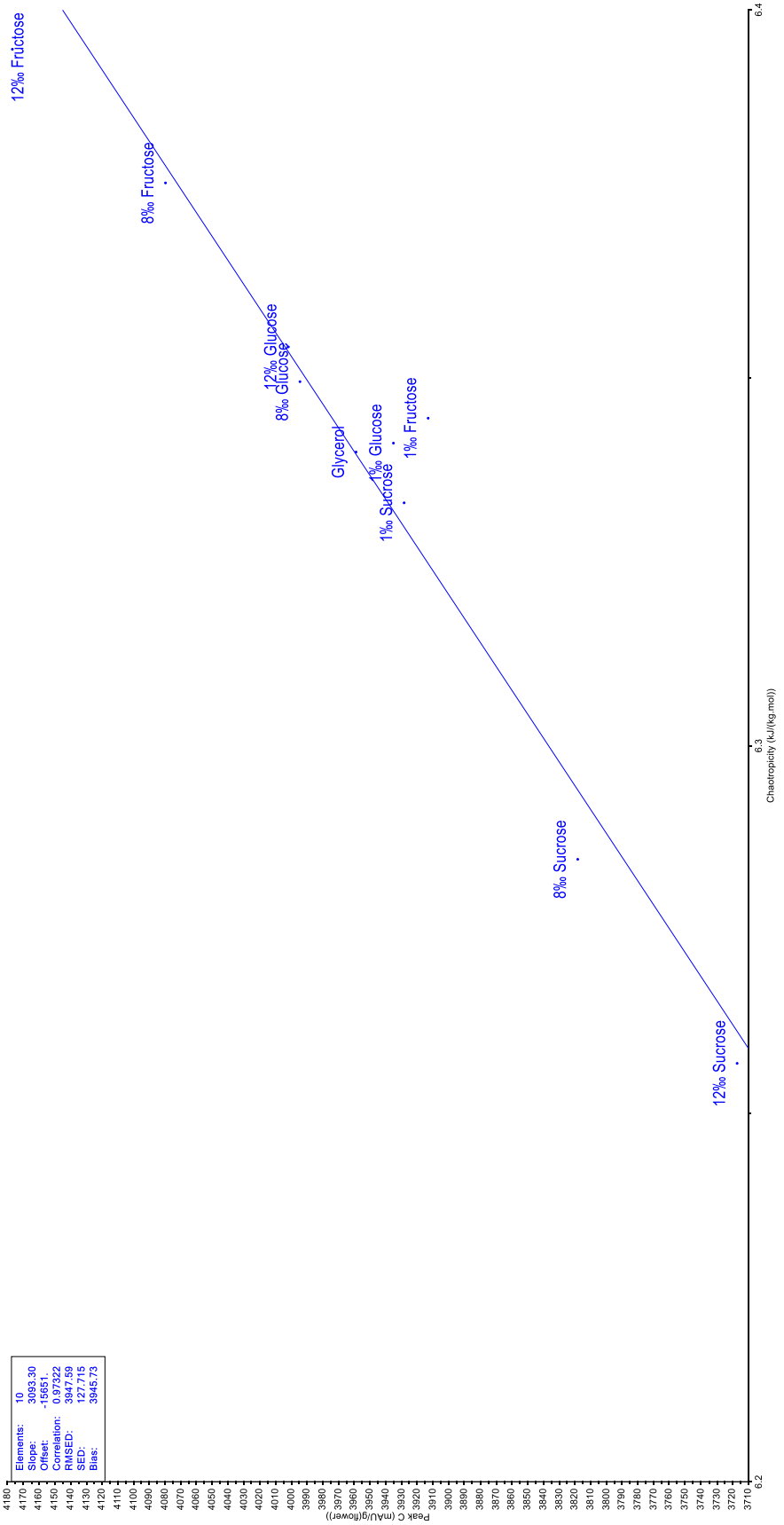


Figure 9-37: Unscrambler® correlation between Peak C height in C. officinalis *extracts prepared with sugar/glycerol solvents and chaotropicity of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-31: Peak D height of C. officinalis *extracts prepared with sugar/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared with neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Sucrose/glycerol composite*	*Fructose/glycerol composite*	*Glucose/glycerol composite*
0‰	5276.2 ± 421.31	5276.2 ± 421.31	5276.2 ± 421.31
1‰	5242.3 ± 284.14	5205.3 ± 340.53	5328.3 ± 201.27
8‰	5127.2 ± 253.11	5470 ± 273.23	5426.8 ± 267.91
12‰	5009.7 ± 415.47	5625.3 ± 152.78	5484.9 ± 174.33
20‰	**4148.2 ± 45.619**	5069.2 ± 198.36	5517.2 ± 264.25
40‰	**3434.1 ± 155.77**	**4682.4 ± 287.38**	**4581.8 ± 115.16**
60‰	**3171.9 ± 241.09**	3731.5 ± 893.3	**4402.5 ± 314.48**

Figure 9-38: Unscrambler® correlation between Peak D height in C. officinalis *extracts prepared with sugar/glycerol solvents and chaotropicity of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-32: Peak E height of C. officinalis *extracts prepared with sugar/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Sucrose/glycerol composite*	*Fructose/glycerol composite*	*Glucose/glycerol composite*
0‰	1215 ± 101.41	1215 ± 101.41	1215 ± 101.41
1‰	1195.9 ± 72.871	1189.6 ± 76.069	1223.4 ± 46.014
8‰	1172 ± 81.838	1269.3 ± 49.914	1240.8 ± 35.519
12‰	1137.1 ± 84.885	1284.4 ± 41.608	1233.6 ± 31.463
20‰	**940.09 ± 15.976**	1178.6 ± 43.63	1225.5 ± 61.825
40‰	**785.41 ± 33.053**	1083.6 ± 56.614	**1034.9 ± 17.339**
60‰	**708.55 ± 52.671**	**870.34 ± 201.09**	**999.28 ± 71.83**

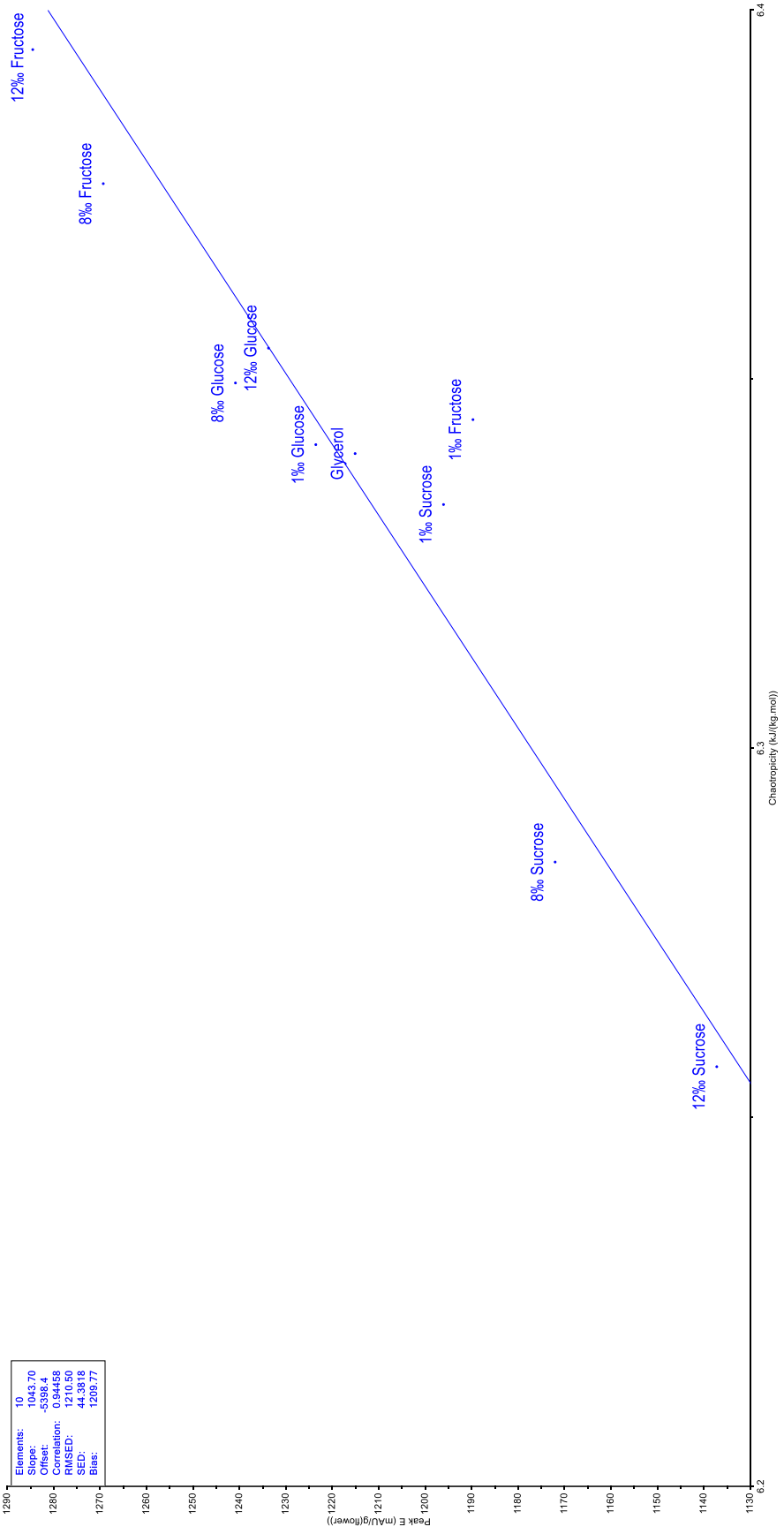


Figure 9-39: Unscrambler® correlation between Peak E height in C. officinalis *extracts prepared with sugar/glycerol solvents and chaotropicity of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-33: Peak F height of C. officinalis *extracts prepared with sugar/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared with neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Sucrose/glycerol composite*	*Fructose/glycerol composite*	*Glucose/glycerol composite*
0‰	35682 ± 2832.9	35682 ± 2832.9	35682 ± 2832.9
1‰	35075 ± 1813.5	35267 ± 2281.4	35315 ± 807.13
8‰	34492 ± 2141	36742 ± 1485	35887 ± 1256.8
12‰	32924 ± 2324.9	37722 ± 1308.8	35875 ± 1029
20‰	**27462 ± 561.63**	34350 ± 1061.4	35745 ± 1151.5
40‰	**22228 ± 943**	**31758 ± 1646.4**	**30593 ± 625.01**
60‰	**19743 ± 1374.5**	25316 ± 5773.1	**29354 ± 2012.2**

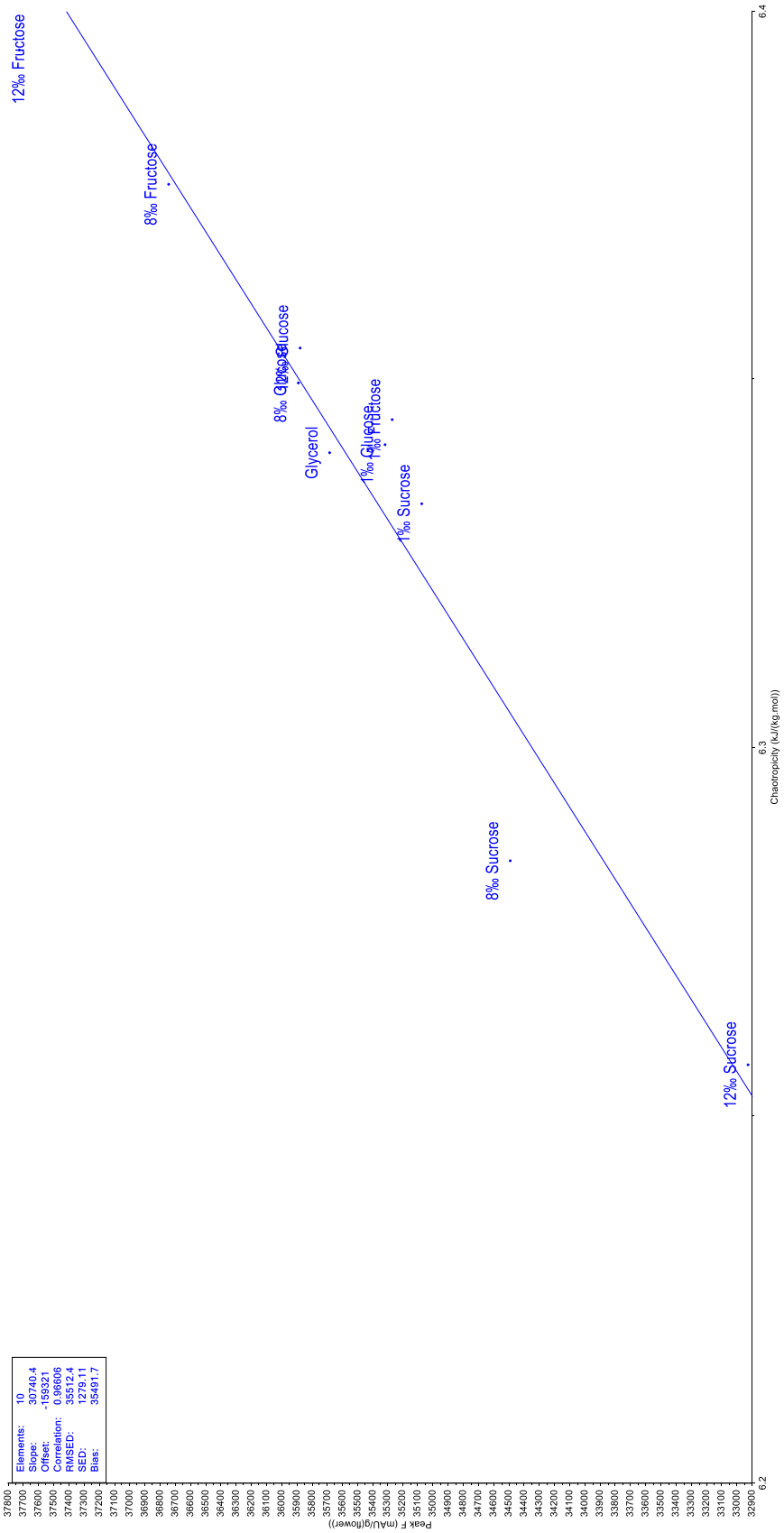


Figure 9-40: Unscrambler® correlation between Peak F height in C. officinalis *extracts prepared with sugar/glycerol solvents and chaotropicity of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-34: Peak G height of C. officinalis *extracts prepared with sugar/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Sucrose/glycerol composite*	*Fructose/glycerol composite*	*Glucose/glycerol composite*
0‰	13260 ± 1108.7	13260 ± 1108.7	13260 ± 1108.7
1‰	13043 ± 752.23	13218 ± 861.28	13319 ± 302.29
8‰	12725 ± 745.49	13821 ± 650.46	13608 ± 515.48
12‰	12333 ± 882.83	14142 ± 440.77	13739 ± 425.17
20‰	**10336 ± 119.48**	12798 ± 402.65	13699 ± 525.96
40‰	**8509.4 ± 325.23**	**11825 ± 663.38**	**11591 ± 300.2**
60‰	**7748.8 ± 565.54**	**9315.7 ± 2147.2**	**11113 ± 765.41**

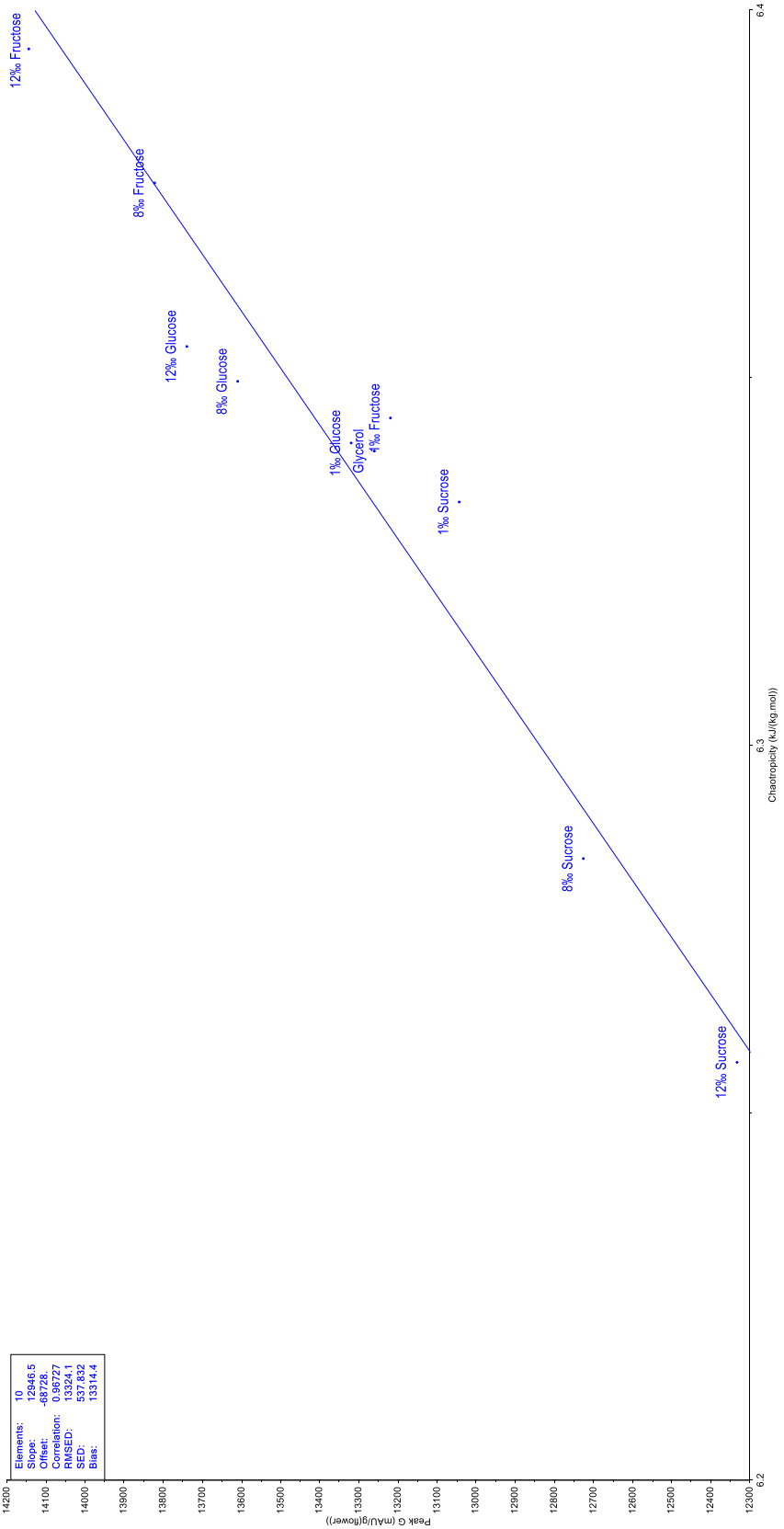


Figure 9-41: Unscrambler® correlation between Peak G height in C. officinalis *extracts prepared with sugar/glycerol solvents and chaotropicity of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-35: Peak H height of C. officinalis *extracts prepared with sugar/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Sucrose/glycerol composite*	*Fructose/glycerol composite*	*Glucose/glycerol composite*
0‰	32858 ± 2860	32858 ± 2860	32858 ± 2860
1‰	31921 ± 1653.7	32031 ± 2047.6	32756 ± 587.22
8‰	31651 ± 2028.5	33646 ± 1122.9	33487 ± 649.24
12‰	30306 ± 2202.7	34727 ± 550.43	33747 ± 1101.8
20‰	25181 ± 459.73	31378 ± 860.2	33069 ± 1355.2
40‰	**20311 ± 914.59**	**28861 ± 1364.1**	**27990 ± 968.47**
60‰	**18019 ± 1270.9**	**22613 ± 5195.6**	**26688 ± 1597.5**

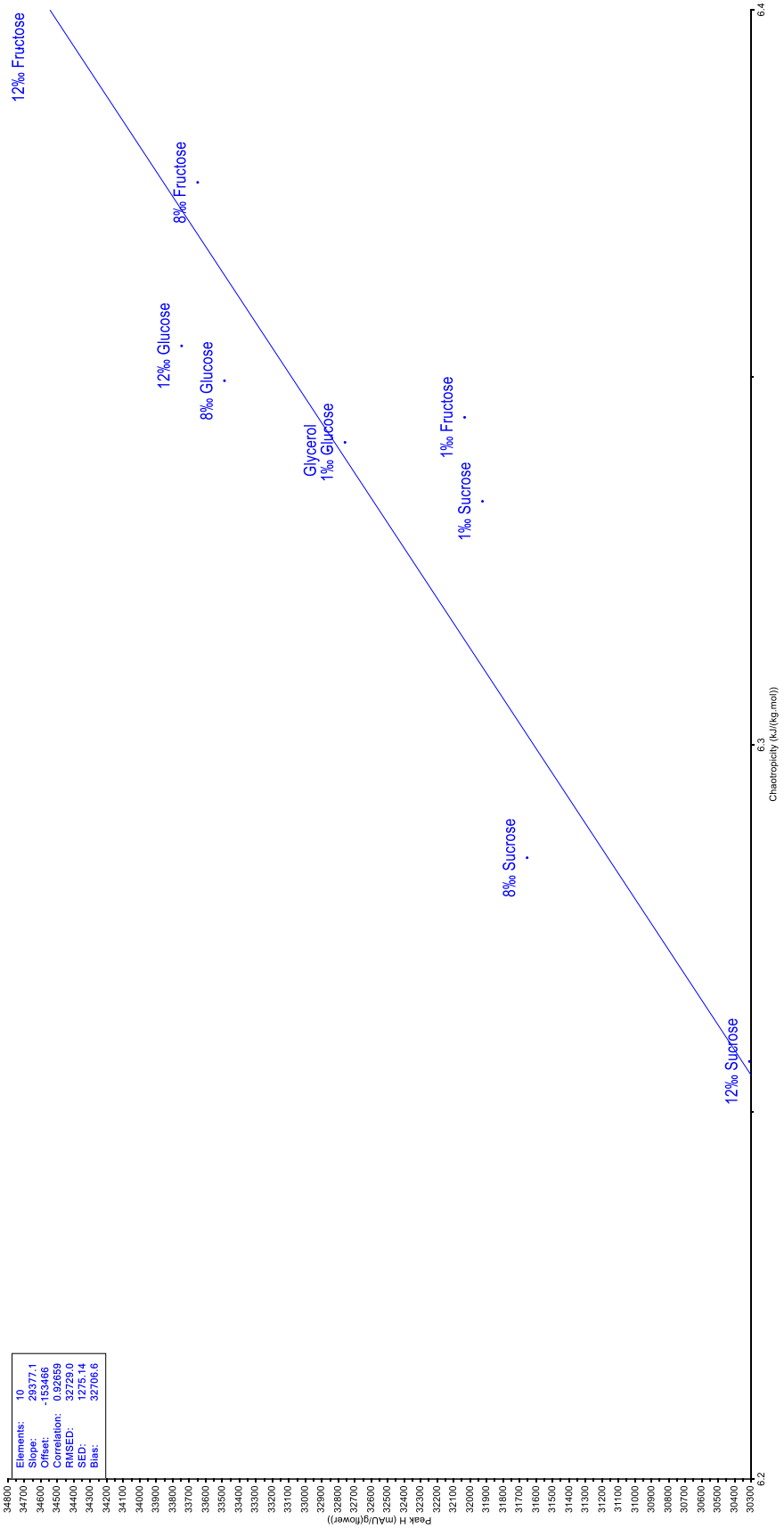


Figure 9-42: Unscrambler® correlation between Peak H height in C. officinalis *extracts prepared with sugar/glycerol solvents and chaotropicity of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-36: Peak I height of C. officinalis *extracts prepared with sugar/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared with neat glycerol. Units are mAU.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Sucrose/glycerol composite*	*Fructose/glycerol composite*	*Glucose/glycerol composite*
0‰	1886.4 ± 151.83	1886.4 ± 151.83	1886.4 ± 151.83
1‰	1880.6 ± 112.78	1824.7 ± 118.36	1834.9 ± 48.474
8‰	1810.7 ± 103.28	1906.3 ± 112.8	1870 ± 46.306
12‰	1755.1 ± 142.08	1954.3 ± 44.371	1860.7 ± 48.566
20‰	**1467.9 ± 21.92**	1778.8 ± 68.941	1841.8 ± 69.794
40‰	**1221 ± 39.302**	**1643.3 ± 108.58**	**1584.9 ± 36.224**
60‰	**1104.2 ± 82.381**	**1301.9 ± 298.93**	**1507.9 ± 82.855**

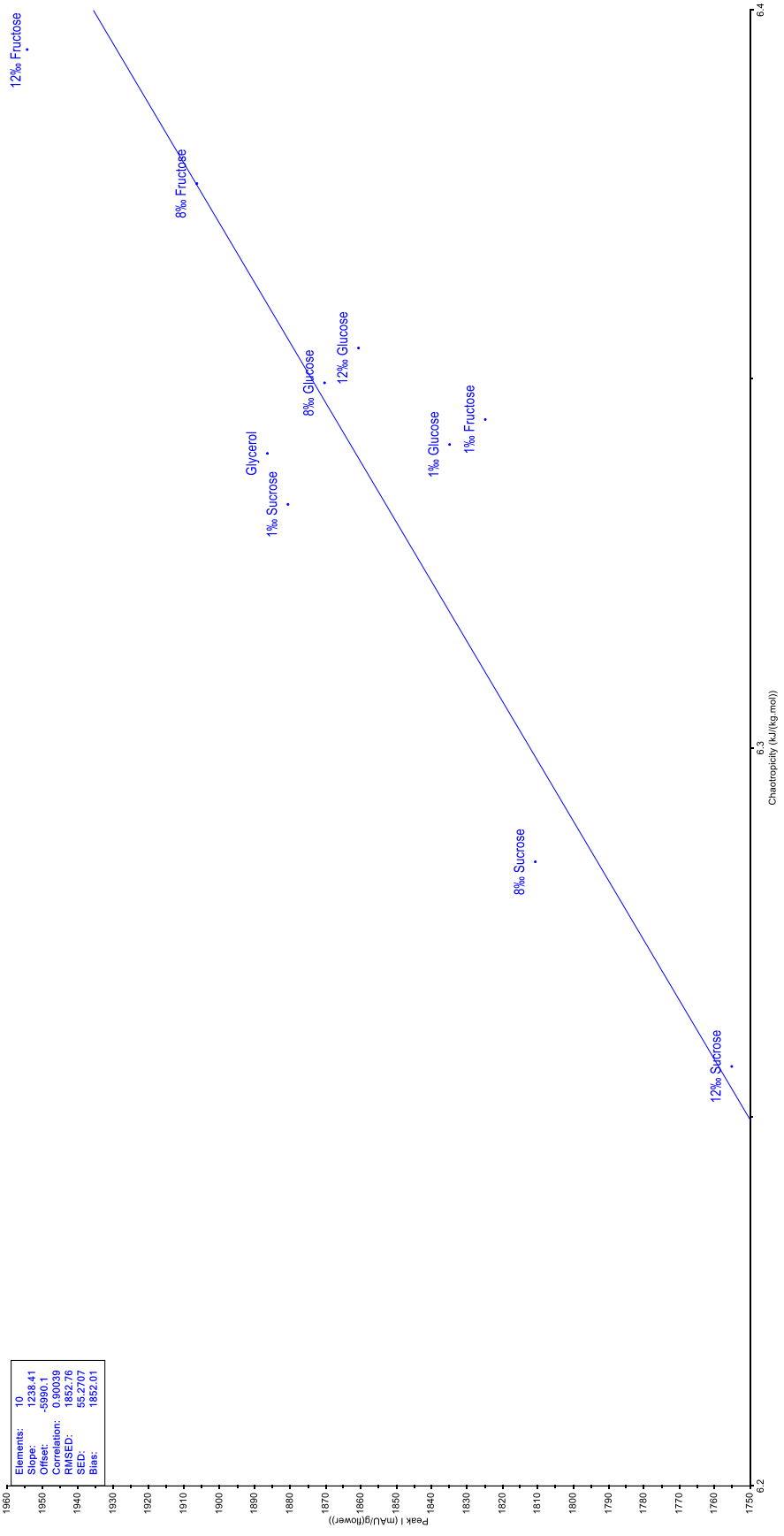


Figure 9-43: Unscrambler® correlation between Peak I height in C. officinalis *extracts prepared with sugar/glycerol solvents and chaotropicity of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-37: Total peak area of C. officinalis *extracts prepared with sugar/glycerol composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared with neat glycerol. Units are mAU.s.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	Sucrose/glycerol composite	Fructose/glycerol composite	Glucose/glycerol composite
0‰	12156 ± 1065.2	12156 ± 1065.2	12156 ± 12156
1‰	11607 ± 183.03	11335 ± 423.23	12440 ± 395.34
8‰	11124 ± 389.14	13103 ± 363.58	12705 ± 286.11
12‰	11238 ± 639.33	13253 ± 278.42	12994 ± 398.63
20‰	**9213.6 ± 267.22**	11731 ± 571.26	12717 ± 411.26
40‰	**7727 ± 293.24**	**10636 ± 346.43**	**10549 ± 429.13**
60‰	**6630.3 ± 330.44**	**8344.9 ± 1920.7**	**9828.7 ± 631.38**

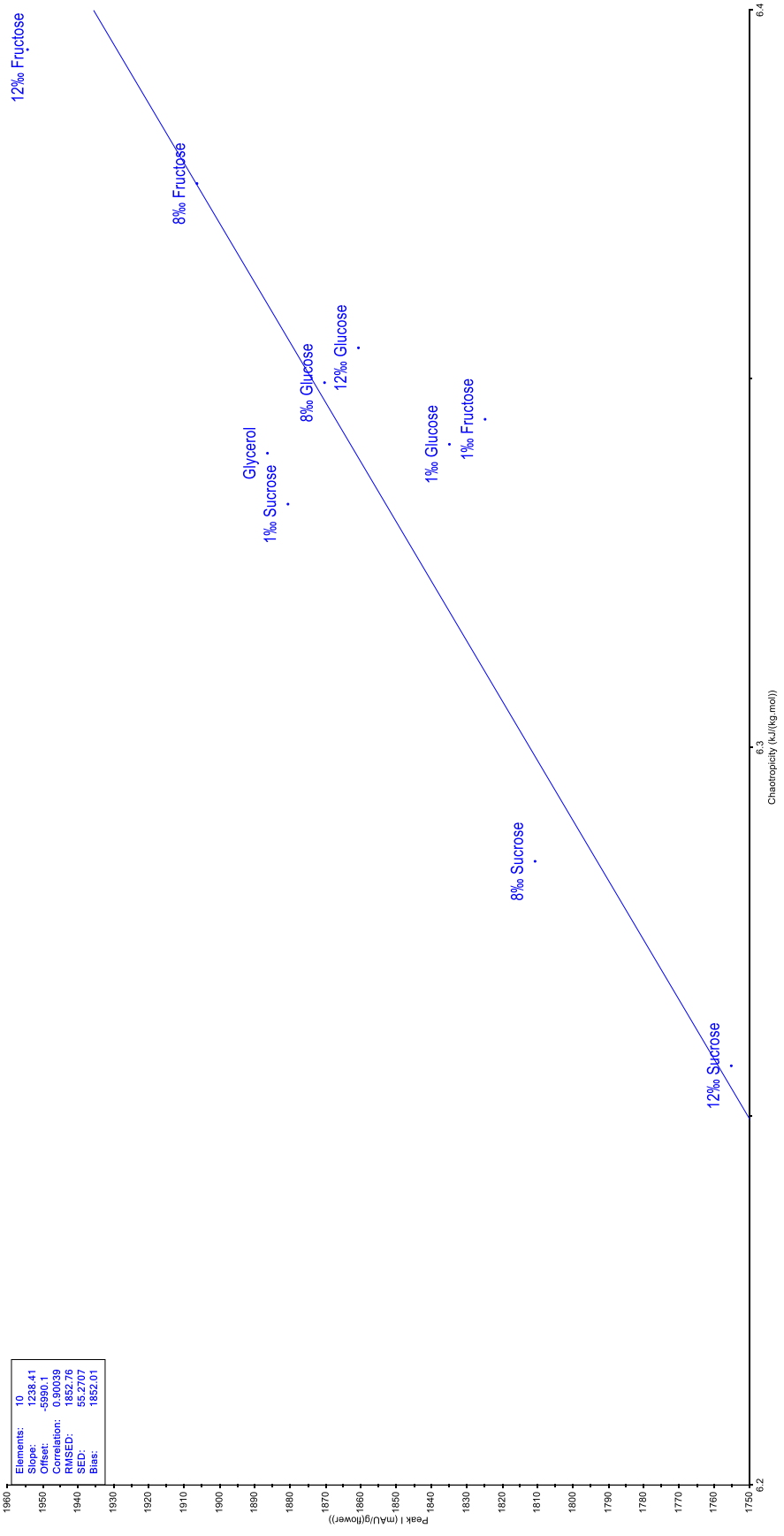


Figure 9-44: Unscrambler® correlation between total peak area in C. officinalis *extracts prepared with sugar/glycerol solvents and chaotropicity of the solvent. Data is expressed as means of replicates (n=3).*

Table 9-38: Chemiluminescence response of C. officinalis *extracts prepared with sugar/glycerol composites. Data is presented as means ± standard deviations of replicates (n=9). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared with neat glycerol. Units are mV.s.g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Sucrose/glycerol composite*	*Fructose/glycerol composite*	*Glucose/glycerol composite*
0‰	29133 ± 4924.2	29133 ± 4924.2	29133 ± 4924.2
1‰	29897 ± 3692.5	28875 ± 4250.3	35024 ± 1242.3
8‰	**22416 ± 4121.1**	33610 ± 2288.7	**38403 ± 3365.6**
12‰	**21147 ± 3453.7**	31884 ± 3156.4	**38697 ± 2120.4**
20‰	**20757 ± 2651.7**	26743 ± 3804.2	**37121 ± 5140.3**
40‰	**18287 ± 3451**	23342 ± 1315.8	26870 ± 788.69
60‰	**13131 ± 3278.6**	**17991 ± 4780.4**	24297 ± 3373.6

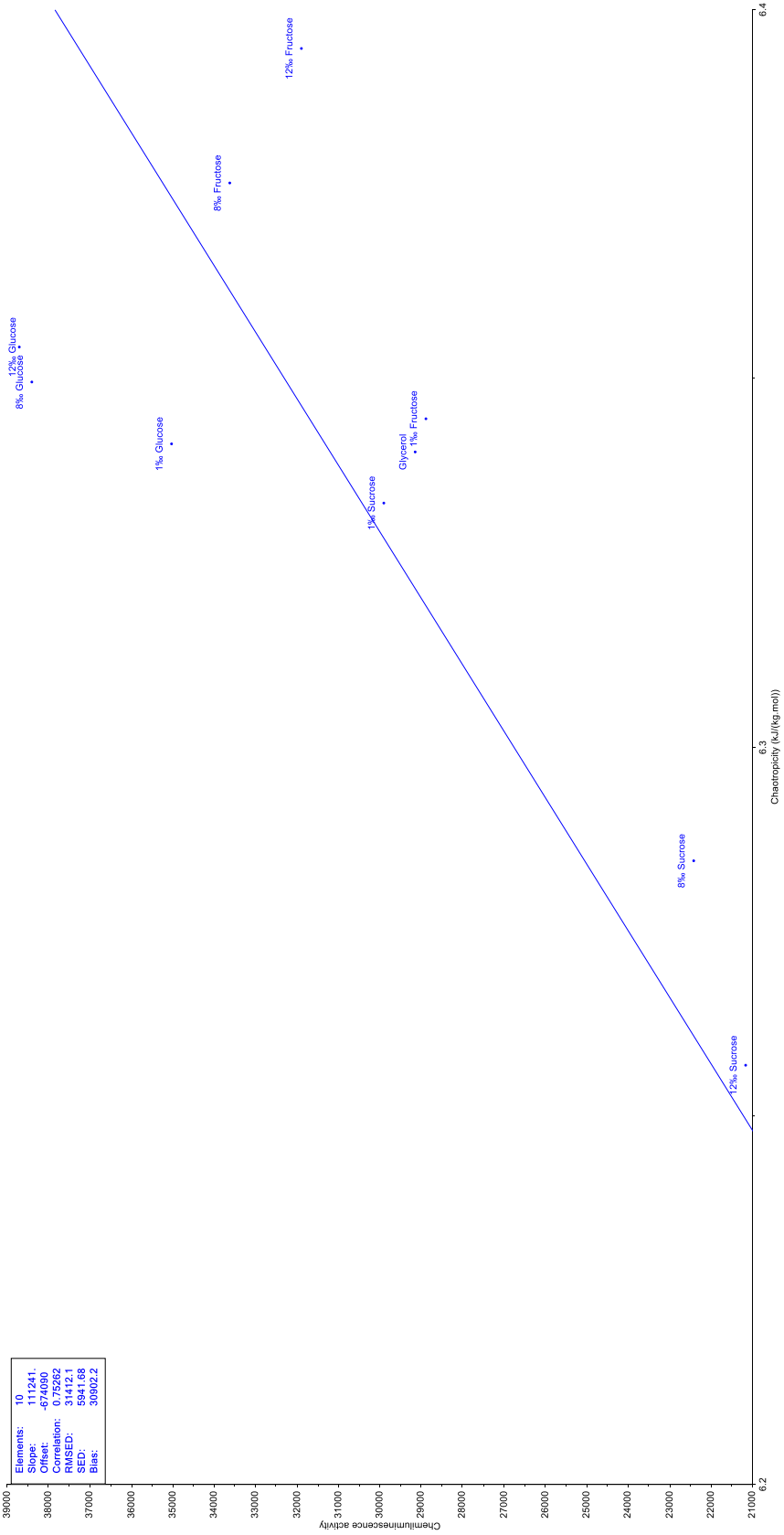


Figure 9-45: Unscrambler® correlation between chemiluminescence response in C. officinalis *extracts prepared with sugar/glycerol solvents and chaotropicity of the solvent. Data is expressed as means of replicates (n=9).*

Table 9-39: DPPH radical scavenging activity of C. officinalis *extracts prepared with sugar/glycerol composites. Data is presented as means ± standard deviations of replicates (n=9). Bold values indicate statistical significance ($p<0.05$) when compared with extracts prepared with neat glycerol. Units are mg(quercetin).g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Sucrose/glycerol composite*	*Fructose/glycerol composite*	*Glucose/glycerol composite*
0‰	0.73533 ± 0.092259	0.73533 ± 0.092259	0.73533 ± 0.092259
1‰,	0.655 ± 0.049432	0.74743 ± 0.034355	0.79404 ± 0.050248
8‰,	**0.64531 ± 0.029999**	0.79136 ± 0.047365	0.81117 ± 0.048595
12‰,	0.61313 ± 0.031308	0.79238 ± 0.035527	0.84262 ± 0.07027
20‰,	**0.57337 ± 0.027124**	0.67756 ± 0.031885	0.78321 ± 0.055782
40‰,	**0.44263 ± 0.018621**	0.62345 ± 0.025218	**0.64419 ± 0.033905**
60‰,	**0.37826 ± 0.016915**	**0.48234 ± 0.019832**	**0.59702 ± 0.033271**

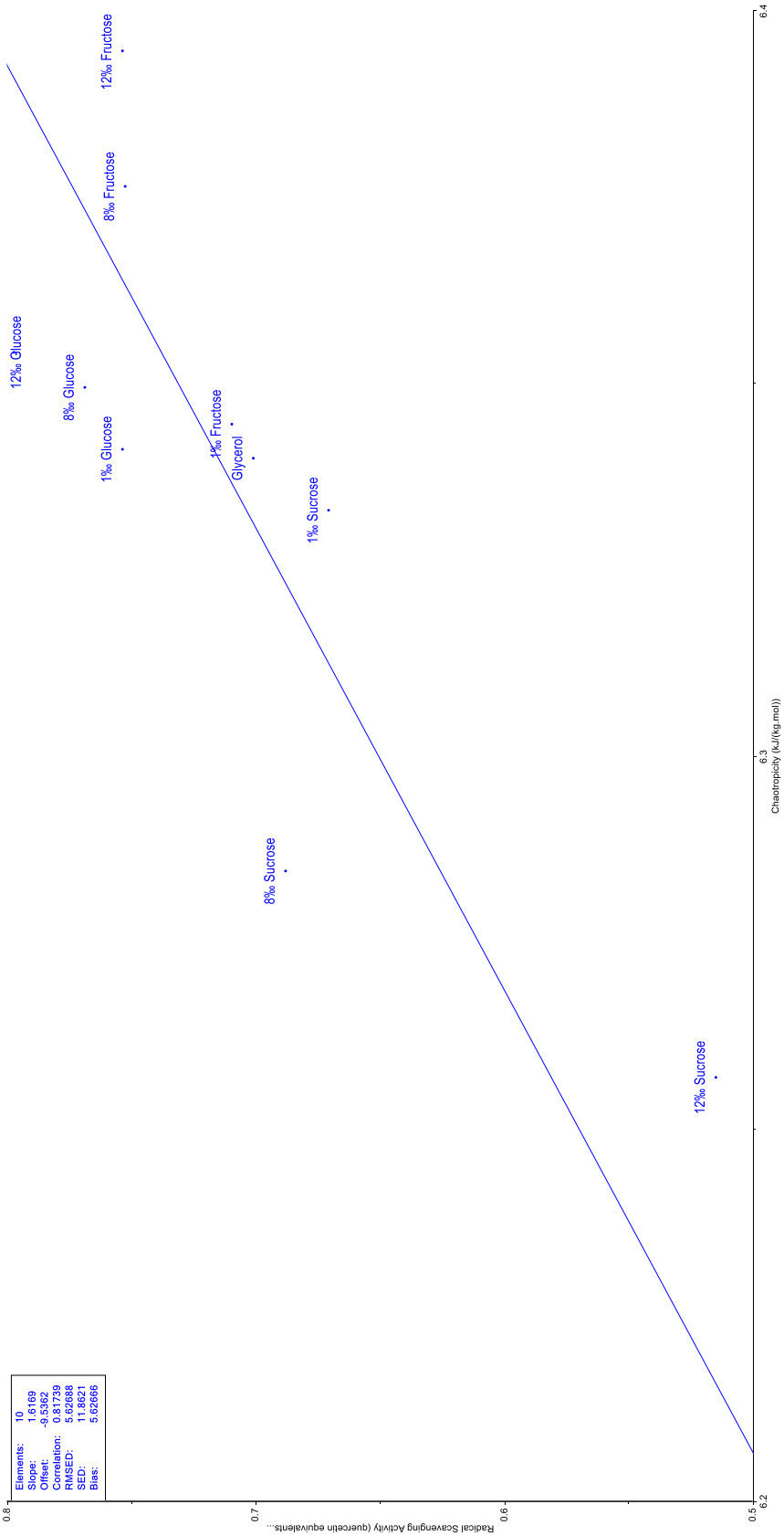


Figure 9-46: Unscrambler® correlation between DPPH radical scavenging activity in C. officinalis *extracts prepared with sugar/glycerol solvents and chaotropicity of the solvent. Data is expressed as means of replicates (n=9).*

Table 9-40: Phenolic content of C. officinalis *extracts prepared with acid/glycerol and sugar/glycerol composites. Data is presented as means ± standard deviation of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol. Units are mg(GAE).g(flower)$^{-1}$.*

Acid Concentration (mol/mol)	*Sucrose/glycerol composite*	*Fructose/glycerol composite*	*Glucose/glycerol composite*
0‰	1.8289 ± 0.12883	1.8289 ± 0.12883	1.8289 ± 0.12883
1‰	1.8003 ± 0.059767	**1.964 ± 0.14286**	1.7907 ± 0.091774
8‰	1.8741 ± 0.091045	**2.3121 ± 0.25449**	**1.9502 ± 0.12347**
12‰	1.7728 ± 0.089417	**2.4046 ± 0.23855**	**1.9748 ± 0.092264**
20‰	**1.5367 ± 0.047104**	**1.9928 ± 0.090434**	**2.0266 ± 0.18285**
40‰	**1.2707 ± 0.033546**	**2.1094 ± 0.11862**	**1.627 ± 0.075973**
60‰	**1.1183 ± 0.06156**	1.656 ± 0.28128	**1.5622 ± 0.096706**

APPENDIX C

Table 9-41: Height of Peak A in C. officinalis *extracts prepared with PS/glycerol and PS/water composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol and water respectively. Units are mAU.g(flower)$^{-1}$.*

PS Concentration (‰ mol/mol)	*PS20/glycerol composites*	*PS80/glycerol composites*	*PS20/water composites*	*PS80/water composites*
0‰	24420 ± 5016.2	24420 ± 5016.2	72567 ± 9858.3	72567 ± 9858.3
1‰	**32812 ± 2227.7**	20237 ± 905.59	60036 ± 1011.3	63716 ± 11563
8‰	31680 ± 2131.1	21765 ± 1200.6	60331 ± 4463.2	60531 ± 5141.8
12‰	**32859 ± 2216.9**	25044 ± 1336.5	70762 ± 5650.8	60502 ± 2264.2
20‰	29117 ± 250.22	22019 ± 386.21	39426 ± 22528	

Table 9-42: Height of Peak B in C. officinalis *extracts prepared with PS/glycerol and PS/water composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol and water respectively. Units are mAU.g(flower)$^{-1}$.*

PS Concentration (‰ mol/mol)	*PS20/glycerol composites*	*PS80/glycerol composites*	*PS20/water composites*	*PS80/water composites*
0‰	997.19 ± 65.722	997.19 ± 65.722	4138.7 ± 519.91	4138.7 ± 519.91
1‰	1286.2 ± 131.27	993.85 ± 28.566	3858 ± 96.739	4191.1 ± 881.06
8‰	**1423.1 ± 97.617**	1118 ± 90.878	4875.6 ± 435.9	4039.3 ± 122.53
12‰	**1696 ± 114.28**	**1235.9 ± 102.99**	**5412.7 ± 162.73**	3797.5 ± 335.7
20‰	**1369.3 ± 22.096**	**1268.1 ± 114.29**	3462.9 ± 2262.5	

Table 9-43: Height of Peak C in C. officinalis *extracts prepared with PS/glycerol and PS/water composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol and water respectively. Units are mAU.g(flower)$^{-1}$.*

PS Concentration (‰ mol/mol)	PS20/glycerol composites	PS80/glycerol composites	PS20/water composites	PS80/water composites
0‰	2362.2 ± 98.359	2362.2 ± 98.359	4126.9 ± 470.64	4126.9 ± 470.64
1‰	2567.5 ± 177.28	2449.2 ± 66.975	4591.4 ± 183.67	4609 ± 738.67
8‰	**2701.7 ± 22.994**	2453.4 ± 126.34	4505.1 ± 343.22	4452.1 ± 191.08
12‰	**2911.6 ± 212.92**	**2555.9 ± 68.888**	4454.7 ± 326.78	4119.4 ± 438.08
20‰	2382.4 ± 92.105	2446.5 ± 104.9	2802.3 ± 1558.6	

Table 9-44: Height of Peak D in C. officinalis *extracts prepared with PS/glycerol and PS/water composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol and water respectively. Units are mAU.g(flower)$^{-1}$.*

PS Concentration (‰ mol/mol)	PS20/glycerol composites	PS80/glycerol composites	PS20/water composites	PS80/water composites
0‰	3340.6 ± 154.38	3340.6 ± 154.38	5346.2 ± 520.17	5346.2 ± 520.17
1‰	**3833.5 ± 278.86**	3559.8 ± 123.95	**7084.8 ± 366.15**	6901.5 ± 1169.2
8‰	**4153.8 ± 59.319**	**3663.3 ± 178.49**	**6877.6 ± 437.61**	**6785.2 ± 260.09**
12‰	**4438.6 ± 270.28**	**3975.8 ± 68.594**	**6994 ± 425.6**	6191.4 ± 674.02
20‰	**3637.1 ± 124.49**	**3744 ± 67.304**	4220.3 ± 2235.2	

Table 9-45: Height of Peak E in C. officinalis *extracts prepared with PS/glycerol and PS/water composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol and water respectively. Units are mAU.g(flower)$^{-1}$.*

PS Concentration (‰ mol/mol)	PS20/glycerol composites	PS80/glycerol composites	PS20/water composites	PS80/water composites
0‰	839.55 ± 67.368	839.55 ± 67.368	1051.4 ± 132.49	1051.4 ± 132.49
1‰	896.08 ± 74.562	**949.73 ± 24.54**	**1596.1 ± 225.39**	**1720.4 ± 288.71**
8‰	**1005.2 ± 55.895**	**984.41 ± 37.102**	**1552.4 ± 117.71**	**1688.6 ± 111.3**
12‰	**1087.3 ± 64.434**	**1064.5 ± 19.56**	**1503.4 ± 121.69**	1145.9 ± 173.2
20‰	889.75 ± 26.672	**1019.5 ± 27.317**	864.67 ± 505.05	

Table 9-46: Height of Peak F in C. officinalis *extracts prepared with PS/glycerol and PS/water composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol and water respectively. Units are mAU.g(flower)$^{-1}$.*

PS Concentration (‰ mol/mol)	PS20/glycerol composites	PS80/glycerol composites	PS20/water composites	PS80/water composites
0‰	23410 ± 926.32	23410 ± 926.32	37545 ± 4971.9	37545 ± 4971.9
1‰	25530 ± 2068.8	24483 ± 559.89	40966 ± 2822.5	42278 ± 6490.9
8‰	**26514 ± 644.72**	24538 ± 1132.2	39564 ± 3109	41017 ± 2134.9
12‰	**28628 ± 1589.9**	**26486 ± 634.94**	39170 ± 2332.6	37364 ± 4502.9
20‰	23224 ± 1134	**25071 ± 887.66**	24875 ± 13079	

Table 9-47: Height of Peak G in C. officinalis *extracts prepared with PS/glycerol and PS/water composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol and water respectively. Units are mAU.g(flower)$^{-1}$.*

PS Concentration (‰ mol/mol)	PS20/glycerol composites	PS80/glycerol composites	PS20/water composites	PS80/water composites
0‰	8410.8 ± 345.14	8410.8 ± 345.14	13039 ± 1540.6	13039 ± 1540.6
1‰	**9624.3 ± 731.66**	8804.3 ± 273.19	**16285 ± 1088.5**	16169 ± 2655.1
8‰	**10578 ± 322.95**	**9155.1 ± 432.7**	15474 ± 929.64	**16168 ± 836.52**
12‰	**11481 ± 687.04**	**9921.4 ± 169.96**	15520 ± 1073.5	14858 ± 1742.1
20‰	**9293.8 ± 438.13**	**9499.2 ± 332.16**	9788.1 ± 5171.9	

Table 9-48: Height of Peak H in C. officinalis *extracts prepared with PS/glycerol and PS/water composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol and water respectively. Units are mAU.g(flower)$^{-1}$*

PS Concentration (‰ mol/mol)	PS20/glycerol composites	PS80/glycerol composites	PS20/water composites	PS80/water composites
0‰	23157 ± 825.35	23157 ± 825.35	28677 ± 4516.2	28677 ± 4516.2
1‰	27310 ± 2754.8	24582 ± 1005.1	**38918 ± 4026.8**	40522 ± 6156.9
8‰	**31024 ± 2193.3**	25797 ± 1098.9	**40226 ± 2914.3**	**41050 ± 3465.5**
12‰	**33873 ± 1641.7**	**29119 ± 527.07**	**40795 ± 2000.6**	**40048 ± 5037.1**
20‰	**27615 ± 1049.5**	**28305 ± 634.48**	26228 ± 14620	

Table 9-49: Height of Peak I in C. officinalis *extracts prepared with PS/glycerol and PS/water composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol and water respectively. Units are mAU.g(flower)$^{-1}$*

PS Concentration (‰ mol/mol)	PS20/glycerol composites	PS80/glycerol composites	PS20/water composites	PS80/water composites
0‰	1229 ± 72.624	1229 ± 72.624	1659.9 ± 218.91	1659.9 ± 218.91
1‰	**1473.7 ± 157.35**	1283.2 ± 23.852	**2535.6 ± 194.53**	**2560.7 ± 413.15**
8‰	**1638.1 ± 95.757**	**1384.4 ± 63.448**	**2535 ± 70.786**	**2528.7 ± 177.76**
12‰	**1754 ± 78.306**	**1465.3 ± 50.13**	**2479.1 ± 120.57**	**2374.2 ± 344.65**
20‰	**1429.4 ± 38.059**	**1432.4 ± 33.248**	1544.3 ± 845.1	

Table 9-50: Total peak areas of C. officinalis *extracts prepared with PS/glycerol and PS/water composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol and water respectively. Units are mAU.s.g(flower)$^{-1}$*

PS Concentration (‰ mol/mol)	PS20/glycerol composites	PS80/glycerol composites	PS20/water composites	PS80/water composites
0‰	7888.7 ± 267.81	7888.7 ± 267.81	15848 ± 1379.4	15848 ± 1379.4
1‰	**9197.7 ± 699.11**	8302.4 ± 234.04	17166 ± 1114.3	19013 ± 3047.1
8‰	**9880.6 ± 509.71**	**8692.9 ± 446.9**	18651 ± 1351.5	17934 ± 609.5
12‰	**10798 ± 534.37**	**9231.3 ± 41.863**	**18952 ± 675.09**	15965 ± 1700.9
20‰	**9015.1 ± 264.94**	**8825.7 ± 294.98**	11394 ± 6415.7	

Table 9-51: Chemiluminescence response of C. officinalis *extracts prepared with PS/glycerol and PS/water composites. Data is presented as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol and water respectively. Units are mV.s.g(flower)$^{-1}$.*

PS Concentration (‰ mol/mol)	*PS20/glycerol composites*	*PS80/glycerol composites*	*PS20/water composites*	*PS80/water composites*
0‰	8924.5 ± 1700	8924.5 ± 1700	23265 ± 2972.8	23265 ± 2972.8
1‰	**11920 ± 3285.9**	**13385 ± 394.53**	**33268 ± 3285.9**	27419 ± 4954.3
8‰	**12585 ± 2636.3**	**12391 ± 284.94**	23716 ± 4055.9	**31900 ± 2144.4**
12‰	**13058 ± 2619.6**	**16736 ± 299.8**	27400 ± 1903.5	**30250 ± 2812.6**
20‰	**16224 ± 9055.5**	**13511 ± 1329.9**	13757 ± 9055.5	

Table 9-52: Radical scavenging activity of C. officinalis *extracts prepared with PS20/glycerol and PS80/glycerol composites. Data is presented as means ± standard deviations of replicates (n=9). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol and water respectively. Units are mg(quercetin).g(flower)$^{-1}$.*

PS Concentration (‰ mol/mol)	*PS20/glycerol composites*	*PS80/glycerol composites*	*PS20/water composites*	*PS80/water composites*
0‰	5149.6 ± 503.39	5149.6 ± 503.39	4662.4 ± 874.87	4662.4 ± 874.87
1‰	4904.9 ± 395.68	**5852.8 ± 267.81**	4632.5 ± 352.15	4898.5 ± 877.52
8‰	**5560.8 ± 402.92**	**6006.8 ± 523.42**	**5212.9 ± 459.73**	**5280.7 ± 280.69**
12‰	**5771.5 ± 453.97**	**5671.8 ± 475.34**	**5454.8 ± 429.98**	**5537.1 ± 275.2**
20‰	**4690.7 ± 321.02**	**5874.7 ± 753.16**	4691.5 ± 310.45	

Table 9-53: Phenolic content of C. officinalis *extracts prepared with PS composites. Data is presented as means ± standard deviations of replicates (n=9). Bold values indicate statistical significance (p<0.05) when compared with extracts prepared with neat glycerol and water respectively. Units are mg(GAE).g(flower)$^{-1}$*

PS Concentration (‰ mol/mol)	*PS20/glycerol composites*	*PS80/glycerol composites*	*PS20/water composites*	*PS80/water composites*
0‰	1.3442 ± 0.063658	1.3442 ± 0.063658	2.5364 ± 0.18121	2.5364 ± 0.18121
1‰	**1.571 ± 0.090016**	**1.4349 ± 0.055279**	**3.1548 ± 0.19318**	**3.5187 ± 0.58624**
8‰	**1.6083 ± 0.12044**	**1.8501 ± 0.089376**	**4.172 ± 0.38347**	**3.9349 ± 0.17851**
12‰	**1.9249 ± 0.12359**	**2.0087 ± 0.065121**	**4.3063 ± 0.27725**	**3.5363 ± 0.23934**
20‰	**1.7805 ± 0.098424**	**1.8989 ± 0.066533**	2.5304 ± 1.2038	

APPENDIX D

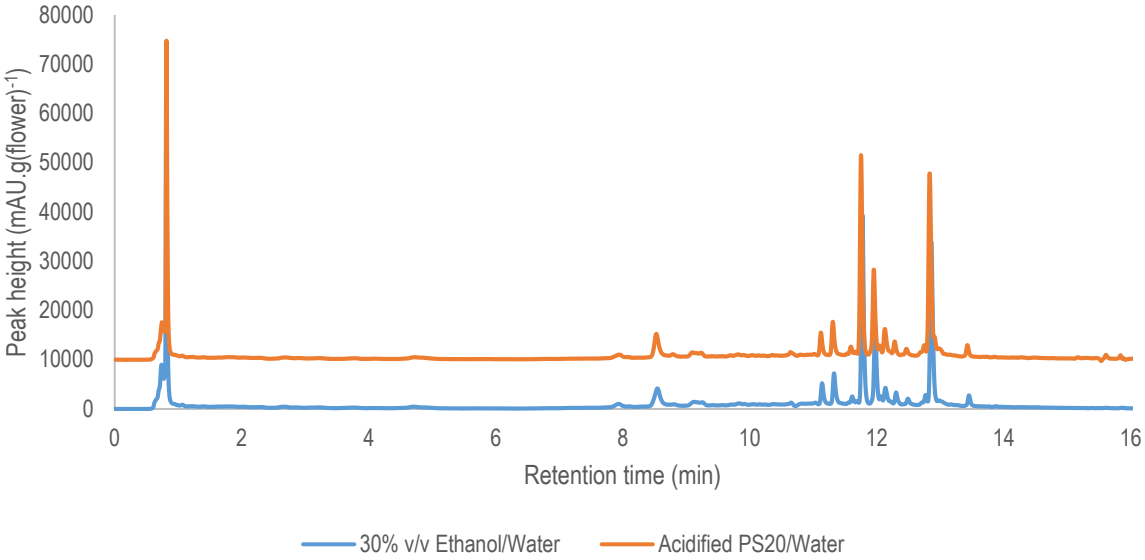


Figure 9-47: UHPLC chromatograms of macerated C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Units are mAU.g(flower)$^{-1}$. Sequential chromatograms are offset by 10,000 mAU.*

Table 9-54: Height of Peak A in ultrasonic and macerated C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is expressed as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with a hydroethanolic extract prepared under similar conditions. Units are mAU.g(flower)$^{-1}$.*

Solvent	*Ultrasonic extraction*	*Maceration extraction*
Ethanol/water	4406.6 ± 391.41	6126.6 ± 386.15
AAP	**6773.1 ± 174.35**	5473 ± 520.51

Table 9-55: Height of Peak B in ultrasonic and macerated C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is expressed as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with a hydroethanolic extract prepared under similar conditions. Units are mAU.g(flower)$^{-1}$.*

Solvent	*Ultrasonic extraction*	*Maceration extraction*
Ethanol/water	4144.9 ± 329.18	3584.3 ± 206.44
AAP	**5363.2 ± 482.95**	**4498.9 ± 153.97**

Table 9-56: Height of Peak C in ultrasonic and macerated C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is expressed as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with a hydroethanolic extract prepared under similar conditions. Units are mAU.g(flower)$^{-1}$.*

Solvent	*Ultrasonic extraction*	*Maceration extraction*
Ethanol/water	4598.7 ± 155.99	4385.4 ± 442.34
AAP	4826.7 ± 53.046	4112.8 ± 395.42

Table 9-57: Height of Peak D in ultrasonic and macerated C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is expressed as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with a hydroethanolic extract prepared under similar conditions. Units are mAU.g(flower)$^{-1}$.*

Solvent	*Ultrasonic extraction*	*Maceration extraction*
Ethanol/water	6810.3 ± 133.41	6431 ± 561.47
AAP	**7420.1 ± 154.17**	6181.9 ± 496.28

Table 9-58: Height of Peak E in ultrasonic and macerated C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is expressed as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with a hydroethanolic extract prepared under similar conditions. Units are mAU.g(flower)$^{-1}$.*

Solvent	*Ultrasonic extraction*	*Maceration extraction*
Ethanol/water	40570 ± 1011	39237 ± 3040.5
AAP	40924 ± 593.51	36818 ± 3062.5

Table 9-59: Height of Peak F in ultrasonic and macerated C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is expressed as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with a hydroethanolic extract prepared under similar conditions. Units are mAU.g(flower)$^{-1}$.*

Solvent	*Ultrasonic extraction*	*Maceration extraction*
Ethanol/water	16510 ± 263.66	15241 ± 1273.3
AAP	**17342 ± 162.2**	14670 ± 1133.9

Table 9-60: Height of Peak G in ultrasonic and macerated C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is expressed as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with a hydroethanolic extract prepared under similar conditions. Units are mAU.g(flower)$^{-1}$.*

Solvent	*Ultrasonic extraction*	*Maceration extraction*
Ethanol/water	35636 ± 213.99	34056 ± 1803.7
AAP	**34380 ± 369.73**	31722 ± 2630.9

Table 9-61: Height of Peak H in ultrasonic and macerated C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is expressed as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with a hydroethanolic extract prepared under similar conditions. Units are mAU.g(flower)$^{-1}$.*

Solvent	*Ultrasonic extraction*	*Maceration extraction*
Ethanol/water	2432.2 ± 56.352	2196.8 ± 173.89
AAP	2560.5 ± 147.1	2155.5 ± 164.71

Table 9-62: Height of Peak I in ultrasonic and macerated C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is expressed as means ± standard deviations of replicates (n=3). Bold values indicate statistical significance (p<0.05) when compared with a hydroethanolic extract prepared under similar conditions. Units are mAU.g(flower)$^{-1}$.*

Solvent	*Ultrasonic extraction*	*Maceration extraction*
Ethanol/water	66.536 ± 3.7316	52.738 ± 4.4233
AAP	**2656.4 ± 239.84**	**609.48 ± 61.135**

Table 9-63: Total phenolic content in ultrasonic and macerated C. officinalis *extracts prepared with hydroethanolic and AAP solvents. Data is expressed as means ± standard deviations of replicates (n=9). Bold values indicate statistical significance (p<0.05) when compared with a hydroethanolic extract prepared under similar conditions. Units are mAU.g(flower)$^{-1}$.*

Solvent	*Ultrasonic extraction*	*Maceration extraction*
Ethanol/water	2.632 ± 0.14468	3.0494 ± 0.12394
AAP	**3.1104 ± 0.17313**	**3.8824 ± 0.17148**

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