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Enrichment of Biologically Active Compounds from Selected Plants Using Adsorptive Bubble Separation

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Dedicated to the memory of my late father, for instilling in me the desire to learn and the praise for the good values of Life

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List of Abbreviations

a	Activity
AAc.	Acetic Acid
ABS	Alkyl Benzene Sulfonate
ABSM	Adsorptive Bubble Separation Methods
ACN	Acetonitrile
α	Alfa
β	Beta
$C_{12}E_7$	Heptaethyleneglycol Dodecyl Ether
c.m.c.	Critical Micelle Concentration
C	Catechine
C_B	Concentration in the Bulk
°C	Degree Celsius
C_I	Concentration at the Interface
C_{IS}	Concentration in the Initial Solution
C_{Foam}	Concentration in the Foam
CBD	Cannabidiol
CBN	Cannabinol
Caff.	Caffeine
δ	Chemical Shift
d	Derivate
Δ	Delta
ϕ	Diameter
DAD	Diode Array Detector
DCM	Dichlormethane
E_R	Relative Enrichment Ratio
$\%E_R$	Percent Enrichment Ratio
EC	Epicatechin
ECG	Epicatechin Gallate
ECNI	Electron Capture Negative Ionisation Detector
EGC	Epigallocathecin
EGCG	Epigallocatechin Gallate
EI	Electron Impact Detector
FID	Flame Ionisation Detector

FL	Faradiol Lauritic Acid Ester
FM	Faradiol Myristic Acid Ester
FL	Faradiol Palmitic Acid Ester
FTIR	Fourier Transform Infrared
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
i	Component *i*
K	Konstant
K’	Konstant
λ	Wavelenght
LD_{50}	Letal Dosis per 50% of Tested Animals
ln	Natural Logaritm
MeOH	Methanol
MeOH-d_4	Deuterated Methanol
MS	Mass Spectrometry
m/z	Mass to Charge Ratio
NMR	Nuclear Magnetic Resonance
PB	Plateau Border
pI	Isoeletric Point
RP	Reversed Phase
R	Gas Constant
SDS	Sodium Dodecylsulfate
Γ	Surface Concentration
γ	Surface Tension
Σ	Summe
T	Absolute Temperature
TFAc.	Trifluor Acetic Acid
THC	Tetrahydrocannabinol
TLC	Thin Layer Chromatography
Try.	Tryptanthrin
UV	Ultra Violet
Vis	Visible
V_{IS}	Volume of Initial Solution
V_{Foam}	Volume of Foam

1. Introduction

Adsorptive Bubble Separation Methods (ABSM) are among the less familiar separation methods. The principle of ABSM is based on differences in properties of the materials to be separated. Manifestations of the methods can be found in the nature: in sea foam and bubbling marshes. Foam fractionation is a separation method belonging to the ABSM. In foam fractionation, dissolved material is selectively adsorbed on the surface of rising bubbles and then is partially segregated by the foam. Among human endeavours, an early example of this process is the pouring of beer. Certain components of beer can concentrate in the foam in a sufficient degree to alter the flavour of that foam (Karger *et al.*, 1967; Lemlich, 1972a; Lemlich, 1972b).

The importance of foams in the industry and in the technical field was known long before the formulation of a precise scientific analysis. In 1838, F. M. Ascherson presented a treatise to the Scientific Academy of Paris, dealing with protein brines ability to form protein layers at gas-liquid interfaces. The principle of utilising the foaming capacity of certain fluids to achieve separation or concentration of their components and purification of one component is not new (Ostwald and Mischke, 1939). Ostwald submitted in 1918 a patent for registration on "Process for the Vaporization of Fluids with the Purpose of Enrichment, Separation or Drying of Dissolved or Emulsified Materials Through Foam Fractionation ..." which was issued by the German Federal Patent Office on the 16th of October 1920 (Ostwald, 1920).

Several factors have been experimentally investigated for their influence on foam fractionation and include basic variables with influence on the process of foam fractionation. Examples are the works of several authors. Ostwald and Siehr (1936, 1937) and Ostwald and Mischke (1940) proposed certain equipment designs, while Rubin and Gaden (1962) and Lemlich (1968a, 1972b) made a comprehensive review of the modes of operation for the equipments. Maas (1974) as well as Karger and DeVivo (1968) described the importance of concentration of the molecule to be separated. Somasundaran (1972), Townsend and Nakai (1983) and Parthasarathy *et al.* (1988), outlined the importance of hydrophobicity of the molecule in question. Robertson and Vermeulen (1969) discussed the influence of concentration of surfactant, concentration of auxiliary materials and foam height. Moreover, Ahmad (1975) reported the effect of pH and Kitchener and Cooper (1959) extensively discussed viscosity effects. Kishimoto (1962) disserted on the effect of gas flow rate and Schonfeld and Kibbey (1967) described the effect of reflux ratio.

In the past, foam fractionation has been confined to the concentration of surface active materials from dilute mixtures, in foams formed by the mixtures and thus, providing a large gas-liquid interface in which the surface active materials are selectively adsorbed and carried off by the overflow, or by skimming of the foam. The generation of the foams in such mixtures could be accomplished incidentally by shaking or by the release of dissolved gas, or deliberately by sparging air or other gas at the bottom of the liquid pool (Lemlich, 1968a). The large-scale removal of detergents from sewage and the laboratory separation of proteins are examples of the early use of this technique.

With the commercial introduction in the middle 1940's of synthetic detergents as alkyl benzene sulfonate (ABS), foaming in sewage treatment plants became a problem of major magnitude (Degens, 1954). The detergent caused foaming of sewages, stimulating the use of a foam fractionation process in a continuous pilot plant, for the removal of ABS which provided that 80% of the detergent could be removed from the sewage by foam fractionation (Jenkins *et al.*, 1972). The concentration and purification of proteins, on the basis of their surface properties has been considered for long time. One of the earliest applications was the ingenious use of air to concentrate small amounts of protein in starch wash water from industrial starch manufacturing (Ostwald and Siehr, 1937). This protein was ultimately dried and used for animal feed.

Further developments of foam fractionation refined the technique and enabled the separation of non-surface active solutes. The separation of non-surface active solutes can be accomplished by the addition of a suitable surfactant that either combines with the solute in question or simply co-adsorbs at the bubble surface. Schnepf *et al*. (1959) and Sebba (1962) have described the combination of the surfactant, termed the "collector" with the substance in question, termed the "colligent". Rubin and Gaden (1962) presented an extensive review of substances separated by foam fractionation for example proteins, enzymes, micro-organisms, fatty acids, cellulose esters and dye wastes.

In recent years, as the technology became more sophisticated, researchers have begun re-evaluating many active principles present in plant extracts, using new separation techniques and analytical equipments. Such techniques include solvent extraction, super-critical extraction, liquid and gas membranes separation, as well as foam fractionation (Singh and Singh, 1996). Analytical equipments based on spectroscopic and chromatographic methods are

widely used for both qualitative and quantitative analysis. The most commonly encountered analytical equipments are Ultraviolet and Visible Spectroscopy (UV-Vis), Fourier Transform Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR), Mass spectroscopy (MS), High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC) and Thin Layer Chromatography (TLC), (Penner, 1998; Rounds and Nielsen, 1998).

The active principles contained in plants can be used for alleviating and healing of various diseases and conditions. The importance of plants for the well being of the Mankind was recognized even by ancient civilizations that began to categorize and document plants for medical use. By trial and error, the ancient civilizations acquired the knowledge to determine which plants possessed medical value (Fleischner, 1985). Recently, foam fractionation has been examined from a fundamental point of view. This fundamental approach has been stimulated by a great recognition of the potentialities this technique possess to become an attractive replacement to more costly separation techniques (Lockwood *et al.,* 2000). Foam fractionation shows particular promise for being environmental friendly, energy saving and economical in terms of fixed and running costs means, for removing substances present at low concentrations, from large volumes of liquids (Uraizee, 1990; Noble *et al.*, 1998).

Topic of the Thesis

This Thesis presents the work performed in order to study the prospects of enrichment of active principles from some plants that are of medical importance, using foam fractionation. Foam fractionation is essentially governed by mass transfer phenomena that occurs under flowing conditions and is characterised by diffusion in the bulk of the liquid and adsorption at the gas-liquid interface of the bubbles. Therefore, a successful separation must take into account several parameters and conditions. The separation of materials from one another by foam fractionation lays on their physicochemical properties, equipments and mode of operation used, as well as conditions in which the process occurs. While the physicochemical properties of the materials determine those materials capacity to be separated by foam fractionation, the right choice of types of columns and auxiliary devices used, together with operational conditions, are of paramount importance to achieve an optimised enrichment.

The present work was initiated by an extensive survey of the literature on the plants containing the active principles under investigation and on the process of foam fractionation. The second part concerned the analytical characterisation and extraction of these active principles

with various solvents. The following part concerned the development of methods for the enrichment of the active principles from aqueous dilute solutions prepared from the extracts. For the purpose of method development, several types of fractionation columns were used. In addition, the influence of parameters and conditions of the process were investigated in accordance with the theory available in the field of foam fractionation and further findings and observations made during the experiments. The last part consisted in the analytical quantification of the achieved enrichments.

2. Theoretical Background

The ABSM comprise various methods of separating dissolved or suspended materials, by means of adsorption or attachment at the surface of bubbles rising through the liquid (Lemlich, 1972c). The size of materials separated by the various ABSM range from molecular and some times colloidal in foam fractionation to macroscopic in froth flotation (Lemlich, 1968a). The rising bubbles may or may not form a foam or froth atop the liquid.

Under the generic name of ABSM there is a division in two major categories: foam separation and non-foaming adsorptive bubble separation. These categories have their corresponding subdivisions. A comprehensive description of the main categories and corresponding subdivisions is given in the works from Karger *et al.* (1967), Mahne (1971) and Lemlich (1972a). The main distinction between foam separation and non-foaming adsorptive bubble separation is that the first requires generation of foam or froth to carry off the materials while the latter does not. Both foam separation and non-foaming bubble separation are further subdivided. A schematic representation generally accepted as the nomenclature for ABSM is presented below.

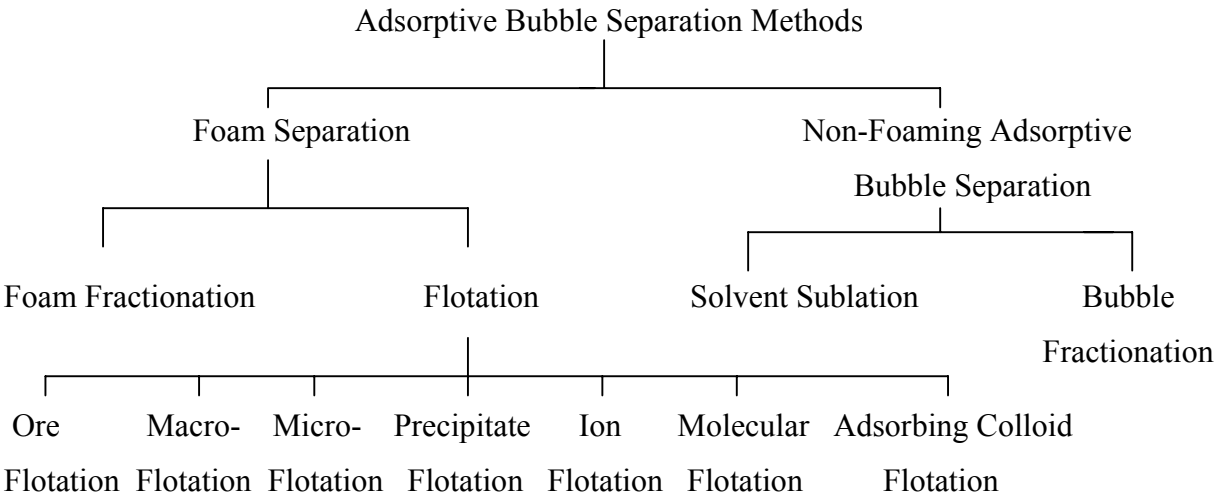


Figure 2.1 Schematic Representation of Adsorptive Bubble Separation Methods (Karger *et al.*, 1967)

2.1 Non-Foaming Adsorptive Bubble Separation and Foam Separation

Non-foaming bubble separation includes two subdivisions. Bubble fractionation and solvent sublation:

- Bubble fractionation (Dorman and Lemlich, 1965) is the adsorption of materials that are molecular or particulate, at the surface of rising bubbles followed by re-deposition at, or just under the surface of the liquid pool.

- Solvent sublation (Sebba, 1962) is the adsorption of materials that are molecular or particulate at the surface of rising bubbles followed by re-deposition at, either the interface of, an immiscible liquid resting atop the main liquid.

Foam separation includes two subdivisions: flotation and foam fractionation. Flotation is the removal of particulate material by foaming. Flotation is further subdivided in ore flotation, macroflotation, microflotation, precipitate flotation, ion flotation, molecular flotation and adsorbing colloid flotation.

- Ore flotation (Gaudin, 1957) is the separation of mineral particles by foaming.
- Macroflotation (Karger *et al.*, 1967) is the separation of macroscopic particles by foaming.
- Microflotation (Dognon and Dumontel, 1931) is the separation of microscopic particles by foaming, especially microorganisms and colloids. The separation of the latter is also termed colloid flotation.
- Precipitate flotation (Baarson and Ray, 1963) is the separation in which a precipitate is formed and separated by foaming, being the precipitating agent other than the surfactant.
- Ion flotation (Sebba, 1962) is the separation of surface inactive ions with a surfactant that is also a collector, in which an insoluble product is formed and removed by foaming as a scum.
- Molecular flotation (Karger *et al.*, 1967) is the separation of surface inactive molecules with a surfactant that is also a collector, in which an insoluble product is formed, by foaming.

- Adsorbing colloid flotation (Mokrushin, 1968) is the separation of dissolved materials by adsorption on colloid particles by foaming.

Finally, foam fractionation, the second subdivision of foam separation, is further being dealt in detail as the main substance of this work, for being the method utilised for the enrichment.

2.2 Foam Fractionation

Foam fractionation is based on the tendency of certain molecules (or colloids) present in highly dilute aqueous solutions, to preferentially adsorb at the large gas-liquid interface of foams, generated by injecting gas bubbles that flow through the solution. In order to explain the phenomena behind foam fractionation, two basic assumptions must be made:

a) Ability of the molecules in question to selectively adsorb at the gas-liquid interface
b) Existence of a large gas-liquid interface for the adsorption

The ability of a molecule to adsorb at a gas-liquid interface is defined by the physicochemical properties of the molecule. The nature of both dilute aqueous solutions and the gas-liquid interface created by such solutions is today common knowledge. Like pure water, dilute aqueous solutions have a polar nature and the gas-liquid interface formed between such solutions and gases is of non-polar nature. The ability of a dissolved molecule to transfer from the bulk of a polar liquid to a non-polar interface, depends on the possession by these molecules of an hydrophobic and an hydrophilic part or for being hydrophobic (Adamson, 1960; Kishimoto, 1962; Lemlich, 1972a, 1972b; Maas, 1974; Townsend and Nakai, 1983; Parthasarathy *et al.*, 1988; Uraizee, 1996). This transfer process is also known as a partition process and is directly connected to the physicochemical properties of the molecule (Lemlich, 1972c, Noble *et al.*, 1998; Nord, 2000).

A large gas-liquid interface provides a large surface for adsorption, which is an important requirement for mass transfer to take place. In foam fractionation, the large surface for adsorption is created with the generation of foam by bubbling a gas through an aqueous dilute solution. The foaming of solutions depends on the presence in solution of surface active solutes which lower the surface tension (Maas, 1974). Foam is a disperse system that contains a

high surface area formed by bubbles that possess a certain life-time (Kitchener and Cooper, 1959). Adsorption of any kind is a time dependent process, consequently, the life-time of the bubbles is an important factor for adsorption.

Adsorption is the phenomena governing the enrichment by foam fractionation. A large variety of factors have an influence on the adsorption and as a result, on the effectiveness of the enrichment. Due to their importance for this work, adsorption and basic variables like concentration of molecule and auxiliary substances, pH value, rheology of solution and operation variables like column geometry, position of gas inlet, mode of operation, bubble size and distribution, coalescence and drainage, gas flow rate and foam height will further be discussed in this chapter.

2.2.1 Adsorption

Foam fractionation is possible due to either the material to be separated or a complex of it with another material, selectively adsorbs at the gas-liquid interface. The principle of the adsorption of materials at the gas-liquid interface is important to understand the theory and mechanisms by which the various materials interact in a dilute solution leading to the separation.

Adsorption of materials at the gas-liquid interface occurs when the interaction among solvent water molecules is greater than among the solute molecules and hence, the existence of solute molecules interfere with the water molecules and their existence is more favourable at the gas-liquid interface than at the bulk of the liquid (Somasundaran, 1972; Charm, 1972). As the size of a hydrophobic molecule, or of the hydrophobic part for surface active molecules with an hydrophilic and an hydrophobic part increases, they interfere with the water molecules to a greater extent, being even less favourable for them to stay in the bulk (Somasundaran, 1972). Therefore, an increase in size causes an increase in adsorption. On the other hand, smaller the hydrophobic part, or the surface active molecule, faster the adsorption. (Tamura *et al.*, 1998).

For organic molecules, the increase of hydrocarbon chain length and presence of halogens in the molecules increases hydrophobicity while the presence of polar groups, heteroatoms as oxygen and nitrogen, ramifications, double and triple bonds decreases the hydrophobicity

(Davies and Riedel, 1963; Wilkinson, 2002). Molecules that only possess a hydrophobic part are non-surface active and their separation by foam requires the careful addition of a surface active material as the presence of such materials in the solution represents a competition for the molecules in question (Somasundaran, 1972; Keller *et al.*, 1997). Another factor that is also detrimental to the adsorption of the desired molecule is the presence of another hydrophobic molecules in solution.

Several theoretical models are available for describing the adsorption of materials at the gas-liquid interface. The most popular, developed by Lemlich (1968a, 1972a) is based on the Gibbs Adsorption Theorem (Gibbs 1876; republished in 1928). The equilibrium adsorption of a dissolved material at the gas-liquid interface is given by Gibbs as the equation:

$$d\gamma = - RT \Sigma\Gamma_i \, d \ln a_i \qquad (2.1)$$

Where:
γ- is the surface tension
R- is the gas constant
T- is the absolute temperature
Γ_i- is the surface concentration of the component *i*
a_i- is the activity of the component *i*

The adsorption of dissolved materials at the interface reduces the surface tension of the solution. In practice, there are difficulties in measuring small changes in γ accurately and it requires the knowledge of activity coefficients, so as to obtain activities, which severely limits the utility of Equation (2.1). As a result, it is usually practical to apply only in special cases. Foremost among these is the case of a single non-ionic surface active solute dissolved in pure water at a sufficient low concentration so that the activity coefficient is constant. For this case Equation (2.1) simplifies to Equation (2.2).

$$\Gamma = - \frac{1}{R \cdot T} \cdot \frac{d\gamma}{d \ln C_B} \qquad (2.2)$$

Where:

Γ- is the surface concentration of surfactant

C_B- is the concentration of surfactant in the bulk

In accordance with Equation (2.2) at concentrations below the critical micelle concentration (c.m.c), when Γ becomes constant means that the surface reached saturation. Methods like foam fractionation operate best at low solute concentration molar solutions (Karger and DeVivo, 1968). At high concentrations, aggregates or micellae are formed (Maas, 1974). The concentration from which the micelles start to form is the c.m.c. At concentrations above c.m.c. Equation (2.2) does not apply, as micelles constitute another species. In accordance with the simple adsorption theory, the lower part of the curve is linear and can be expressed by the Equation (2.3).

$$\Gamma = KC_B \tag{2.3}$$

The upper portion of the curve levels off at saturation. The dependence of an incremental Γ from C_B, translates the partition that takes place and can be utilized for separation (Maas, 1974). A generalization of Langmuir's Theory for the unimolecular layers adsorption to the multimolecular layers adsorption, permits to obtain a relationship that represents the general shape of the Langmuir isotherm (Brunnauer, Emmett and Teller, 1938). For non-ionised materials, the entire curve can be approximated by a Langmuir isotherm (Davies and Riedel, 1963) and can be expressed by Equation (2.4).

$$\Gamma = KC_B / (1+K'C_B) \tag{2.4}$$

Where:

K - is a constant

K′- is a constant

A typical curve representing Equation (2.3) and (2.4) is presented in Figure 2.2.

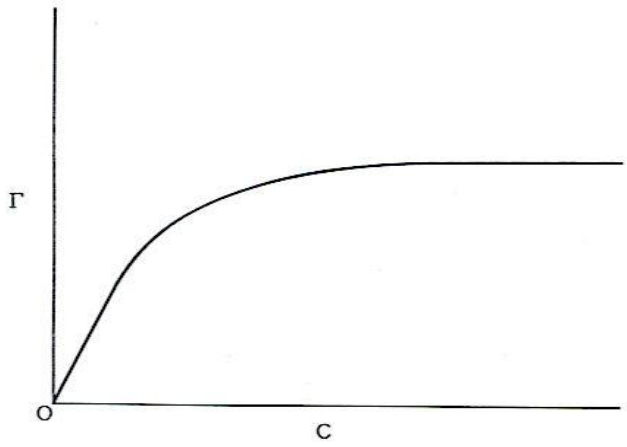
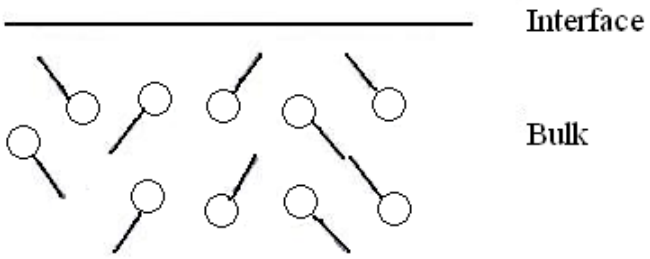
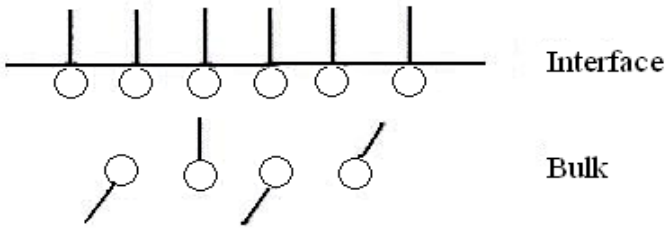


Figure 2.2 Curve Showing the Effect of Solute Concentration in Solution on the Solute Concentration at the Surface for the Adsorption Process (Lemlich, 1972a)

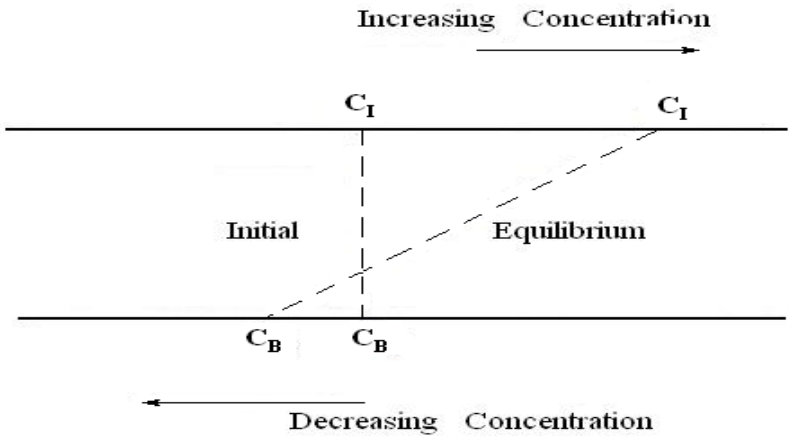
The adsorption process takes place due to the interference between water molecules from the solution and molecules of the solutes to be separated and the preference of solute molecules to the gas-liquid interface. When the foam fractionation process begins, there is a difference in chemical potential between the bulk of the liquid and the gas-liquid interface. There is no concentration gradient between the bulk and the interface, hence, the driving force for diffusion of the molecules to be adsorbed is at a maximum. At equilibrium, a concentration gradient exists and the driving force is at zero (Charm, 1972). An illustration of this process is presented in Figure 2.3.



(A)



(B)



(C)

Figure 2.3 Representation of Interface and Bulk Phase Concentration Resulting from Adsorption, During the Foam Fractionation Process (Charm, 1972 ; de Vries, 1972) (A) Initiation of the Process. (B) Equilibrium. (C) Whole Process. C_I – Concentration at the Interface. C_B – Concentration at the Bulk. (⬭—) Surfactant. (⬭) – Surfactant Hydrophilic Part (—) Surfactant Lipophilic Part

A rising bubble is different from a flat stagnant fluid surface since the bubble has a life-time and the materials to be adsorbed must diffuse to it. Diffusion is a rate process driven by the concentration of molecules in the bulk of the liquid and at the gas-liquid interface (Levich, 1962). For an immobile bubble, the molecules would adsorb uniformly at the surface. For a rising bubble, diffusion is not uniform due to the drag of the liquid sliding at the vicinity of the bubble. Therefore, the adsorbed materials tend to crowd in the lower part of the surface as presented in Figure 2.4.

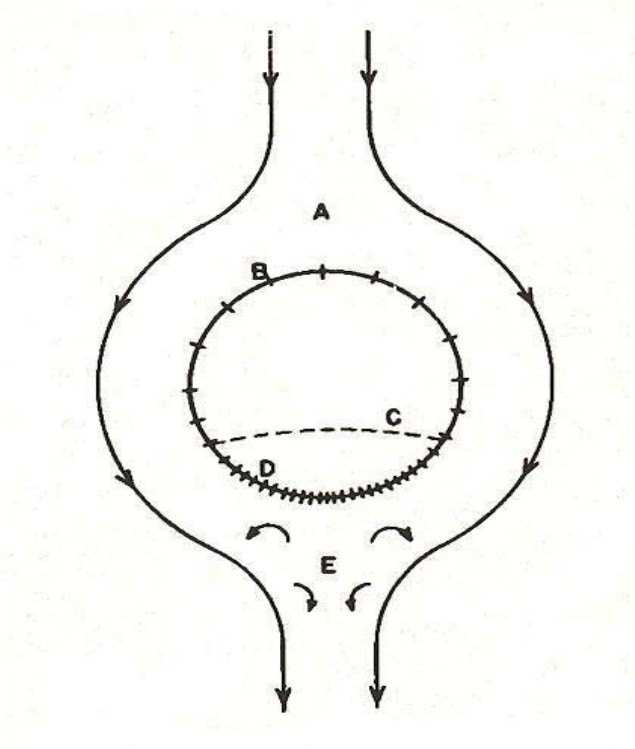


Figure 2.4 Adsorption at the Surface of a Bubble Rising Inside a Liquid Pool. A: Upstream Region; B: Bubble; C: Separation Ring; D: Crowding of Adsorbed Material; E: Wake (Lemlich, 1972c)

Generally, it is difficult in practice to predict Γ quantitatively for complex systems. The reasons other than the difficulties to find values for γ_i and a_i are the variety of interactions between the different molecules in solution as complex formation, micelle formation and competition for the adsorption sites (Lemlich, 1972c).

2.2.2 Foams

In foam fractionation, foam provides for the large gas-liquid interface for adsorption. The characteristic of the foam is therefore, important for the process. Foaming of solutions is dependent on the presence of dissolved surface active solutes. Dynamic foams are complex physicochemical disperse systems with a large surface area and as a consequence foams tend to collapse spontaneously. The average structure, stability, and size of bubbles are important factors for the quality of foams.

2.2.2.1 Types of Foam

There are two types of foams, unstable foams and metastable foams. The unstable foams are constantly collapsing as the liquid drains from between the bubbles and the life-time of the foams is short. The metastable foams do not collapse immediately and as a result the life-time of the bubbles is longer (Kitchener and Cooper, 1959). In unstable foams the bubbles are not drained and have a spherical form that is only slightly distorted by their neighbours, whereas, metastable foams persist long enough for drainage to proceed extensively so that the bubbles press against each other and the films of liquid between them become planar or slightly planar lamellae of almost uniform thickness. Manegold (1953) appropriately calls the two structures "Kugelschaum" and "Polyederschaum", which means spherical and polyhedral foam. The typical bubble is considered to have 12 pentagonal faces. In reality, a typical bubble deviates from this ideal and is in fact an average of shapes. An illustration of a typical foam is presented in Figure 2.5.

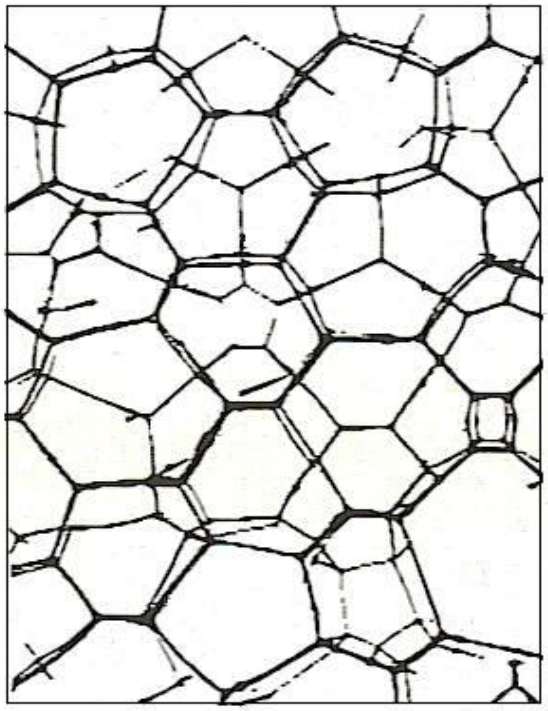


Figure 2.5 Well Drained Polyhedral Foam (Kitchener and Cooper, 1959)

2.2.2.2 Drainage and Thinning

The films between bubbles are almost planar and intersect three at a time to form channels or capillaries that are usually called plateau borders (PB). The PB are essentially randomly oriented and have a curved triangular cross section (Lemlich, 1968a; DeVries, 1972). A representation of the cross section of a PB is presented in Figure 2.6.

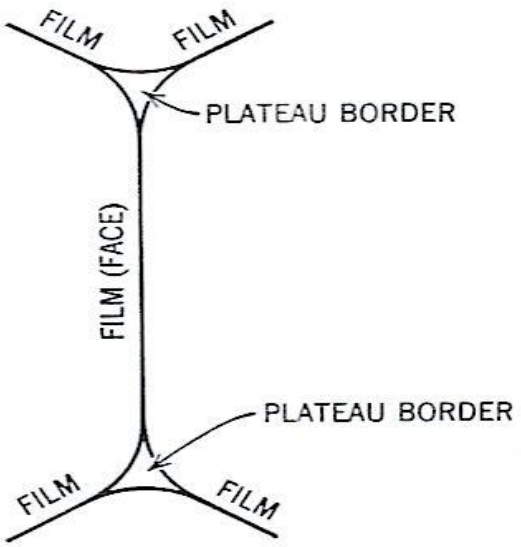


Figure 2.6 A Cross Section of Intersecting Films with the Plateau Borders Between Them (Lemlich, 1968)

The drainage through a foam of low liquid content occurs with the flow of the interstitial fluid through the PB. In foam fractionation, foam drainage is an important process as it provides for a foam overflow with low liquid content. Foam drainage occurs primarily through the interconnecting network of capillaries. The flow is caused by a pressure forcing the liquid from between the bubbles, that Derjaguin (1957) calls the "disjoining pressure". The flow or drainage of interstitial liquid results in the thinning of the liquid membrane. The persistence of foams results from the persistence of the membranes separating the bubbles. Solutions with good foam stability are able to resist excessive localized thinning, while a controlled general thinning proceeds. The persistence of foams, therefrom, a good foam stability pertain special rheological properties as surface elasticity and viscosity (Tamura *et al.*, 1997; Kitchener and Cooper, 1959).

Gibbs showed in his surface elasticity theory that for a solution, film elasticity stems from a depletion of surfactant from the film surface when the surface is stretched. This depletion raises the surface tension that results in the exertion of a restoring force that opposes rupture (Lemlich, 1968a). A complement of this theory was given by Maragoni in his surface elasticity theory, which postulate that the stretching of a film depletes the adsorbed surfactant from the surface due to the inability of surfactant molecules to diffuse instantaneously from the interior of a film to the surface. This is known as the Maragoni effect (Kitchener and Cooper, 1959). Bikerman (1973) reported that the stability of foams depends on viscosity, which is also strongly correlated with the drainage of foams. The most important function of viscosity is to retard liquid drainage from between the bubbles (Miles *et al.*, 1945).

2.2.2.3 Gas Diffusion Coalescence and Bubble size

In foam fractionation, the average size of the bubbles can be an important factor for a better separation. The increase of bubble size results from coalescence of the bubbles that arise from two sources. One is the diffusion of gas from smaller bubbles to larger bubbles as a result of surface tension that makes the pressure in smaller bubbles greater than the pressure in larger bubbles. With time, it results in the growth of the larger bubbles and disappearance of the smaller bubbles. This type of diffusion is important for foam fractionation columns with high residence times. Other source is the rupture of walls separating the individual bubbles (Lemlich, 1968a).

In metastable foams, there is a spontaneous formation of “black spots” characteristic of persistent bubble films. With time, the black spots thin until the critical film thickness of 10^{-5} cm or less, which does not entail an immediate rupture. With further thinning the rupture of the film occurs (DeVries, 1972). In either case in a rising column of foam, a certain extent of loss of surface and subsequent additional drainage constitutes internal reflux that further enriches the overflowing foam.

2.2.3 Equipments and Mode of Operation

2.2.3.1 Foam Fractionation Devices

The devices used in foam fractionation consist essentially of a column that contains the materials to be separated, a gas delivery system consisting of a gas supply, manometer, flow-meter, a sparger, and a foam collector. Auxiliary units include foam breaker, chamber for saturating gas and external reflux device.

- The column for foam fractionation is the device were enrichment of the desired materials takes place (Ostwald and Siehr, 1936). The column is usually made of glass and the height as well as the area of the cross section, depend chiefly on the foam generating method and the pretended foam height.

- The gas delivery systems provides for a controlled delivery of the gas serving for the bubble formation in the foaming liquid. The gas used should be inactive to the constituents present in solution and accordingly, nitrogen gas, oxygen gas, air and in some cases gaseous carbon dioxide are usually used. The manometer measures the pressure of gas available for delivery and the flow-meter measures and allows for the controlled delivery of a flow rate to the foam fractionation column. The sparger is usually a fritted glass bubbler or a group of carefully matched capillary tubes (Lemlich, 1968a; Ahmad, 1975). The foam collector is a simple beaker or flask to collect the foam.

- Auxiliary units can be used according to the requirements. The foam breaker is a device to break the foam produced in the foam fractionation column, usually by mechanical means. The gas saturating chamber saturates the bubble generating gas with solvent vapours in order to reduce evaporation of solvents (Kishimoto, 1962). The external reflux device is a collector connected with the foam fractionation column and which provides for the return of the foamate to the column.

The typical foam fractionation column for laboratory use consists of a reservoir and piping for the gas supply that is connected with a manometer which measures the pressure of the delivered gas. The gas is transported by the pipe to the flow-meter. The adjustable flow-meter allows the supply of a pretended flow rate to the foam fractionation column. The gas is then

delivered to the bottom of the fractionation column through a pipe connecting the flow-meter to the frit which is fastened to the bottom of the column. The injected bubbles flow through a defined volume of dilute solution fed to the column and the foam is produced on the top of the liquid pool. The foam production increases with time and increase of flow rate. The overflow is ultimately collected in a beaker.

A large number of column designs were propose by several authors like Ostwald and Siehr (1936, 1937), Ostwald and Mischke (1940), Kishimoto (1962) and Lemlich (1968). Irrespective of the column design, a number of phenomena occur in the foam fractionation column. In their work with proteins, Uraizee and Narsimhan (1996), depict a schematic diagram of those phenomena. A more generalized illustration is presented in Figure 2.7.

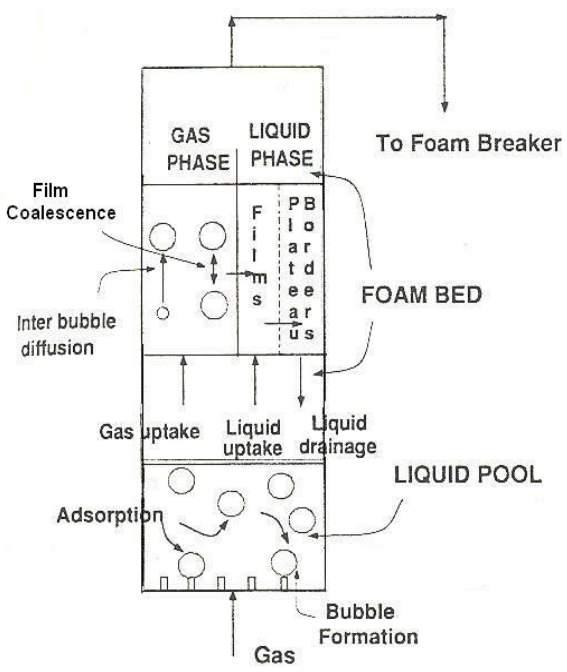


Figure 2.7 Schematic Diagram of the Phenomena Occurring in a Fractionation Column (Uraizee and Narsimhan, 1996)

2.2.3.2 Mode of Operation

There are several modes for operating the foam fractionation column. A foam fractionation column can be a batch or a continuous process and operate in the simple mode, the enrich-

ment mode, the striping mode and the combined mode. Representations of various modes of operation developed by Lemlich (1972a) are presented in Figures. 2.8 and 2.9.

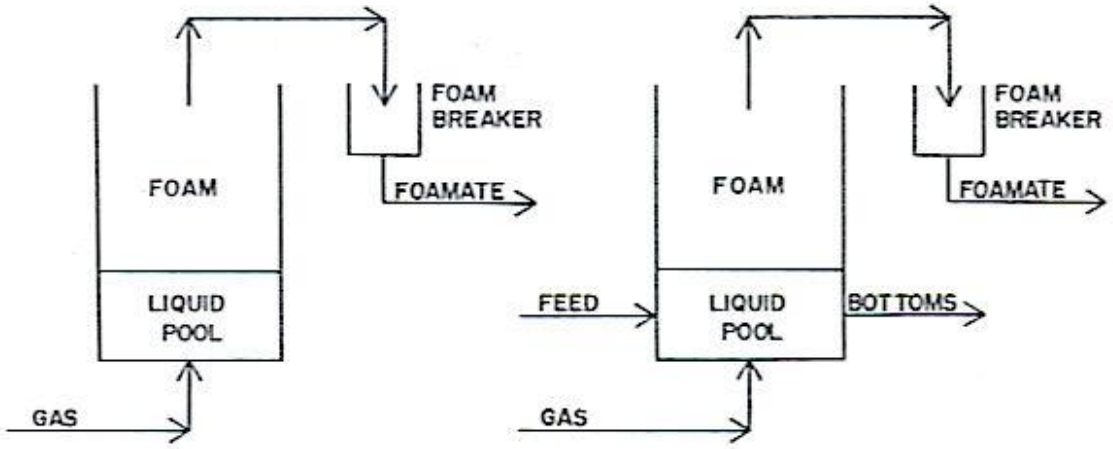


Figure 2.8 Foam Fractionation in the Simple Mode: (A) Batch Process. (B) Continuous Process (Lemlich, 1972a)

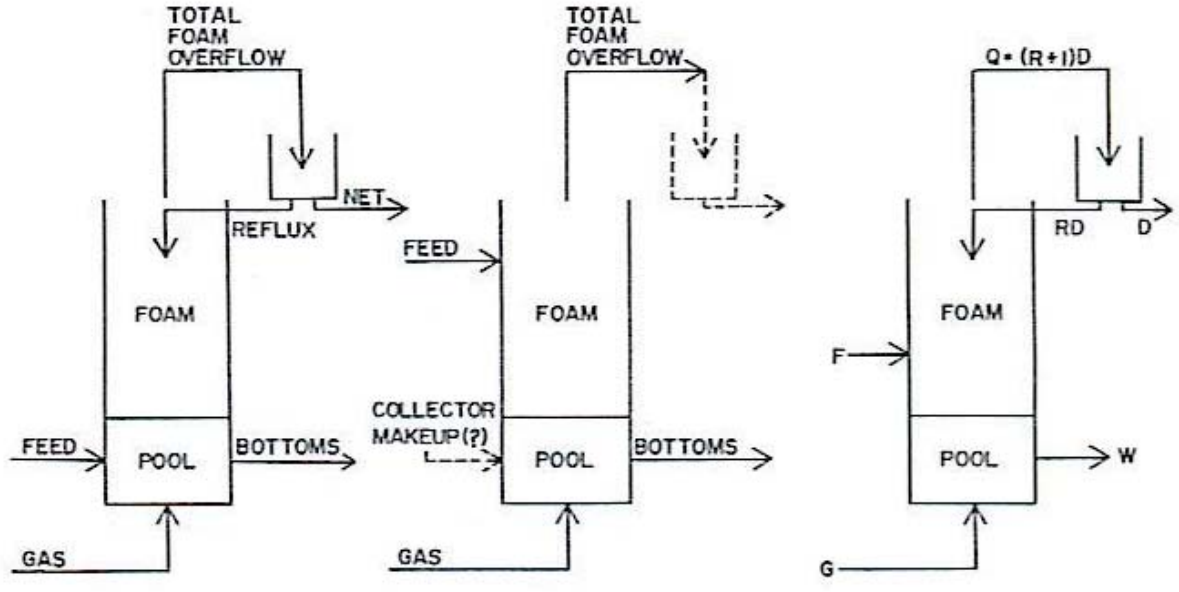


Figure 2.9 Foam Fractionation in a Continuous Process.(A) Enrichment Mode. (B) Striping Mode. (C) Combined Mode (Lemlich, 1972a)

The main features of a foam fractionation column operating in the simple mode entail the continuous withdrawal of foam. This is accomplished by first running the column for a while at gas rates sufficient to drive the foam up to nearly the top of the column. The gas is then slowly as well as successively raised and foam is collected in a beaker (Lemlich, 1968a).

In the enrichment mode, there is an external reflux in which part of the foam is returned to the fractionation column. A portion of the collapsed reflux is fed back to the top of the column and flows in counter-current to the rising foam. Since the reflux is richer than the interstitial liquid, the mass transfer resulting from this counter-current may considerably increase the enrichment (Rubin and Gaden, 1962; Lemlich, 1972b).

In the stripping mode, feed enters the column at some distance above the liquid pool in counter-current to the rising foam. This tends to replace interstitial liquid with interstitial liquid of feed composition. The overall result is an improvement in the degree of stripping. The striping process can be successful in removing surfactant acting as a collector that is in excess, for a colligend that is to be removed (Lemlich, 1972b).

The combined mode is a combination of the enriching and striping modes (Lemlich, 1968).

At laboratory scale, difficult multi-component separations can sometimes be achieved by using tall columns in batch simple operation at very low gas rates that give more internal reflux and drainage (Grieves, 1972; Lemlich, 1968). Schütz (1937) points out that a sudden decrease (or even increase) of foam stability while batch foaming in the simple mode may indicate the substantially complete removal of one component.

2.2.4 Efficiency of Foam Fractionation

The evaluation of efficiency or degree of removal/enrichment of defined components by foam fractionation may be expressed graphically, qualitatively or numerically (Kishimoto, 1962). The graphical, qualitative and numerical expressions of foam fractionation efficiency are usually made for one characteristic of the foam, which varies during the experiment. Such a characteristic is generally the concentration of the components intended to be enriched by foam fractionation at the initiation, at certain times and at the end of the process.

An example of the graphical representation of enrichment by foam fractionation is the illustration by chromatogram comparison of the concentration of those components to be enriched, in the initial solution, foam and residual solution. The qualitative representation of the enrichment by foam fractionation is often made in tables that present the concentration of the components to be enriched in the initial solution, foam and residual solution. Ultimately, numerical representation is the most common expression of efficiency or enrichment by foam fractionation and is defined as the ratio of concentration of the species to be separated, in the foam and in the initial solution (London *et al.*, 1954; Schnepf and Gaden, 1959; Kishimoto, 1962; Bhattacharjee, *et al.*, 1997), as follows:

Relative enrichment:

$$E_R = \frac{C_{Foam}}{C_{IS}} \tag{2.5}$$

And the percent enrichment:

$$\%E = \frac{C_{Foam} \cdot V_{Foam}}{C_{IS} \cdot V_{IS}} \cdot 100 \tag{2.6}$$

Where:

E_R – is the Relative Enrichment Ratio
%E – is the Percent Enrichment
C_{Foam} – is the Concentration of the Component in the Foam
C_{IS} – is the Concentration of the Component in the Initial Solution
V_{Foam} – is the volume of the Foam
V_{IS} – is the volume of the Initial Solution

An enrichment or the efficient operation of the foam fractionation column is achieved for $E_R > 1$.

2.2.5 Parameters Influencing the Efficiency of Foam Fractionation

The parameters that influence foam fractionation include basic variables such as concentration of solutes and surfactants, auxiliary materials, pH value, ionic strength and other operating variables as flow rate, height of liquid pool, foam height and internal reflux ratio.

2.2.5.1 Concentration of Solute and Surfactant

In foam fractionation, the enrichment is largely dependent on the concentration of materials to be separated that are present in the bulk of the dilute solution. Robert and Vermeulen (1969) demonstrated in their foam fractionation study of rare hearth elements that low concentrations of the materials in the bulk is a desired property for extraction. Ahmad (1975) as well as Uraizee and Narsimhan (1996) proved in their work with proteins that low concentrations of materials present in the bulk is the key for an effective separation. They pointed out that there is an optimal concentration range, which is more suitable to achieve a more effective separation. Karger and DeVivo (1968) in their work on the fundamentals of foam fractionation, postulated that the conventional foam fractionation operates at best with concentrations between 10^{-3} to 10^{-7} molar solutions. Maas (1974) found that at higher concentrations micelles are formed, which has a negative effect on enrichment.

2.2.5.2 Addition of Organic Solvents

The separation of materials present in a solution by foam fractionation presupposes that these materials are highly diluted in the bulk of the solvent. The solvent traditionally used in foam fractionation is water. Therefore, the foam fractionation of substances that are not soluble in water is problematic. Bartsch (1925), demonstrated in his work with sodium cholate solutions, that for solutions with foaming capacity, the presence of solid particles to be separated is detrimental to the foam ability of these solutions and that those solid particles cannot be separated by foam fractionation. Bartsch (1925) and Young (1994) proposed the addition of small quantities of organic solvents to which the materials in question have affinity and with which they are soluble. The solubilization of the solid particles by organic solvents provides for the presence of these materials in the solution, at their diluted form.

2.2.5.3 Addition of Electrolytes and Polyelectrolytes

In foam fractionation, the addition of electrolytes is used to increase the degree of separation. The effect of electrolytes is in most cases the activation of adsorption of the materials. The concentration and nature of electrolytes and polyecletrolytes have a pronounced effect on the rate of increase of separation (Somasundaran, 1972). It is observed that at low concentration of electrolytes, the adsorption decreases due to the increase of solvent's activity and consequent increase of the solubility of the molecule in question, which is termed "salting in". To a certain extent, higher concentrations of electrolytes increases the adsorption due to the decrease of solvent's activity and in consequence the solubility of the molecules in question decreases, which is termed "salting out" (Young, 1994). Similarly, the addition of polyelectrolytes that can complex with the molecules in question to form hydrophobic species, results in an improved separation.

The increase of adsorption of materials due to the addition of polyelectrolites was described by Robertson and Vermeulen (1969) in their foam fractionation work with rare hearth elements. The effect of addition of electrolytes was discussed by Tamura *et al.* (1997). Their work with sodium dodecylsulfate (SDS) and heptaethyleneglycol dodecyl ether ($C_{12}E_7$) attests that the adsorption rate of materials gradually increases with the addition of electrolytes to reach a peak and then decreases gradually.

2.2.5.4 Addition of Viscosity Enhancers

The importance of viscosity for the improvement of foam separation has already been discussed in the sub-chapter 2.2.2. The increase of viscosity of a solution and the surface is attained with the addition of small amounts of water soluble viscous liquids. Tamura *et al.* (1997) demonstrated that the controlled addition of glycerin to aqueous solutions of SDS and $C_{12}E_7$ increase the viscosity of the solutions and surfaces and serves to slow down the rate of thinning.

2.2.5.5 pH of Solution

In general, the pH value of a solution will determine the sign and magnitude of the charge of a variety of molecules. Therefore, adsorption of those molecules at the gas-liquid interface of dilute aqueous solutions and the extent of their removal by foam fractionation can be positively influenced by the solution's pH value. For some types of molecules, a remarkable degree of separation can be achieved by choosing appropriate pH conditions. This effect is due to the different functional groups that some types of molecules possess. For those molecules, there will be some pH, known as the isoelectric point (pI), where the net charge of the molecule is zero. At this point the solubility of such molecules is at the minimum. The pI is different for the different molecules (Young, 1994). Ahmad (1975) and Uraizee and Narsimhan (1996) proved in their works with proteins, that the enrichment is greater at the pH corresponding to the pI.

2.2.5.6 Ionic Strength

The adsorption of materials at the gas-liquid interface may increase with the increase of ionic strength. Foam fractionation of certain materials can be assisted to some extent by an increase in ionic strength, provided that the c.m.c is not lowered below the concentration of the material in solution and that the effect of ionic strength on the other foam properties is not of detrimental nature. Somasundaran (1972) discussed the influence of ionic strength in the separation of several materials.

2.2.5.7 Gas Flow Rate

The gas flow rate has a marked effect on the enrichment ratio attained in foam fractionation. A low gas flow rate is in general beneficial for the enrichment, even though the rate of separation will be lower at lower flow rates. During the process of foam fractionation at low flow rates, provision must be taken that there is a sufficient gas flow to maintain the foam height that is essential for a good separation – the optimum flow rate being determined by the concentration of the surfactant and the stability of the foam. Various authors, including Schnef *et*

al. (1959), Kishimoto (1962) well as Grieves *et al.* (1970) have found that for different substances high enrichment and low foam density is obtained at low flow rates.

2.2.5.8 Height of Liquid Pool

In foam fractionation, the height of liquid pool above the sparger can affect the enrichment ratio. The variation of height of liquid pool implies a change of contact time between the solution and the rising bubbles before they reach the top of the liquid pool. This change may affect the mass transfer of the desired materials that occurs between the solution and surface of the rising bubbles. To a certain extent, increase in pool height results in an increase of bubbles residence time in the pool and thus, more time for the bubbles to approach adsorption equilibrium, hence, improve the enrichment ratio. Parthasarathy *et al.* (1987) and Bhattacharjee *et al.* (1997) ascertained in their works with proteins and microbial cells, the effect of pool high above the sparger for the enrichment.

2.2.5.9 Height of Foam Tower

As mentioned in 2.2.2 foam is important to obtain a separation during foam fractionation. Different foam heights show a significant effect on the separation. An increase in foam height to a certain extent brings about a drastic change in the mass transfer process due to the increase in the interfacial transfer area and the overall increase of drainage and internal reflux. Therefore, foam height is essential to obtain a good enrichment. Ahmad (1975) and Uraizee and Narsimhan (1996) showed during their work with proteins, the influence of foam height on enrichment.

2.2.5.10 Internal Reflux Ratio

The degree of enrichment achieved in foam fractionation is generally dependent on the reflux ratio. The concentration of materials to be separated both in the overhead and in the pool increase with an increase in internal reflux ratio, unless or until the surface of the foam is saturated with the material. Shonfeld and Kibbey (1967) as well as Grieves *et al.* (1970) proved in

their work with several substances the proportionality between internal reflux ratio and enrichment.

A General Overview of the Use of Foam Fractionation

Foam fractionation is used for the separation of a variety of materials from highly diluted aqueous solutions. However, hitherto, the technique is mainly used for the separation of two classes of materials. The largest class of materials separated by foam fractionation are undoubtedly proteins. The technique is used as early as 1937 and the aim of the separation is usually protein recovery from food industry effluents for eventual reuse. The technique shows a great potential to be a source of low cost protein. Foam fractionation provides for good efficiencies with dilute solutions of surface active and hydrophobic materials, nevertheless, suffers from requiring a strict control of the foaming process (Singh and Singh, 1996).

Recently, another application of the technique for the separation of proteins is developing. A growing number of amateur aquarists are successfully using the technique to remove excess proteins from aquariums (Kim, 2003). Another class of materials for which the technique is widely used are detergents. The use of plant scale foam fractionation units for the separation of detergents from sewage dates back to the late fifties (Ludwig *et al.*, 1962). Nowadays, with the introduction of biodegradable detergents, foam fractionation of sewage is decreasing. However, there are areas where "hard" detergents are still in use and where the technique can be largely applied. The utilisation of foam fractionation to isolate active principles from medical plants for pharmaceutical use is to date a laboratory curiosity however, there is a potential for the extensive use of the technique in this field.

2.3 Plant Materials Under Investigation

The plants under investigation in this work are: *Calendula officinalis* L., *Camellia sinensis* L., *Isatis tinctoria* L. and *Cannabis sativa* L.. These plants contain compounds generally termed active principles for their biological and pharmacological activity, which can be used for medical purposes. The common feature of the active principles under investigation in this work is the presence in their molecules of aromatic groups. The physicochemical properties of the molecules play a key role in foreseeing the behaviour of theses molecules during foam

fractionation. Therefore, the knowledge of the properties of the active principles is important to set the most favourable conditions while trying to achieve an enrichment using the technique of foam fractionation.

2.3.1 *Calendula Officinalis* L.

2.3.1.1 General

The *Calendula officinalis* plant belongs to the genus *Calendula*, from the family Asteraceae also known as Compositae. The plant is indigenous to the Mediterranean region and is now widely spread to Central and South Europe, North Africa, Asia and North and Central America (Boulos, 1983; Isaac, 1994). Within the genus Calendula, *Calendula officinalis* and *Calendula avensis* are the types that have medical value. *Calendula officinalis* L., has some vernacular names as marigold, ringelblume, maravilha, zubaydah and fleur the souci officinal. The plant is widely cultivated as a garden plant and is cultivated commercially in the Balkans, Eastern Europe, The Netherlands, Germany and North America (Bisset, 1994; Wichtl, 1997).

Calendula officinalis is an annual herb, branched from the base, aromatic and grows up to 30 to 50 cm. The stem is angular, hairy and solid, the leaves are light green, either spatulate or oblanceolate, 10 to 15 cm long and 3 to 4 cm wide, covered with hair. The flowers of cultivated plants are generally multi-seriate and a single plant can produce up to 50 flowers. The flowers are 2 to 5 cm wide and the corolla are oblong-spatulate and brigth yellow, dark orange or orange-yellow in colour and occasionally with a partly bent yellowish-brown or orange brown ovary. Flowers with lobed corolla are described as “full” and are the most cultivated type (Backer and Brink 1965; Isaac, 1994).

Figure 2.10 *Calendula Officinalis* L.

Cultivated *Calendula officinalis* is typically a plant that grows in warmer climates, but still, not too sensible to the cold. The harvest of the flowering tops begins in May and ends in August. Manual harvest allows for one to two harvests each week, however mechanical equipments are usually used. After the harvest, the flower heads, flowers and calyxes are dried and cleaned. The cleaned most valuable *Calendula officinalis* is generally termed *Calendula* flos and consists of 50 to 75% total weight of flowers (Isaac, 1994 and 2000).

2.3.1.2 Chemical Composition

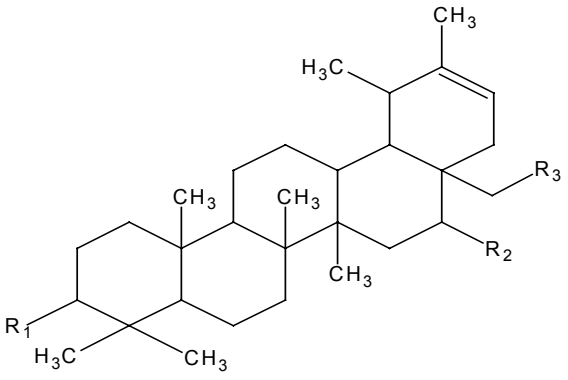
Calendula officinalis L. contains several constituents that exhibit biological and pharmacological properties. Some main constituents of the plant are triterpene glycosides, carotenoids, flavonol glycosides, polyscharides, sterols, cumarins, etheric oils and relatively high quantities of triterpene alcohols (Zitterl-Eglseer *et al.*, 2000c). The constituents found in larger quantities are triterpene glycosides, carotenoids, polysacharides and triterpene alcohols (Zitterl-Eglseer *et al.*, 1997 and 2000a; Isaac, 2000). Triterpene alcohols are, due to specific biological and pharmacological properties, the active principles under consideration in this work

and will be dealt with more detail in the next paragraph. A general description of the other major constituents will be made further in this chapter.

2.3.1.3 Active Principles

Triterpene alcohols from *Calendula* flos are monols, diols and triols, constituted by ψ-taraxen, taraxen, lupene, oleane and ursene. About 10% of the monols and 98% of diols and 1% of triols are esterified, the monols with acetic acid and the diols mainly with the fatty acids lauritic, myristic and palmitic acids. The diolesters are 98% bound 3-monoesters and only 2% are diesters. The triterpenediol-3-monoesters are mainly monoesters from faradiol. The triterpenediol-3-monoesters make between 2 to 4% of the dry mass of *Calendula* flos, from which 85% are faradiol esters, 6% calenduladiol esters, 5% of brine esters and small quantities of maniladiol and arnidiol esters (Issac, 2000; Zitterl-Eglseer *et al.*, 2000a, 2000c).

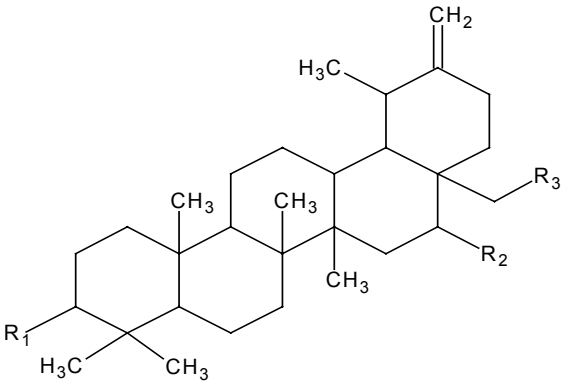
Faradiol esters are composed by faradiol-3-lauritic acid ester, faradiol-3-myristic acid ester and faradiol-3-palmitic acid ester, being the two latter present in higher quantities. These are the main active principles of the plant. Faradiol esters are very stable at high temperatures, and along the time, are not light sensible and are highly lipophilic. (Kasprzyk and Pyrek, 1968; Issac, 2000; Zitterl-Eglseer *et al.*, 1997 and 2000a, 2000b). Figures. 2.11 and 2.12 depict the structures and properties of the main triterpene alcohols.



ψ–Taraxasterol: R_1=OH; R_2=R_3=H

Faradiol: R_1=R_2=OH; R_3=H

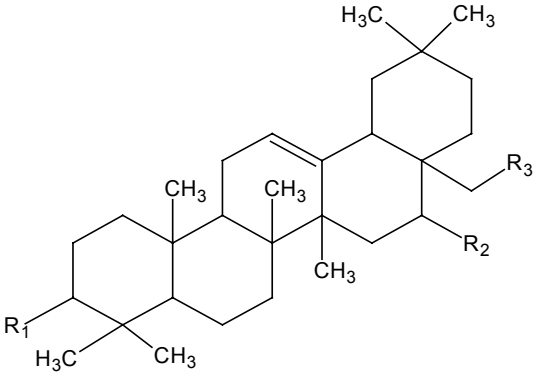
Heliantiol B_0: R_1=R_2=R_3=OH



Taraxasterol: R_1=OH; R_2=R_3=H

Arnidiol: R_1=R_2=OH; R_3=H

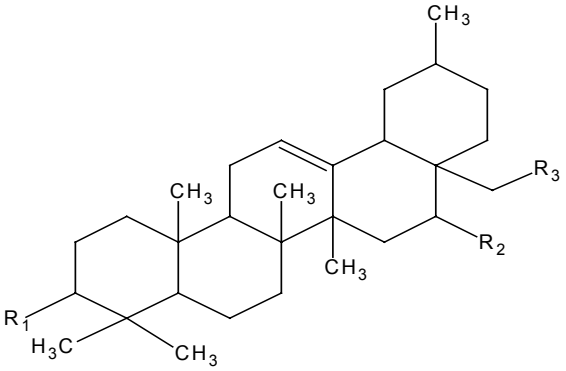
Helantriol B_1: R_1=R_2=R_3=O



β-Amyrin: R1=OH; R_2=R_3=H

Maniladiol: R_1=R_2=OH; R_3=H

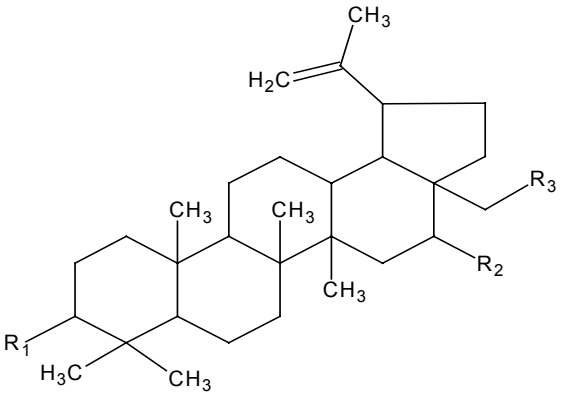
Longispinogenin:R_1=R_2=R_3=OH



α- Amyrin: R_1=OH; R_2=R_3=H

Brein: R_1=R_2=OH; R_3=H

Ursatriol: R_1=R_2=R_3=OH

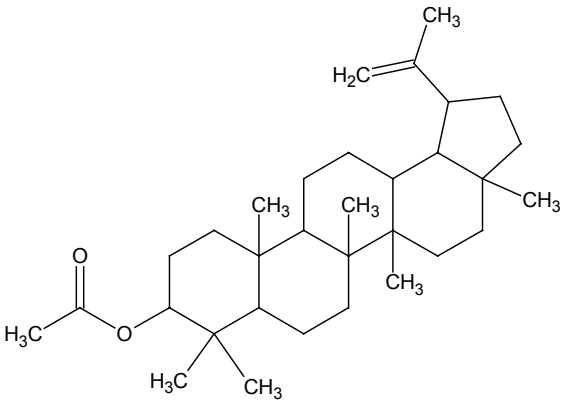


Lupeol: R_1=OH; R_2=R_3=H

Calenduladiol:R_1=R_2=OH; R_3=H

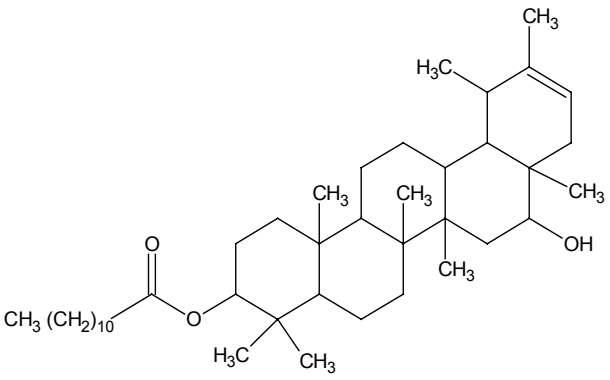
Heliantriol: R_1=R_2=R_3=OH

Figure 2.11 Molecular Structure of Triterpene Alcohols from *Calendula Officinalis* L.



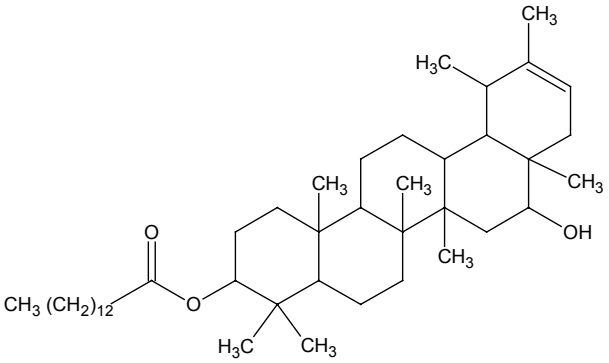
Lupeol Acetate

Molecular Formula = $C_{32}H_{52}O_2$
Formula Weight = 468.754
Composition = C(81.99%) H(11.18%) O(6.83%)
Molar Refractivity = 141.31 ± 0.4 cm^3
Molar Volume = 461.3 ± 5.0 cm^3
Parachor = 1144.7 ± 6.0 cm^3
Index of Refraction = 1.524 ± 0.03
Surface Tension = 37.9 ± 5.0 dyne/cm
Density = 1.01 ± 0.1 g/cm^3
Dielectric Constant = Not available
Polarizability = 56.01 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass = 468.39673 Da
Nominal Mass = 468 Da
Average Mass = 468.76654 Da



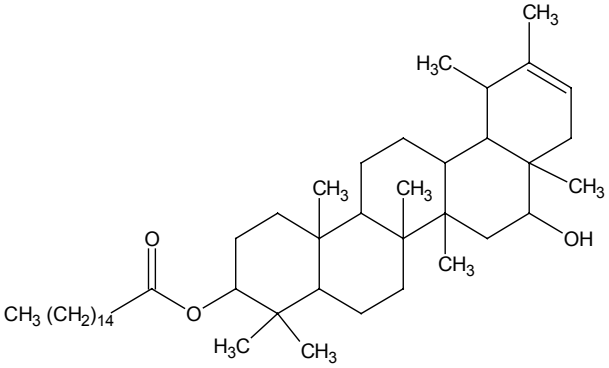
Faradriol Lauritic Acid Ester

Molecular Formula = $C_{42}H_{72}O_3$
Formula Weight = 625.019
Composition = C(80.71%) H(11.61%) O(7.68%)
Molar Refractivity = 189.27 ± 0.4 cm^3
Molar Volume = 617.7 ± 5.0 cm^3
Parachor = 1558.8 ± 6.0 cm^3
Index of Refraction = 1.524 ± 0.03
Surface Tension = 40.5 ± 5.0 dyne/cm
Density = 1.01 ± 0.1 g/cm^3
Dielectric Constant = Not available
Polarizability = 75.03 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass = 624.548145 Da
Nominal Mass = 624 Da
Average Mass = 625.035733 Da



Faradiol Myristic Acid Ester

Molecular Formula = $C_{44}H_{76}O_3$
Formula Weight = 653.073
Composition = C(80.92%) H(11.73%) O(7.35%)
Molar Refractivity = 198.53 ± 0.4 cm^3
Molar Volume = 649.8 ± 5.0 cm^3
Parachor = 1639.0 ± 6.0 cm^3
Index of Refraction = 1.523 ± 0.03
Surface Tension = 40.4 ± 5.0 dyne/cm
Density = 1.00 ± 0.1 g/cm^3
Dielectric Constant = Not available
Polarizability = 78.70 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass = 652.579445 Da
Nominal Mass = 652 Da
Average Mass = 653.089711 Da



Faradiol Palmitic Acid Ester

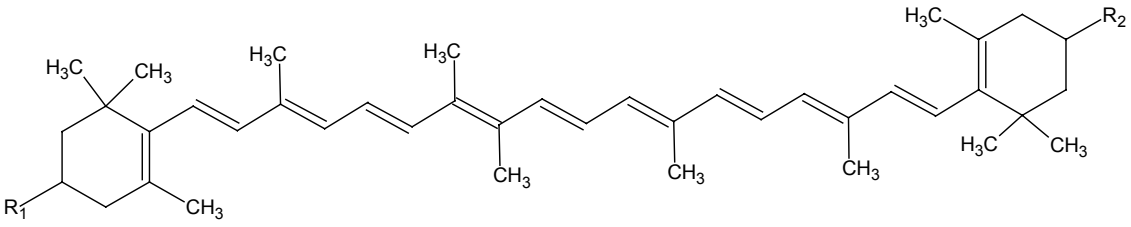
Molecular Formula = $C_{46}H_{80}O_3$
Formula Weight = 681.126
Composition = C(81.11%) H(11.84%) O(7.05%)
Molar Refractivity = 207.79 ± 0.4 cm^3
Molar Volume = 681.8 ± 5.0 cm^3
Parachor = 1719.1 ± 6.0 cm^3
Index of Refraction = 1.521 ± 0.03
Surface Tension = 40.4 ± 5.0 dyne/cm
Density = 0.99 ± 0.1 g/cm^3
Dielectric Constant = Not available
Polarizability = 82.37 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass = 680.610745 Da
Nominal Mass = 680 Da
Average Mass = 681.143688 Da

Figure 2.12 Molecular Structure of Lupeol Acetate and Faradiol Monoesters from *Calendula Officinalis* L. and Respective Properties Calculated with ACD/Labs Software

2.3.1.4 Other Components

Triterpene glycosides from *Calendula officinalis* are described as a to f oleanol acid glycosides or simply saponins. These saponins are surface active substances and make between 2 to 10% of the dry mass of the *Calendula* flos. Carotenoids are the constituents with a strong colour, yellow and orange. The varieties with yellow colour are rich in xanthophylle and the varieties with the orange colour are rich in β-caroten. Carotenoids (Figure 2.13) are sensible to light and degrade during storage. They are highly lipophilic and make between 1.5 to 3% of the dry mass of *Calendula* flos. Carotenoids possess valuable anticarcinogenic activity, however, they are not active principles under consideration in this work. The highly lipophilic character of carotenoids make them undesirable substances (Steinegger and Hänsel, 1988; Isaac, 1994; Della Logia, 2000).

Flavonoid glycosides are mainly quercitine and isorhametine. The flavonoid content in *Calendula* flos is between 0.3 and 0.8% of the dry mass. This class of compounds are generally hydrophilic, yet, due to complexation may become hydrophobic. The polysacharides are mainly the lipophilic pectins and hemicelluloses, the hydrophilic rhamnoarabinogalactans and arabinogalactans. The lipophilic and hydrophilic polysaccharides make each, about 15% of the dry substance of *Calendula* flos. The sterols, cumarins, proteins, chlorophyll and eteric oils contents in *Calendula* flos dry mass is considerably low (Varljen *et al.*, 1989; Isaac, 1994; Chew *et al.*, 1996; Piccaglia *et al.*, 1997; Della Logia, 2000; FAO, 2001).



β-caroten : $R_1=R_2=H$

Xanthophylle: $R_1=R_2=OH$

Figure 2.13 Molecular Structure of β-Caroten and Xanthophylle from *Calendula Officinalis* L.

2.3.1.5 Biological and Pharmacological Activity

Calendula flos preparations for topical applications is widespread both in dermatology and in cosmetics. One of the most relevant pharmacological features of the preparations is the anti-inflammatory and wound-healing activity (Isaac, 1992; Della Loggia, 1994), which is well documented in many experimental and clinical studies (Shipochliev *et al.*, 1981; Peryox *et al.*, 1981; de Carvalho *et al.*, 1991; Livre *et al.*, 1992).

Several components of *Calendula* flos have been proposed by some authors as the active principles responsible for this activity, although without demonstration. Della Loggia *et al.* (1990) demonstrated by means of the croton oil model that the most lipophilic components possess the same anti-inflamatory and wound-healing activity as the equivalent amount of the usual total extract. The same authors demonstrated that the activity concentrates in the fraction containing mostly triterpene alcohols. The active fraction containing the most lipophilic components is constituted mainly of triterpene alcohols, from which the faradiol esters represent about 75% and show the highest activity. Among the faradiol esters, faradiol myristate and faradiol palmitate show the highest activity. The active principles of the *Calendula* flos with respect to the anti-inflamatory and wound-healing activity of *Calendula officinalis* L. is proved to derive from the triterpene alcohols, being faradiol esters the most relevant (Della Loggia *et al.*, 1994 and 2000; Zitterl-Eglseer *et al.*, 1997; Isaac, 2000)

2.3.1.6 Toxicology

Calendula flos extracts for topical application present a very low acute toxicity. In humans, it is only contraindicated in cases of known allergy to plants of the Astracea family. The acute toxicity of *Calendula* flos extracts in laboratory test animals is very low. The lethal doses per 50% of tested animals (LD_{50}) is 4640 mg kg^{-1} (CTFA, 1980; ESCOP, 1996).

2.3.2 *Camellia Sinensis* L.

2.3.2.1 General

The *Camellia sinensis* plant belongs to the genus *Camellia* of the family Theacea that comprises more than 80 species. *Camellia sinensis* is classified in two varieties, *assamica* and *sinensis*. The plant is indigenous from the area including northern Myanmar and South-east China. The plant gradually expanded first to India and Sri Lanka and further into many temperate, tropical and subtropical countries (De Candlle, 1953; Sealy, 1958; Hashimoto, 1988). The vernacular name of *Camellia sinensis* is tea. The plant is cultivated commercially in more than 20 countries mainly in Asia, Africa and South America. The main producers are India, China and Sri Lanka (FAO, 1993; Chu, 1997).

Camellia sinensis is an evergreen shrub, much branched, with thick dark green leaves, 5.5 to 6.1 cm in length and 2.2 to 2.4 cm in width. In the wild, the plant reaches the size of a tree. The cultivated plant is a vegetatively propagated and three to four years after transplant, the young plants are cut into a semi-cylindrical shape, convenient for field work (Hänsel *et al.*, 1992; Chu, 1997).

Figure 2.14 *Camellia Sinensis* L.

Camellia sinensis is a plant cultivated in a broad area, ranging from temperate to tropical regions. The plant is adaptable. and can tolerate winter temperatures as low as –12 °C. The valuable parts of the plant are young buds and the first two leaves. The harvest starts early summer and is made four times a year usually in April, June, August and October. Harvesting is mainly manual and made in intervals of seven days. The harvested plant is then subject to processing and after processing are classified as unfermented, semi-fermented and fermented or green tea, oo-long tea and black tea. During the fermentation process, the plant loses part of the valuable components. Green tea is the one with more medical value for containing the components of medical value in greater quantities. The quality of green tea is dependent on the quantity of the desired components (Chu, 1997; Schreck, 2000).

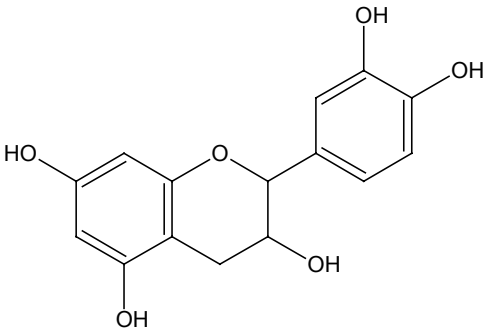
2.3.2.2 Chemical Composition

Camellia sinensis contains various components that possess biological and pharmacological properties. Some of the main components are relatively large quantities of polyphenols, alkaloids, polysaccharides, proteins, aminoacids, lipids and small quantities of organic acids, saponins, vitamins and minerals (Herrman, 1983; Chu and Juneja, 1997). Polyphenols are due to their specific biological and pharmacological properties, the active principles under consideration in this work and will be dealt with more detail in the next paragraph. A general description of the other major components will be made further in this chapter.

2.3.2.3 Active Principle

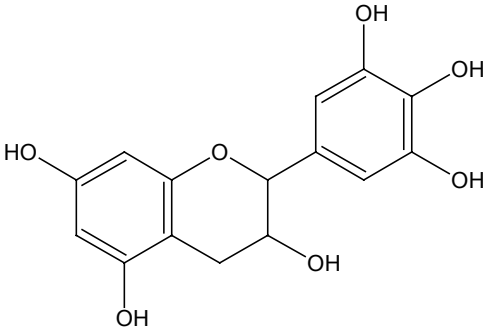
Camellia sinensis polyphenols are constituted by tannine glycosides, condensed flavonoids and flavonids that are composed by flavones, flavonols and catechins. Polyphenols make about 30% of *Camellia sinensis* dry mass, from which catechins are by far present in larger quantities. Catechins, the active principle of the plant are (+)- catechin [(+)-C)], (-)- epicatechin [(-)-EC], (+)- gallocatechin [(+)-GC], (-)- epigallocatechin [(-)-EGC], (-)- epicatechin gallate [(-)-ECG], (-)- gallocatechin gallate [(-)- GCG] and (-)- epigallocatechin gallate [(-) EGCG)]. The quantities of catechins, the ratio between components and the quantity of each individual component vary among others with factores like cultivation, soil, precipitation and time of harvesting (Selectchemie; Chu and Juneja, 1997).

Catechins are unstable along the time, easily oxidable, are light and heat sensible, degrade at pH values over 6.5 and are highly reactive. They react with several types of molecules showing the properties of metal chelators, oxidative radical scavengers, nitrosation inhibitores among other properties. Catechins are hydrophilic molecules and the hydrophilicity decreases with molecular weight (Scholz and Bertram, 1995; Ninomya *et al.*, 1997; Koketsu, 1997; Zhu *et al.*, 1997; Chung *et al.*, 1998; Robb and Brown, 2001). Figure 2.15 presents the structures of catechins.



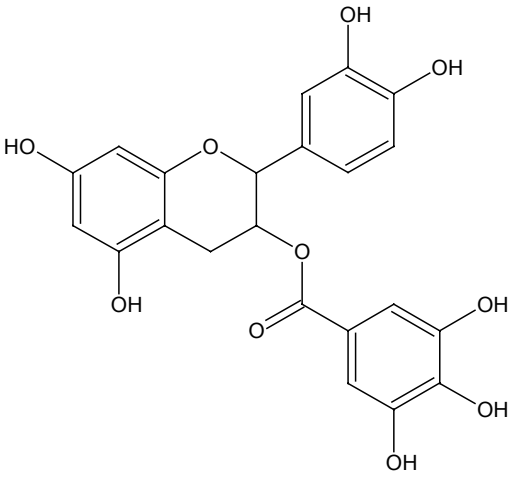
(+)-Catechin

Molecular Formula = $C_{15}H_{14}O_6$
Formula Weight = 290.268
Composition = C(62.07%) H(4.86%) O(33.07%)
Molar Refractivity = 73.59 ± 0.3 cm^3
Molar Volume = 182.1 ± 3.0 cm^3
Parachor = 558.2 ± 4.0 cm^3
Index of Refraction = 1.741 ± 0.02
Surface Tension = 88.1 ± 3.0 dyne/cm
Density = 1.593 ± 0.06 g/cm^3
Dielectric Constant = Not available
Polarizability = 29.17 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass = 290.07904 Da
Nominal Mass = 290 Da
Average Mass = 290.273047 Da



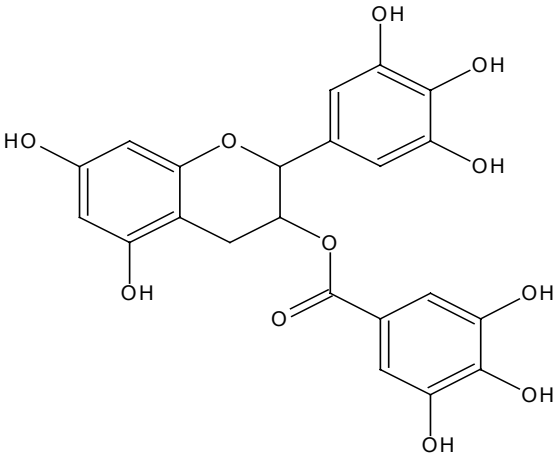
(-)- Epigallocatechin

Molecular Formula = $C_{15}H_{14}O_7$
Formula Weight = 306.267
Composition = C(58.82%) H(4.61%) O(36.57%)
Molar Refractivity = 75.47 ± 0.3 cm^3
Molar Volume = 180.6 ± 3.0 cm^3
Parachor = 573.2 ± 4.0 cm^3
Index of Refraction = 1.775 ± 0.02
Surface Tension = 101.4 ± 3.0 dyne/cm
Density = 1.695 ± 0.06 g/cm^3
Dielectric Constant = Not available
Polarizability = 29.92 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass = 306.073955 Da
Nominal Mass = 306 Da
Average Mass = 306.272352 Da



(-)- Epicatechin Gallate

Molecular Formula = $C_{22}H_{18}O_{10}$
Formula Weight = 442.372
Composition = C(59.73%) H(4.10%) O(36.17%)
Molar Refractivity = 106.90 ± 0.4 cm^3
Molar Volume = 244.4 ± 5.0 cm^3
Parachor = 828.6 ± 6.0 cm^3
Index of Refraction = 1.825 ± 0.03
Surface Tension = 132.0 ± 5.0 dyne/cm
Density = 1.80 ± 0.1 g/cm^3
Dielectric Constant = Not available
Polarizability = 42.37 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass = 442.09 Da
Nominal Mass = 442 Da
Average Mass = 442.37943 Da



(-)- Epigallocatechin Gallate

Molecular Formula = $C_{22}H_{18}O_{11}$
Formula Weight = 458.372
Composition = C(57.65%) H(3.96%) O(38.40%)
Molar Refractivity = 108.43 ± 0.4 cm^3
Molar Volume = 241.2 ± 5.0 cm^3
Parachor = 843.8 ± 6.0 cm^3
Index of Refraction = 1.857 ± 0.03
Surface Tension = 149.7 ± 5.0 dyne/cm
Density = 1.90 ± 0.1 g/cm^3
Dielectric Constant = Not available
Polarizability = 42.98 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass = 458.084915 Da
Nominal Mass = 458 Da
Average Mass = 458.378735 Da

Figure 2.15 Molecular Structure of Some Catechins from *Camellia Sinensis* L. and Respective Properties Calculated with ACD/Labs Software

2.3.2.4 Other compounds

The main alkaloid present in *Camellia sinensis* is caffeine. Caffeine is a trimethyl derivate of purine 2, 6-diol. Caffeine contents in green tea leaves reaches 5% of the dry mass and is highly hydrophilic. Caffeine (Figure 2.16) is a cardiac stimulant and diuretic, stimulates the cerebral cortex, causes irritation of the gastrointestinal tract and sleepless for certain people. The two latter effects together with caffeine high hydrohillic character, makes caffeine an undesired component for the purpose of this work (Selectchemie; Chu and Juneja, 1997; Chu *et al.*, 1997).

Polysaccharides are mainly the lipophilic celluloses and starches which when heated above 60 °C polymerises forming disperse colloids in aqueous solution, becoming viscous and foamable. Figure 2.17 presents the structure of starch. The polysaccharides content of green tea dry mass is about 40%, from which the greater part is cellulose. Green tea contains chlorophyll, proteins, peptides and amino acids. The proteins and peptides are insoluble due to the binding with tannins that occurs during the process of green tea manufacturing. Chlorophyll is also insoluble. The main amino acid is theanine. Proteins, peptides and amino acids make between 4.5 to 6% of the dry mass of green tea, from which about 2% is theanine. Lypids contents is mainly oleic acid and the presence of lipids in green tea dry mass is about 4%. Saponins and vitamins are also present in small quantities (Selectchemie; Ikegaya *et al.*, 1997; Chu and Juneja, 1997; Chu *et al.*, 1997, FAO, 2001). Saponins stability is pH dependent (Backleh, 2001) and degrade at pH values lower than 2 and higher than 12.

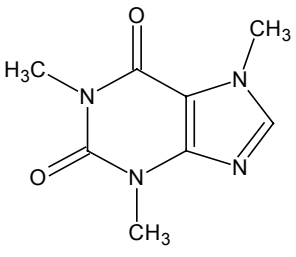


Figure 2.16 Molecular Structure of Caffeine from *Camellia Sinensis* L.

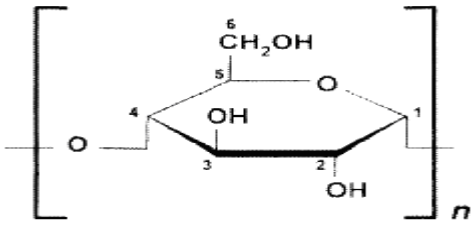


Figure 2. 17 Anhydroglucose Unit the Building Block for Amylose and Amylopectin, the Constituents of Starch.

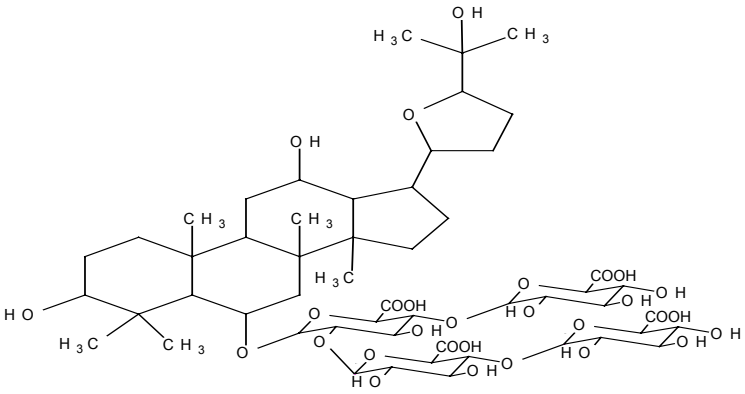


Figure 2.18 Molecular Structure of Saponin from *Camellia Sinensis* L.

2.3.2.5 Association of Polyphenols to Various Substances

It is well documented that catechins form several complexes in the reaction with different molecules like caffeine and polysaccharides. Studies of such molecular interactions are not only of scientific interest, but they are also of considerable practical interest (Ninomiya *et al.*, 1997).

2.3.2.6 Polyphenol - Caffeine Complexation

In aqueous media, polyphenols readily associate with caffeine forming hydrophobic complexes. These complexes are usually termed “tea cream” and can be observed in practice. “Tea cream” occurs when a freshly prepared hot tea infusion is cooled, hence, changing from

the familiar clarity into a turbid infusion (Rutter and Stainsbury, 1975; Ramachandran and Dhanaraj, 1988; Chao and Chiang, 1999; Robb and Brown, 2001; Liang *et al.*, 2002). Circunstancial evidence suggests that the complexation is principally mediated by hydrogen bonding and apolar interactions that manifest themselves in solution as hydrophobic effects. The stoicheiometry of these complexes is determinant to hydrophobicity and only when there are sufficient polyphenol hydroxyl groups to hydrogen bound with caffeine and vice-versa, hidrophobicity reaches a maximum. If then more caffeine is added, the excess caffeine interacts with the bonded caffeine decreasing the solubility of the complexes (Collier *et al.*, 1975; Martin *et al.*, 1986; Ninomiya *et al.*, 1997).

It is reported that in the complexation of caffeine with catechins that lack the ester linkages as (-)- EC, (+)- C and (-)-EGC tendency to form a complex is not as strong as with esterified catechin as (-)- ECG and (-)- EGCG. Since esterified catechins have extra hydroxyl groups compared with the non-esterified, extra intermolecular hydrogen bonding sites are provided for Caffeine. These more compact larger structures are believed to be less soluble structures than non-esterified catechins. Figures 2.19 and 2.20 show the lattice structure and in-plane hydrogen bonding of the caffeine – polyphenol complex (Gaffney *et al.*, 1986; Martin *et al.*, 1986; Robb and Brown, 2001).

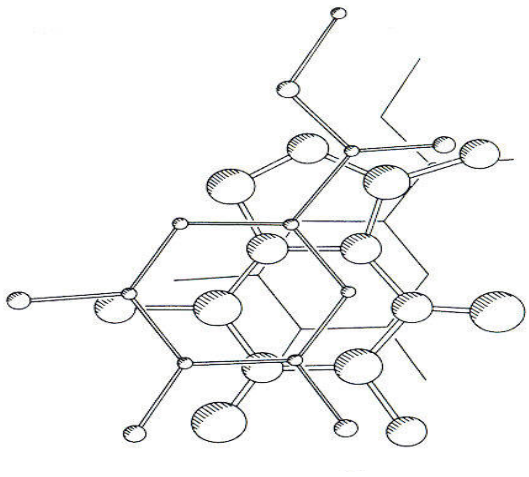


Figure 2.19 Layer Lattice Structure: Caffeine – Methyl Gallate (Martin *et al.*, 1986)

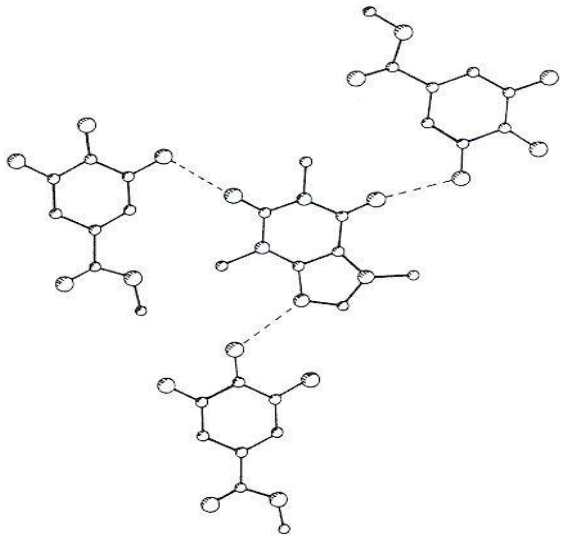


Figure 2.20 Planar Hydrogen Bonds: Caffeine – Methyl Gallate (Martin *et al.*, 1986)

2.3.2.7 Polyphenol – Polysaccharides Complexation

In aqueous media, the association of polyphenols with polysaccharides is enhanced, where the polysaccharide through the development of secondary structures can sequester the hydrophobic aryl residues of the polyphenolic substrate. Studies made with α– and β– cyclodextrins as model polysaccharides suggest that the complexation of the polyphenols with polysaccharides is via encapsulation or inclusion of the polyphenols in the saccharide cavity due to noncovalent interactions. These inclusion complexes are termed "clathrate compounds". Low temperature and high concentration are determinant for the hydrophobicity of the complex caffeine - polysaccharide. Hydrophobicity occurs when the concentration of both caffeine and polysaccharides approaches saturation. At lower concentrations, the solubility of the molecules is five to seven times greater than each molecule alone (Gaffney *et al.*, 1986; Yamamoto *et al.*, 2001).

2.3.2.8 Polyphenol – Caffeine – Polysaccharides Ternary System

The coexistance of polyphenol, caffeine and polysaccharides in aqueous media results in a partition of polyphenols between caffeine and polysaccharides. Therefore, the complexation

of polyphenols with caffeine is likely to be modified by the presence of polysaccharides (Gaffney *et al.*, 1986).

2.3.2.9 Biological and Pharmacological Activity

The biogical and pharmacological activity of green tea has been found by several authors as deriving from the polyphenols, particularly the catechins. Catechins possess a wide range of activities which include strong antioxidative capacity (Kajimoto, 1963; Yen and Chen, 1995), dental caries prevention (Sakanaka, 1997), renal failure prevention (Sakanara and Kim, 1997), anticarcinogenic (Kim and Masuda, 1997; Fujiki *et al.*, 1998), hypocholesterolemic (Yang and Koo, 1997), antimutagenic (Kada *et al.*, 1989; Kennedy *et al.*, 1998), and antiviral capacity (Hirose *et al.*, 1994).

Several activities displayed by catechins stem from their oxide radical scavenger capacity. The degree of scavenging capacity of individual catechins was examined by various authors. In several antioxidative biological assays, individual catechins show different antioxidation capacities. The different responses of individual catechins can be related to the bioavailability of each catechin. The differences have been also attributed to the difference in the reduction potentials, stabilities and relative partition coefficients of catechins in different media (Koketsu, 1997; Chung *et al.*, 1998; Robb and Brown, 2001).

2.3.2.10 Toxicoloy

There is no record toxicity of green tea polyphenols for Humans. The lethal doses for laboratory test mice is very low. The estimated LD_{50} value for females is 3090 mg kg^{-1} and more than 5000 mg kg^{-1} for males (Takahashi and Ninomiya, 1997).

2.3.3 *Isatis Tinctoria* L.

2.3.3.1 General

Isatis tinctoria L. belongs to the family Brassicaceae. The plant originates from Southeastern Europe and Southern Asia and is now distributed in Europe, Asia and Northern America. The vernacular names of the plant are woad, waid and pastel. The plant was formerly extensively cultivated in Western Europe and parts of Asia (List, 1976; Delmas *et al.*, 1992; Danz *et al.*, 2001).

Isatis tinctoria is an herbaceous biennial plant, deep-rooted and 0.6 to 1.2 m tall. The leaves growing round and out of the erect stem are lanceolate, have no petiole and are blue-green in colour. The flowers are yellow, small and numerous. The seeds are contained in one-celled winged pods, which turn dark brown or black at maturity (King, 1966; Callihan *et al.*, 1984; Delmas *et al.*, 1992).

Figure 2.21 *Isatis Tinctoria* L.

Isatis tinctoria is a plant that grows in temperate and subtropical climates. The plant flowers from May to July and the harvest of the leaves takes place when the plants begin to flower.

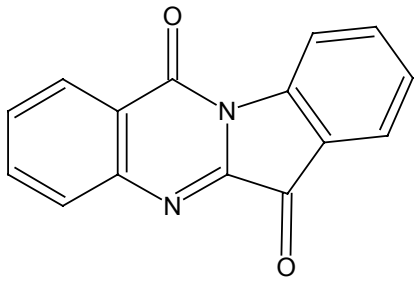
The harvest can be made four to five times per season with few weeks interval. After harvest, the leaves are dried (Callihan, 1984; Russel and Lorenz, 1988; Delmas *et al.*, 1992).

2.3.3.2 Chemical Composition

Isatis tinctoria contains an active principale that owns a number of biological and pharmacological properties. The main components of the plant are polysaccharides, indigo precursors, flavonoid glycosides and alkaloids (Delmas *et al.*, 1992). Alkaloids are due to their biological and pharmacological activity, the active principles under consideration in this work.

2.3.3.3 Active Principle

Isatis tinctoria contains an active principle the alkaloid tryptanthrin, which is an indolo[2,1-b] quinazoline-6-12-dione. There are large differences of tryptanthrin amount in the dry mass of *Isatis tinctoria*. The variation ranges from traces to about 0.01% of the dry mass. Tryptanthrin (Figure 2.22) is substance slightly basic, polar, however poorly water soluble but soluble in lipophilic solvents, it is light and heat sensitive and degrades along the time (Scovill *et al.*, 2002; Danz *et al.*, 2001).



Tryptanthrin

Molecular Formula = $C_{15}H_8N_2O_2$
Formula Weight = 248.236
Composition = C(72.58%) H(3.25%) N(11.29%) O(12.89%)
Molar Refractivity = 70.19 ± 0.5 cm^3
Molar Volume = 170.1 ± 7.0 cm^3
Parachor = 479.5 ± 8.0 cm^3
Index of Refraction = 1.762 ± 0.05
Surface Tension = 63.1 ± 7.0 dyne/cm
Density = 1.45 ± 0.1 g/cm^3
Dielectric Constant = Not available
Polarizability = 27.82 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass = 248.058578 Da
Nominal Mass = 248 Da
Average Mass = 248.241418 Da

Figure 2.22 Molecular Structure of Tryptanthrin from *Isatis Tinctoria* L. and Properties Calculated with ACD/Labs Software

2.3.3.4 Other Constituents

The polysaccharide contained in *Isatis tinctoria* is mainly cellulose and the flavonol glycoside is isoscoparine. Others components are saponins, chlorophyll, proteins and indigo precursors, mainly isatan b and indican. Celluloses, chlorophyll and indigo precursors make a large part of the plant's dry mass. Isoscoparine is present in reduced quantities. Cellulose, chlorophyll and isoscoparine are lipophilic and indigo precursors solubility in water ranges from poorly soluble to fairly soluble. Proteins de-natured by heating are insoluble. (Delmas *et al.*, 1992; Scovill *et al.*, 2002; Seilnacht, 2003; FAO, 2001). Indigo and indigo precursors, mainly isatan b (Figure 2.23), constitute undesired compounds in this work, due to the relative hydrophilic character and/or extremely high amounts in the plant.

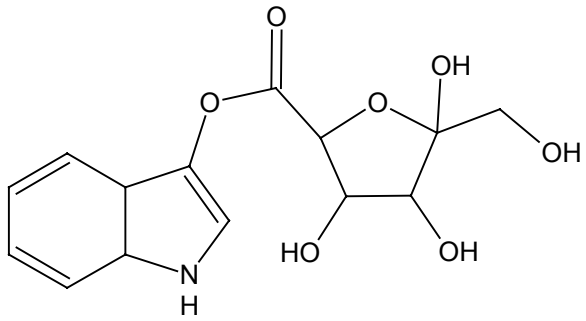


Figure 2.23 Molecular Structure of the Indigo Percursor Isatan B

2.3.3.5 Important Conversions of the Main Components

Isatis tinctoria contains large amounts of indigo percursores. In the past, indigo was an important pigment both in Western Europe and Asia. In these regions indigo was virtually the only source of blue dye. Indigo was extract from the plant in a soluble form by fermentation, oxidation and reduction (Grieve, 2003; Chaucer, 2003; Seilnacht, 2003). The process is as described in Figure 2.24.

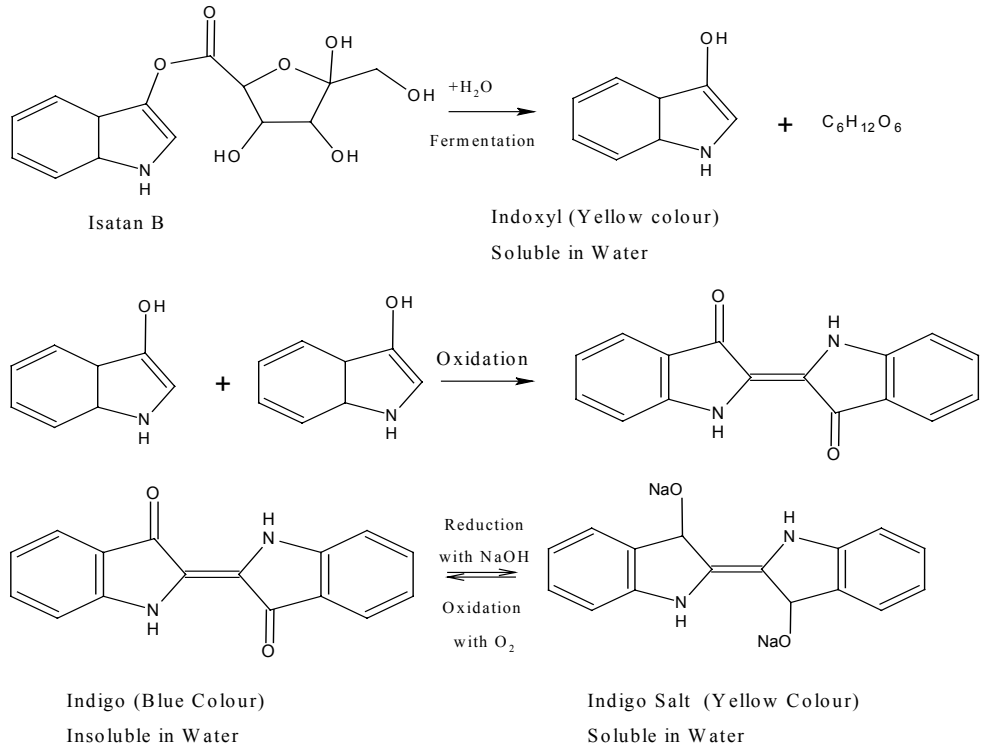


Figure 2.24 Conversion of Indigo Percursors to Indigo

2.3.3.6 Biological and Pharmacological Activity

Isatis tinctoria contains the alkaloid tryptanthrin that have biological and pharmalogical activity. Several studies indicate that tryptanthrin shows various types of activities as anti-ainflammatory, fungicidal and insecticidal activity, inhibition of nitric oxide synthase expression and potent anti-microbial activity. Typtanthrin antimicrobial activity includes action against the mycrobacterium tuberculosis the bacteria that causes tuberculosis, action against trypanosoma brucei the protozoa that causes the sleeping sickness and action against the plasmodium falciparium, the intracellular parasite that causes malaria (Hartleb and Seifert, 1994; Danz *et al.,* 2000; Scovill *et al.*, 2002).

2.3.3.7 Toxicity

Tryptanthrin is a new class of active principle, and there is no reliable data available on the toxicity of the compound.

2.3.4 *Cannabis Sativa* L.

2.3.4.1 General

The genus *Cannabis* belongs to the family Cannabinaceae. The plant is native to Central Asia and is now a widespread cultivar in subarctic, temperate, subtropical and tropical regions. There are two varieties of *Cannabis sativa*. *Cannabis sativa* L. spp. *sativa*, cultivated for fiber and oil production and *Cannabis sativa* L. spp. *indica* which is the variety with medical value and is mainly cultivated at the subtropical and tropical regions. Some vernacular names of *Cannabis sativa* L. spp. *indica*, further referred simply as *Cannabis sativa*, are marihuana, hemp, henf, granja and pot. The cultivation of the plant is in most countries clandestine, however there is legal cultivation for medical purposes (Duke, 1983; Schmidt, 1992; Reichling and Saller, 1999).

Cannabis sativa is an annual herb with a short stem and much branched. The leaves are dark green and the leaf arrangement is opposite, becoming alternate at the ends of the branches. Each leaf is palmated and usually divided, with five or seven toothed leaflets. The flowers are small and a plant may have male and female flowers, which are usually separated. The flowers germinate auxiliary and terminally and the male flowers form panicles, while the female form dense clusters at the ends of the branches. The male flowers have pole sacks and the female an ovary. The fruit is shining brown and contains a seed (Duke, 1983; Reed, 2002).

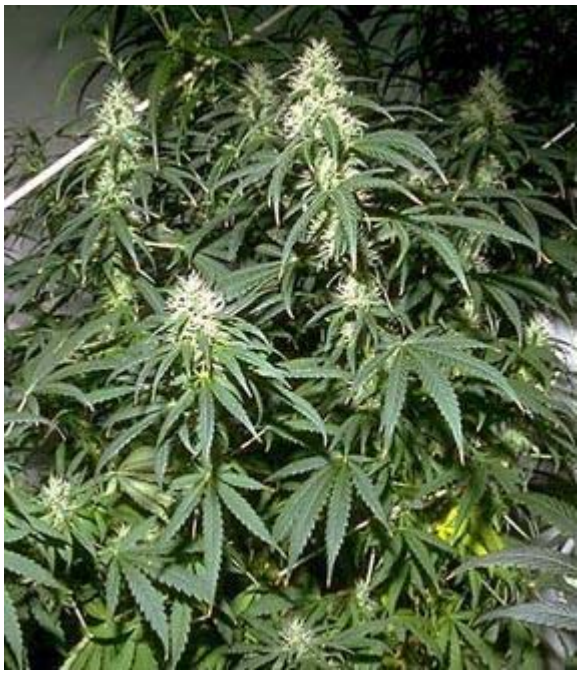


Figure 2.25 *Cannabis Sativa* L.

Cultivated *Cannabis sativa* is a plant very adaptable to soil and climatic conditions. The cultivation is more viable in subtropical and tropical regions where the cultivated plant flowers the year round. Harvest is made four to five months after planting and the harvested parts are the leaves of the tops of branches as well as the flowers and fruits of the female plants. After harvesting the plant is processed by drying. (Duke, 1983; Schmidt, 1992).

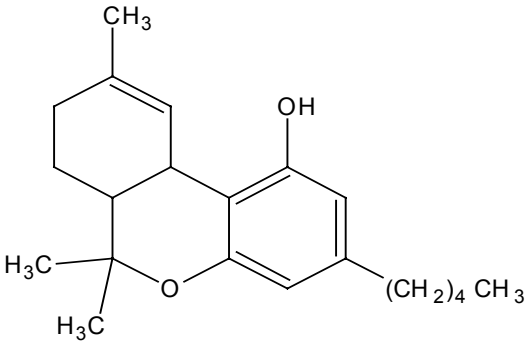
2.3.4.2 Chemical Composition

To date, more than five hundred components of *Cannabis sativa* have been identified and characterised. From them a large part also exists in other plants, with exception to cannabinoids, that possess particular biological and pharmacological activities. *Cannabis sativa* contains reduced amounts of cannabinoids. The main components of *Cannabis sativa* are monoterpenes, sesquiterpenes, betacaryophylen, humulen α-pinen, β-pinen, limonene and myrcen. For being the active principles under consideration in this work, cannabinoids will be dealt in detail in the next paragraph (Grotenhermen, 1997). A general description of the other components will be made further in this chapter.

2.3.4.3 Active Principle

Cannabis sativa contains more than sixty different cannabinoids, regarded as C_{21} terpenes, their carboxylic acids and transformation products. The major cannabinoids are Δ^9– tetrahydrocannabinol, cannabinol and cannabidiol, cannabivarin, canabigerol, cannabicylol, cannabicromene, cannabidiolic acid, cannabinolic acid and cannabielsoic acid (Mechoulam, 1970, and 1973; Gaoni and Mechoulam, 1971; Agurell *et al.*, 1984).

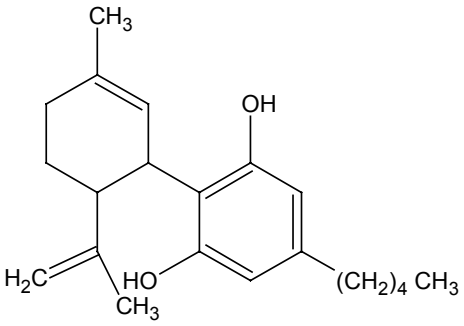
The amount of cannabinoids vary considerably from sample to sample and is dependent on plant genetics, region of origin, growing conditions, processing after harvest and age of the sample. The proportions between the cannabinoids also vary from sample to sample. In the usual sample, Δ^9– tetrahydrocannabinol concentration ranges from 0.3 to 4% of dry mass, however, cultivated and selected cannabis sativa may contain 15 to 25% of Δ^9– ltetrahydrocannabinol. The concentration of cannabinol, cannabidiol and cannabivarin in the usual plant can reach each of them, 3% of the dry mass. Cannabigerol, cannabichromene, cannabicylol, cannabinolic acid and cannabielsoic acid contents varies between 0.1 and 0.2% of the dry mass. The main active principle of *Cannabis sativa* is Δ^9– tetrahydrocannabinol (Δ^9– THC), which is psychoactive, followed by cannabinol (CBN) and cannabidiol (CBD) which are not psychoactive, but nevertheless have other type of activity. Δ^9– tetrahydrocannabinol, cannabinol and cannabidiol are light sensible, heat sensible, unstable in strongh acidic and alkaline media, oxidizable, degrade with time and are lipophilic. (Mechoulam. 1970; Agurell *et al.*, 1986; Hollister, 1986; Adams and Martin, 1996; Saller, 1999).



Δ^9-Tetrahydrocannabinol

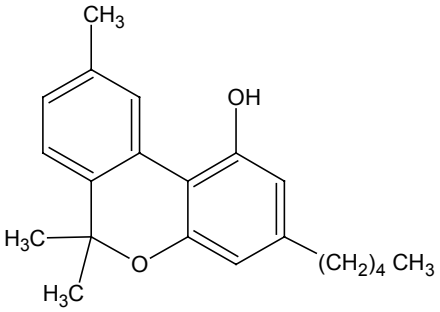
Formula Weight = 314.462
Composition = C(80.21%) H(9.62%) O(10.18%)
Molar Refractivity = 95.49 ± 0.3 cm^3
Molar Volume = 309.7 ± 3.0 cm^3
Parachor = 753.7 ± 6.0 cm^3
Index of Refraction = 1.528 ± 0.02
Surface Tension = 35.0 ± 3.0 dyne/cm
Density = 1.015 ± 0.06 g/cm^3
Dielectric Constant = Not available
Polarizability = 37.85 ± 0.5 $10^{-24} cm^3$
Monoisotopic Mass = 314.22458 Da
Nominal Mass = 314 Da
Average Mass = 314.469663 Da

Molecular Formula = $C_{21} H_{30} O_2$



Cannabidiol

Molecular Formula = $C_{21}H_{30}O_2$
Formula Weight = 314.462
Composition = C(80.21%) H(9.62%) O(10.18%)
Molar Refractivity = 97.01 ± 0.3 cm^3
Molar Volume = 306.6 ± 3.0 cm^3
Parachor = 769.0 ± 6.0 cm^3
Index of Refraction = 1.545 ± 0.02
Surface Tension = 39.5 ± 3.0 dyne/cm
Density = 1.025 ± 0.06 g/cm^3
Dielectric Constant = Not available
Polarizability = 38.45 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass = 314.22458 Da
Nominal Mass = 314 Da
Average Mass = 314.469663 Da



Cannabinol

Molecular Formula = $C_{21}H_{26}O_2$
Formula Weight = 310.430
Composition = C(81.25%) H(8.44%) O(10.31%)
Molar Refractivity = 94.87 ± 0.3 cm^3
Molar Volume = 292.5 ± 3.0 cm^3
Parachor = 743.0 ± 4.0 cm^3
Index of Refraction = 1.561 ± 0.02
Surface Tension = 41.6 ± 3.0 dyne/cm
Density = 1.061 ± 0.06 g/cm^3
Dielectric Constant = Not available
Polarizability = 37.61 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass = 310.19328 Da
Nominal Mass = 310 Da
Average Mass = 310.43776 Da

Figure 2.26 Molecular Structure of Δ^9 – Tetrahydrocannabinol, Cannabinol and Cannabidiol from *Cannabis Sativa* L. and Respective Properties Calculated with the ACD/Labs Sofware

2.3.4.4 Other components

The main components of *Cannabis sativa* are the monoterpenes, sesquiterpenes, betacaryophylen, humulen α– pinen, β– pinen, limonene, myrcen, the celluloses, proteins and chlorophyll. These components make a high percentage of the plant's dry mass.

2.3.4.5 Important Reactions of Active Cannabinoids

The inter-conversion and degradation of the active cannabinoids when exposed to different conditions, is a matter of great interest as it can alter the quantities and proportions of the active principles in the plant extracts and thus, the activity of the cannabinoids as a whole (Fairbairn *et al.*, 1976; Narayanaswami *et al.*, 1978). The conditions that affect the stability of active cannabinoids are described in 2.3.4.3. Mechoulam (1970) demonstrated that in the presence of oxygen and a strong alkaline media, cannabidiol is oxidized to monomeric and dimeric hydroquinones (Figure 2.26) and that Δ^9– tetrahydrocannabinol converts to cannabinol (Figure 2.27).

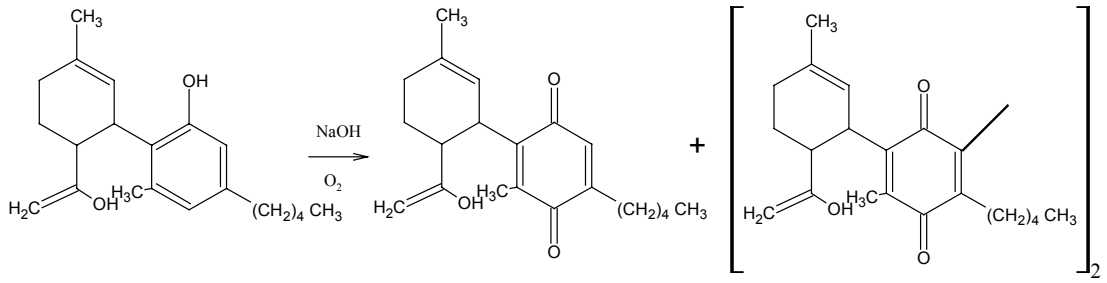


Figure 2.27 Conversion of Cannabidiol into Monomeric and Dimeric Hydroquinones

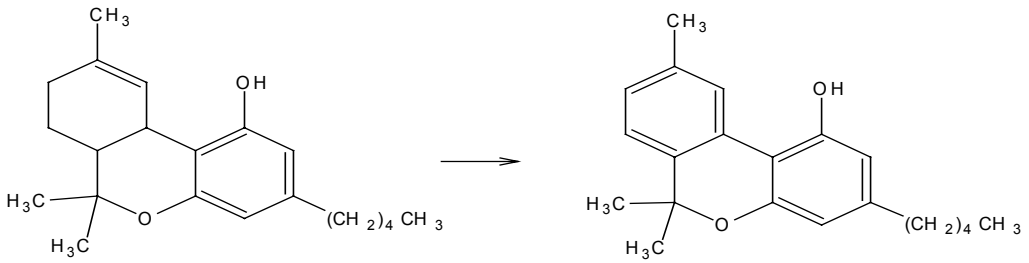


Figure 2.28 Conversion of Δ^9–Tetrahydrocannabinol into Cannabinol

2.3.4.6 Biological and Pharmacological Activity

The biological and pharmacological activity of orally ingested cannabinoids is well documented in the literature. The effects of active cannabinoids derive from specific effects of each cannabinoid. The primary active principle of *Cannabis sativa* is the psychoactive Δ^9–THC, CBN and CBD are not psychoactive. The psychoactivity of Δ^9–THC is caused by the activation of the cannabinoid receptors in the brain and Δ^9–THC alone can reproduce the psychoactive effect of the *Cannabis sativa* plant. Sometimes, CBN and CBD are present in such quantities that they might modulate or strengthen the effects of Δ^9– THC; or cause effects of their own (Mechoulam, 1970; Hampson *et al.*, 1998; Saller, 1999).

Several medical trials on the effects of the active principles of *Cannabis sativa* showed the potential of these compounds to treat and alleviate the following diseases and conditions (Carlini *et al.*, 1973; Heim, 1982; Formukong *et al.*, 1988a and 1988b; Thomas *et al.*, 1996; Hampson *et al.*, 1988; Beaver *et al.*, 1997; Malfait *et al.*, 2000; Strauss, 2000):

- Alleviation of intraocular pressure associated with glaucoma and antiemetic for the alleviation of nausea and vomiting associated with anticancer therapy
- Appetite stimulating effect to treat nervous anorexia and cachexia associated with AIDS wasting syndrome
- Treatment of neurological and movement disorders caused by the over stimulation of brain receptors concerned with memory, cognition and motor coordination
- Anticonvulsive to alleviate convulsions associated with epilepsy
- Analgesic and anti-inflammatory, to treat chronic inflammatory diseases like arthritis
- Anticataleptic to alleviate psychotropic effects

The first three effects are attributed to Δ^9– tetrahydrocannabinol and to a lesser extent to cannabinol, while the latter are attributed to Δ^9– tetrahydrocannabinol, cannabinol and cannabidiol. The activity of cannabidiol is in those cases compared or superior of that showed by Δ^9– tetrahydrocannabinol. An effect attributed to cannabidiol alone is the inhibition of the adverse cataleptic effect of Δ^9– tetrahydrocannabinol. Research on the potential effects of cannabinoids is still under way and the goal of the cannabis research is to draw a separation

between specific biological and pharmacological activities of cannabinoids and the undesirable psychotropic effects.

2.3.4.7 Toxicology

The toxicity of cannabinoid extracts is surrounded by some controversy. There is no record of adverse effects caused by cannabinol and cannabidiol. Medical studies done on healthy humans with the ingestion of pure Δ^9– tetrahydrocannabinol, or where only Δ^9– tetrahydrocannabinol was taken in consideration, indicate that despite the potent psychoactivity of Δ^9– tetrahydrocannabinol, the compound has remarkably low lethal toxicity. Lethal doses in Humans is not known. The most important unwanted acute effects of Δ^9– THC are anxiety and panic attacks, increased heart rate, changes in blood pressure, conjuntival injection, changes in behaviour, temporary mental impairment for some individuals and dependence. However, the adverse effects of Δ^9– tetrahydrocannabinol do not preclude the legitimate therapeutic use of cannabinoids. The effects of smoked cannabis are to date, a matter of great controversy and a number of studies are still under way (Mechoulam, 1970; Benowitz and Jones, 1981; Jones *et al.*, 1981; Fehr and Kalant, 1983; Jones, 1987; Grotenhermen, 1999).

3. Materials Equipments and Methods

3.1 Materials

3.1.1 Samples of Plant Materials

The *Calendula officinalis* sample also designated *Calendula* flos was supplied by the Raps Institute, Freising, Germany and was composed of dried and grounded flower heads of the *Calendula officinalis* plant. The sample was yellow greenish in colour and had a characteristic smell. The storage of the sample was at room temperature and in the dark.

The *Camellia sinensis* sample, also designated as green tea, was supplied by the Raps Institute, Freising, Germany and was constituted by dried leaves of the *Camellia sinensis* plant. The sample was dark olive green in colour. Sample storage was at room temperature and in the dark.

Isatis tinctoria samples were supplied by the Raps Institute, Freising, Germany and by the Handelsbüro Stiller, Neudietendorf, Germany. The sample supplied by the Raps Institute comprised dried and roughly ground leaves of *Isatis tinctoria* and was dark olive green in colour. The second sample encompassed 3 batches. The first batch comprised dried and finely grounded leaves of *Isatis tinctoria*. The second batch consisted of slightly dried leaves of *Isatis tinctoria*. Both samples were dark olive green in colour. The third sample comprised dried, fermented and roughly ground leaves of *Isatis tinctoria*. The colour of the sample was dark brown. All samples were stored at room temperature and in the dark.

Cannabis sativa samples were supplied by the Thueringer State Institute of Agriculture, Jena, Germany and by the BAFA, Malsch, Germany. The sample supplied by the Thüringer State Institute of Agriculture consisted on dried branches of cannabis plants and was light brown in colour. The sample supplied by BAFA consisted of roughly grounded and slightly dried leaves of branch tops, flowers and fruits. The colour of the sample was dark olive green and had a strong and unpleasant smell. All samples were stored at room temperature and in the dark.

3.1.2 Standards

The standard used for characterisation and quantification of faradiol esters present in *Calendula* flos was the internal standard lupeol acetate. Lupeol acetate, not available for sale at the time of the experiments can be synthesized by acetilation of lupeol. Lupeol was bought from Sigma, Germany. The synthesis and cleaning of lupeol acetate as well as the preparation of the stock solution were performed in accordance with a modification of the method developed by Zitterl-Egleseer *et al.* (2000). The cleaning, synthesis and preparation of the stock solution were as follows:

Cleaning of *Calendula* flos

1 g of *Calendula* flos was extracted with 10 ml of DCM in the ultra-sonic bath for 30 min. The mixture was subsequently centrifuged at 2000 rpm for 10 min, the solids were decanted and the liquid filtrated under vacuum with filter paper nr. 2, to eliminate the remaining solids. The resulting solution was evaporated in the rotating evaporator under vacuum at 40 °C and diluted in 200 ml of a solution 8 hexan : 2 ethyl acetate, then fed to a chromatographic column containing 50 g of silica gel. The obtained clear solution was evaporated in the rotating evaporator under vacuum at 40 °C. The resulting oily substance, yellow in colour was diluted in 200 µl DCM. A sample was characterised by HPLC-UV, HRGC-FID and HRGC-ECNI/MS

Synthesis of Lupeol Acetate

10 mg of lupeol were dissolved in 1 ml of water free pyridin and 1 ml of anidrous acetic acid. The solution was left to rest at room temperature for 48 hours. After resting, 50 ml of cold water were added to the solution. The resulting mixture was shacked tree times with 100 ml DCM and the organic phase evaporated under vacuum in the rotating evaporator at 40°C. The obtained white powder constituted the internal standard lupeol acetate. A sample of lupeol was diluted in DCM and characterised by HRGC-FID and HRGC-ECNI/MS. At several steps of the synthesis, samples were characterised with HPLC-UV, HRGC-EI/MS and HRGC-ECNI/MS. A sample of the internal standard was characterised with UV-Vis, HPLC-UV, HRGC-EI/MS and HRGC-ECNI/MS

Preparation of the Stock Solution

200 µl of cleaned *Calendula* flos was added to 200 µl of lupeol acetate (3 mg/ml). This mixture was given to a TLC plate by means of a capillary tube and the TLC plate was placed in a

TLC camera containing a solution 8 hexan : 2 ethyl acetate. After column chromatography, the layer between hR_f 30 and hR_f 80 was scratched and 50 ml of DCM were added. The mixture was shacked, 2.5 g of Na_2SO_4 added and the mixture was shacked again. The solution was than centrifuged for 20 min at 2000 rpm. The organic phase was decanted and evaporated in the rotating evaporator at 40 °C under vacuum to 200 µl. This solution constituted the stock solution, which was divided in several parts and each part diluted to give solutions with dilutions between 1-20 times the stock solution concentration. A sample of that solution was characterised with HRGC-FID and HRGC-ECNI/MS. Samples of the solutions at various dilutions were quantified with HPLC-UV.

The standards used for characterisation and quantification of catechins present in green tea were catechin and epigallocatechin gallate standards. Caffeine standard was used for the characterisation and quantification of unwanted caffeine present in the sample. Cathecin and EGCG standards were bought from Sigma, Germany and caffeine standard was bought from Aldrich, Germany. The stock solution for catechins calibration curve was prepared by diluting 2 mg of catechin standard in 10 ml MeOH. The stock solution was than divided in several parts and each part further diluted to give solution concentrations between 2 – 200 mg l^{-1}. One sample was characterised with UV-Vis and HPLC-UV, the other samples at various dilutions were quantified with HPLC-UV. The same procedure was used for caffeine. A stock solution for the characterisation of epigallocatechin gallate by NMR was prepared by dissolving 5 mg of EGCG in MeOH-d_4.

The standard used for characterisation and quantification of tryptanthrin present in *Isatis tinctoria* was tryptanthrin standard and was supplied by Prof. Dr. M. Hamburger, Friedrich-Schiller University, Jena, Germany. The stock solution for tryptanthrin calibration curve was prepared by diluting 2.5 mg tryptanthrin standard in 5 ml MeOH. The stock solution was than divided in several parts and each part was further diluted to give concentrations between 1 – 500 mg l^{-1}. One sample was characterised with UV-Vis and HPLC-UV and the samples at various dilutions were quantified with HPLC-UV. A stock solution for the characterisation of tryptanthrin by FTIR was prepared by diluting 0.5 mg tryptanthrin standard in chloroform.

The standards used for the characterisation and quantification of cannabinoids present in *Cannabis sativa* were CBD, CBN and THC standards. These standards were bought from Sigma, Germany as stock solutions of 1 mg standard per millilitre of MeOH. The calibration

curves for the quantification were prepared by dividing 200 µl of the stock solution in several parts and further diluting each part to give concentrations between 10 – 500 mg l^{-1}. One sample was characterised with UV-Vis, HPLC-UV, HRGC-EI/MS and the other samples at various dilutions were quantified with HPLC-UV.

3.1.3 Chemicals and Solvents

- Acetic Acid: p.a., Merck
- Aceton: p.a, Merck
- Acetonitril: HPLC grade, Merck
- Anidrous Acetic Acid: p.a., Merck
- Chloridric Acid: (1 M and 0.1 M Sol.), p.a., Fluka
- Chloroform: p.a., Merck
- Dichlormethan: HPLC grade, Merck
- Ethyl Acetate: HPLC grade, Merck
- Glycerin: p.a., Merck
- Hexan: HPLC grade, Merck
- Lupeol: HPLC grade, Sigma
- Microliter pippetes : 10 – 100µl; 100 – 1000 µl, Fischer Labworld
- Methanol: HPLC grade, Merck
- Nitrogen Gas: 5.0, Messer Griesheim GmbH
- Pyridin: pur. 99.8%, Fluka
- Saponin: pur., Fluka
- Sodium Chloride: water free, p.a. grade, Merck
- Sodium Hydroxide: (1 M and 0.1 M Sol.), p.a., Fluka
- Sodium Sulfat: water free grade, p.a., Merck
- Silica Gel: 60 (0.053 – 0.200 mm), Merck
- Trifluor Acetic Acid: p.a., Merck
- Water (bi-distilled): CTA Distillation System
- Water (de-ionised): CTA De-ionozation System

3.1.4 Miscellaneous Materials

- Filter Paper: S&S circles Ø 90 mm Schleicher and Schuell
- Filter Paper: S&S folded filters Ø 185 mm Schleicher and Schuell
- Glassware: TLC Camera, Condensers, pasteur pipets and general laboratory glassware
- HPLC Column: Lichrospher 100 RP-18, 5µm, 250 mm X 4 mm ID, Phenomenex
- HPLC Column: Spherisorb ODS2, 3 µm, 150 mm X 4.6 mm ID, Waters
- Membran Filters: opti-solv filters (1 ml and 2 ml Vol.) WiCom GmbH
- Microliter pipets: Volumes: 0 – 10µl; 10 – 100 µl; 100 $^{-1}$000 µl, Brand
- Microliter Syringes : Volumes : 5 and 50 µl, Hamilton
- Security Gard Column: C-18, 4 X 3 mm, Phenomenex
- TLC plates: Silica Gel 60, F_{254}, 5 X 20 cm, 0.25 mm, Merck
- Water Pump: Fisher Labworld

3.2 Foam Fractionation Unit and Equipments

3.2.1 Foam Fractionation Unit

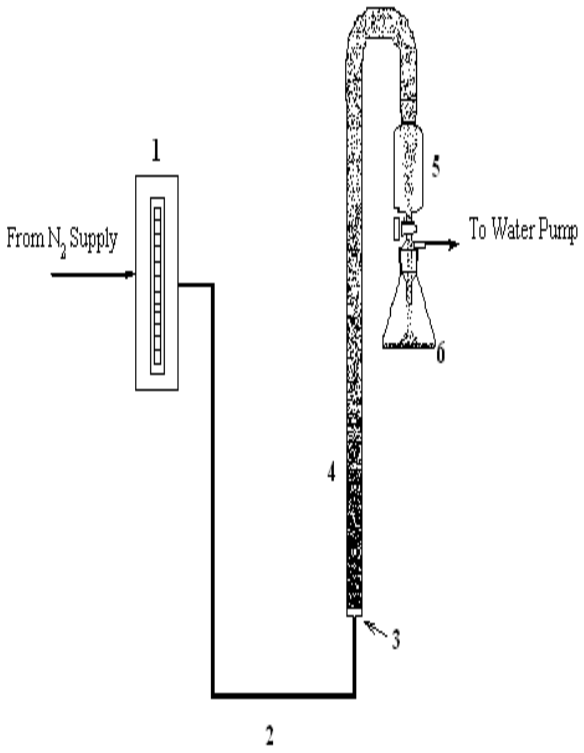


Figure 3.1 Foam Fractionation Unit.

(1) Flow-meter, (2) Pipe, (3) Frit, (4) Glass Column, (5) +(6) Foam Breaker

The columns used for foam fractionation were glass columns with various dimensions as presented in Table 3.1 below.

SIZE	EXTERNAL Ø [mm]	INTERNAL Ø [mm]	LENGTH [cm]	LENGTH [cm]
Small	22.2	18.5	130.0	65.0
Medium	32.0	29.2	130.0	65.0
Large	50.0	45.0	165.0	82.5

Table 3.1 Dimensions of Glass Columns for Foam fractionation

3.2.2 High Performance Liquid Chromatography

- HPLC-UV:
  Pump: Gynkosoft 480
  Detector: Merck Hitachi, L – 4000A
  Injector: Rheodyne 8125 with 20 µl injection port
  Computer Software: Gynkosoft 5.32

- HPLC-UV/DAD:
  Pump: Merck Hitachi, Lachrom L – 7100
  Detector: Merck Hitachi, Lachrom L – 7450
  Autosampler: Merck Hitachi, Lachrom L – 7200
  Degasser: Merck
  Interface: Merck Hitachi, Lachrom D – 7000
  Column Oven: Merck, Lachrom L – 7350
  Peltier Cooling Module: Merck, Lachrom L – 7350/735l
  Computer Software: D – 7000 HSM Version 3.1.1

3.2.3 Gas Chromatography

- HRGC-FID: Dani 8500
  Computer Software: Maestro 2/3

- HRGC-EI/MS
  GC: MFC 500, Carloerba/Krabs
  MS: MSD, HP 5970
  Computer Software: Maestro 2/3

- HRGC-ECNI/MS
  GC: HP 5890 Series II
  MS: Finningan, Model 8200
  Computer Software: Maspec

3.2.3 UV-Vis Spectroscopy

- UV-Vis: Perkin Elmer Lambda 16
  Computer Software: PECSS

3.2.4 Nuclear Magnetic Resonance Spectroscopy

- NMR: Bruker, 2000 Aspect, AC 250
  Computer Software: Mestrec 23

3.2.5 Fourier Transform Infrared Microspectroscopy

- FTIR Spectrometer: Bruker, IFS 28B
  Irscope: Bruker

Computer Software: OPUS 3.1

3.2.6 Other Equipments

- Centrifuge: Hettich, Rotanda
- Electronic Balances
- Flowmeter: Porter Instrument Company
- Gas Supply: Bottled prepurified nitrogen gas
- Heaters
- Manometer: Ewo DIN EN 585
- pH-meter: 761 Calimatic
- Rotating Evaporators: Büchl
- Shaker
- Ultra-sonic Bath: Edmund Bühler, SM 25
- Water Purification Equipment: Millipore, Milli-Q Plus 185
- Ultra-sonic Bath

3.3 Methods

3.3.1 Analytical Characterisation and Quantification

3.3.1.1 Faradiol Esters

- UV-Vis Conditions

Vial Type:	Glass Vial
UV Range:	200 – 400 nm
Sample Volume:	1.5 ml
Solvent:	MeOH

- HPLC-UV (Method as per Zitterl-Egleseer *et al.*, 2000)

Column:	Lichrospher RP – 18, 250 X 4 mm ID, 5 µm

Security Guard Column:	C – 18, 4 X 3 mm, Phenomenex
Solvent A:	MeOH
Solvent B:	Aqueous Trifluor Acetic Acid pH 4
Gradient:	95% A + 5% B to 100% A +0% B in 50 min, than 100% A + 0% B for 45 min
Flow rate:	1.5 ml min^{-1}
Wavelenght:	210 nm
Injected Volume:	20 µl

- HRGC-FID

Column:	DB5 30 m X 0.25 mm ID, 0.25 µm
Carrier gas:	N_2
Splitt:	32 ml min^{-1}
Injected Volume:	1 µl
Injector T°:	260 °C
Detector T°:	280 °C
Oven T° Maximum:	300 °C
Temperature Program:	100 °C (3 min) 10 °C/min 280 °C (15 min)

- HRGC-EI/MS (Full Scan) Conditions

Column:	DB5 30 m X 0.25 mm ID, 0.25 µm
Carrier gas:	He
Injected Volume:	1 µl
Injector T°:	260 °C
Transfer Line T°:	280 °C
Ion Source T°:	250 °C
Electron Beam Energy:	70 eV
Pressure:	4 10^{-5} Bar
Temperature Program:	100 °C (3 min) 10 °C/min 280 °C (15 min)

- HRGC-ENCI/MS (SIM Mode) Conditions

Column:	DB5 30 m X 0.25 mm ID, 0.25 µm
Carrier gas:	He
Injected Volume:	1 µl
Injector T°:	260 °C
Transfer Line T°:	230 °C
Ion Source T° :	200 °C
Electron Beam Energy:	120 eV
Ptressure	4 10^{-5} Bar
Temp. Prog:	100 °C (3 min) 10 °C/min 280 °C (35 min)

3.3.1.2 Catechins

- UV-Vis Conditions

Vial Type:	Glass Vial
UV Range:	200 – 400 nm
Sample Volume:	1.5 ml
Solvent:	MeOH

- HPLC-UV Conditions (Method as per Kuhr and Engelhardt, 1991)

Column:	Lichrospher 100 RP – 18, 250 X 4 mm ID, 5 µm
Security Guard Column:	C – 18, 4 X 3 mm, Phenomenex
Solvent A:	Acetic Acid (2%, aqueous)
Solvent B:	Acetonitril
Gradient:	88% A, 6 min isocratic, in 5 min to 75% A, 15 min isocratic, at starting conditions 15 – 20 min equilibrium
Flow rate:	1 ml min^{-1}
Wavelenght:	278 nm
Injected Volume:	20 µl

- NMR Conditions (Method as per Ninomiya *et al.*, 1997)

Frequency:	^{1}H – 250 MHz; ^{13}C – 260.5 MHz
Sample Volume:	1 ml
Solvent:	MeOH-d_4

3.3.1.3 Tryptanthrin

- UV-Vis Conditions

Vial Type:	Glass Vial
UV Range:	200 – 400 nm
Sample Volume:	1.5 ml
Solvent:	MeOH

- HPLC-UV/DAD Conditions (Method as per Danz *et al.*, 2001)

Column:	Lichrospher 100 RP – 18, 250 X 4 mm ID, 5 µm
Security Guard Col.:	C – 18, 4 X 3mm, Phenomenex
Solvent A:	Acetonitril
Solvent B:	Acetic Acid (2% aqueous)
Gradient:	40% A over 10 min isocratic, rinsing step 40% A to 95% A over 6 min and 95% A for 4 min, added at end of isocratic run
Flow rate:	1 ml min^{-1}
Wavelenght:	254 – 387 nm
Injected Volume:	20 µl

- FTIR Conditions (Method as per Honda *et al.*, 1980)

Scan Speed:	20 KHz
Resolution:	6 cm^{-1}
Sample Volume:	1 ml
Solvent	Chloroform

3.3.1.4 Cannabinoids

- UV-Vis Conditions

Vial Type:	Glass Vial
UV Range:	200 – 400 nm
Sample Volume :	1.5 ml
Solvent:	MeOH

- HPLC-UV Conditions (Method as per Brenneisen, 1984)

Column:	Spherisorb ODS2, 150 mm X 4.6 mm ID, 3 µm
Security Guard Column:	C – 18, 4 X 3 mm, Phenomenex
Solvent A:	MeOH
Solvent B:	Water
Solvent C:	Acetic Acid
Isocratic:	85% A; 14.2% B; 0.8% C isocratic for 20 min each 10 runs, rinsing with MeOH
Flow rate:	1.5 ml min^{-1}
Wavelenght:	230 nm
Injected Volume:	20 µl

- HRGC-EI/MS (Full Scan) Conditions

Column:	DB5 25 m X 0.25 mm ID, 0.25 µm
Carrier gas:	He
Injected Volume:	1 µl
Injector T°:	260 °C
Transfer Line T°:	230 °C
Ion Source T°:	200 °C
Electron Beam Energy:	70 eV
Pressure:	4 10^{-6} Torr
Temperature Program:	150 °C (2min) 15 °C/min 280 °C (10min)

3.3.2 Extraction

The extraction of active principles from those plants under investigation was attempted by several methods, using water as a solvent because foam fractionation is realised in aqueous dilute solution of the active principles. If this extraction failed, extraction by organic solvents with affinity to the active principles was used, followed by the evaporation of the solvents, to obtain a dried extract with high contents of active principles. The extraction of such dried extracts was once again performed with water.

Samples of plant materials were primarily extracted using water by stirring, extraction with reflux and ultra-sonic bath extraction. The water used for extraction was bi-distilled or di-ionised. Extraction took place with water at various temperatures and the pH's varied from 2 to 12. When this resulted ineffective, the extraction was performed with organic solvents by stirring, extraction with reflux, ultra-sonic bath extraction and soxhlet extraction, at room temperature or with heating. The method of choice was the one that provided for higher extraction of the active principles and lower extraction of ballast substances that might negatively influence the foaming process. The extract was then evaporated to dryness and prior to the foam fractionation, was once again extracted with water.

3.3.2.1 Extraction of Faradiol Esters

Extraction of faradiol esters from *Calendula* flos using water as a solvent failed and thus, extraction with organic solvents was attempted. The method of choice was a modification of a method developed by Zitterl-Eglseer *et al.* (2000). The resulting dried extract was once more extracted with water by stirring. A description of the method is as follows:

1 g of grounded *Calendula* flos were extracted with 10 ml of DCM in the ultra-sonic bath for 30 min. Solids were separated from the liquid by filtration. This solution was evaporated to dryness in the rotating evaporator under vacuum at 40 °C. The resulting dried extract was frozen and prior to foam fractionation, was once more extracted with bi-distilled water at 100 °C, in a heated magnetic stirrer, for 5 min The aqueous extract was then vacuum filtered to eliminate fine solids.

3.3.2.2 Extraction of Catechins

Catechins from green tea leaves were extracted with di-ionised water by stirring and with heating in accordance with a modification of the method developed by Kuhr and Engelhardt (1991). A description of the method is presented below. For control purposes, experiments were made to extract catechins from green tea using organic solvents and by the method developed by Todd (1994).

1 g of green tea leaves was extracted for 15 min with 100 ml of boiling de-ionised water, with a heated magnetic stirrer. The brown solution was filtrated under vacuum to eliminate fine solids, let to cool and the total volume was adjusted to 100 ml, with de-ionised water.

3.3.2.3 Extraction of Tryptanthrin

Tryptanthrin extraction from *Isatis tinctoria* with water failed. Therefore, extraction with organic solvents was attempted. Tryptanthrin extraction could only be achieved by reflux using MeOH as a solvent (Danz *et al.*, 2001). The dried extract was again extracted with water by stirring and vacuum filtered. The description of the method is as follows:

1 g of dried and fermented leaves of *Isatis tinctoria* sample was extracted under reflux tree times with 300 ml MeOH (70% v/v) for one hour. The solution so obtained was filtrated and than evaporated to dryness in the rotating evaporator under vacuum and stored in a freezer at –28 °C. Prior to foam fractionation, the dried extract was extracted for 5 min with bi-distilled water at pH 6.5 and 100 °C, in a heated magnetic stirrer. The aqueous extract was then let to cool and vacuum filtered to eliminate fine solids.

3.3.2.4 Extraction of Cannabinoids

Cannabinoids extraction with water from *Cannabis sativa* provided for very low cannabinoid extractions. Extraction with organic solvents was then tried. The method of choice was the extraction by ultra-sonic bath using MeOH as solvent (Brenneisen, 1984). The dried extract was then extracted with water at pH 4. A description of the method is as follows:

1 g of leaves of branch tops, flowers and fruits was dried in a oven at 40 °C, finely ground and extracted with 5 ml of MeOH in the ultra-sonic bath for 15 min The organic solution was filtrated and evaporated to dryness at the rotating evaporator under vacuum and stored in a freezer at –28°C. Prior to foam fractionation, the dried extract was extracted for 5 min with bi-distilled water with pH 4 and 100°C in a heated magnetic stirrer, let to cool and filtered with vacuum to eliminate fine solids.

3.3.3 Foam Fractionation

The enrichment of active principles from the plants under investigation was attempted by performing foam fractionation of those active principles aqueous dilute solutions prepared from plant extracts. The process was realised at various parameters and conditions like various solute and surfactant concentrations, viscosities, pH's, ionic strengths, hydrophobicities, gas flow rates, heights of liquid pool, foam heights and internal reflux ratios. The foam fractionation process took place in the foam fractionation unit as presented in Figure 3.1. Once an enrichment of the active principles was achieved, the process was optimised, by adjusting all parameters and conditions. The optimised process provided for an improvement of the enrichment.

Aqueous dilute solutions of the plant extracts utilised for foam fractionation experiments were prepared by adjusting the volumes of plant extracts prepared as described at 3.3.1, with bi-distilled or di-ionised water to obtain solutions of pretended concentrations and volumes. For those solutions with a weak foaming capacity, surfactants were added previous to foaming. In accordance with solute molecule properties, additives like organic solvents of different polarities, viscosity enhancers, electrolytes, acids and alkalis could be added to the initial solution.

Long glass columns with small cross sections were used for the foam fractionation process. Table 3.1 presents the sizes of the glass columns used. The aqueous dilute solutions were fed to those columns before the initiation of the process. The gas chosen to produce the bubbles should be inactive to the materials present in solution and thus, dried nitrogen gas was used. The gas supplied by a cylinder, was equipped with a manometer and the bubbles were generated by injecting nitrogen gas through a glass frit attached to the bottom of the glass column, into the liquid pool. The manometer measured the pressure of the gas delivered and the flow

rate was measured by the flow-meter. The flow rate could be by adjusting the flow-meter scale.

Column operation was in a simple batch mode, with low gas flow rates, in order to permit long residence time and bubble-liquid contact, higher foam height, maximization of drainage and internal reflux. At the initiation of the process, the gas flow rate was at its minimum and the foam exiting the top of the column was collected in the foam breaker. When no more foam exited the column, the flow rate was gradually increased and more foam collected. The process terminated when increase in flow rate did not produce more foam. The solution might be consecutively foamed by adding more surfactant and other additives. Foam samples were drawn over known intervals of time or over each consecutive foaming. The foam exiting the column and collected in the foam breaker was broken using moderate vacuum generated by a water pump and/or by addition of organic solvents. At the end of each foam fractionation process, the column was thoroughly cleaned, to prevent fat and solids depositions, which negatively affect the process.

Samples of the initial solution, foam samples collected over defined time intervals or over each consecutive foaming and the residual solution, were graphically and quantitatively evaluated (2.2.4) to access the enrichment of the active principles accomplished. In case of no enrichment or a reduced one, the conditions of the foam fractionation process were changed or adjusted and quantification was once again made. The foam fractionation methods of choice for each group of active principles of the plants under investigation are the optimised methods. In general, these methods entailed a dependence of some parameters and conditions, which optimisation enabled the achievement of best enrichment ratios.

3.3.3.1 Foam Fractionation of Faradiol Esters

The enrichment of faradiol esters from *Calendula* flos initiated by foam fractionating faradiol esters aqueous dilute solutions prepared from *Calendula* flos extracts (3.3.3.1), at different concentrations of faradiol esters. Foam fractionation took place in columns of various sizes (Table 3.1). The solutions had only a weak foaming capacity and therefore, various amounts of saponin were added before foaming. The medium size column resulted the most favourable for foam fractionation. The resulting foam was broken with DCM. Samples of the initial solu-

tions, foam and residual solutions were filtered and the organic phase evaporated to 200 µl and quantified using HPLC-UV. No enrichment was detected. The materials deposited inside the glass column after foam fractionation were washed out with DCM and the resulting DCM solution evaporated to 200 µl and a sample quantified. The sample contained higher concentration of faradiol esters than the initial solution.

Different amounts of DCM were added to the solutions prior to foaming and the foaming resulted difficult or even ineffective for higher amounts of DCM. The bubble size and foam stability were poor and thus, various amounts of saponin and glycerine were then added to the solutions. With the addition of 600 µl DCM, 0.22 g saponin and 1 ml glycerine, the foam fractionation was more favourable and a good enrichment was obtained. At the latter conditions, faradiol esters concentrations in the initial solutions were again varied. All samples provided for enrichments, however, the best results were obtained for initial solutions with concentrations of 80 mg l^{-1}. Initial solution pH values were varied from 2 to 12 and the enrichments calculated. The influence of pH value on enrichment was negligible.

Moreover, the height of foam tower, total time of foaming and gas flow rates were adjusted to attain the optimised parameters and conditions for the enrichment. Once the optimised conditions were reached, the foaming was performed and samples were withdrawn over five min time intervals and each broken with DCM, filtrated and the organic phase evaporated to 40 µl. The latter samples, samples of the initial solution and residual solution were then quantified by HPLC-UV, as described above.

Experimental results indicated that faradiol esters enrichment ratios are mainly dependent of the following parameters and conditions: concentration of faradiol esters and saponin in the initial solution, height of foam tower, time of foaming and correspondent gas flow rates. A detailed description of the experiments made to study the dependence of the above mentioned parameters and conditions as well as the optimised process are presented in Table 3.3 A, B, C and D.

Attempt	Fixed Parameters and Conditions	Variable Parameters and Conditions
A	-Total Volume of Aqueous Solution: 200 ml -Total Concentration of Initial Solution: 20 mg l^{-1} FL: 0.97 mg l^{-1}; FM: 10.3 mg l^{-1} FP: 8.63 mg l^{-1} -pH of Initial Solution: pH Bi-distilled water -DCM Amount: 600 µl -Glycerine Amount: 1 ml -Frit Type: Porosity 3 -Column Size: 130cmX32mmX29.2mm -Total Foaming Time: 0-45 min -Total N_2 Flow Rate: 24-330 ml min^{-1}	Variable Saponin Amounts [g] 1. 0.22 2. 0.26
B	-Total Volume of Aqueous Solution: 200 ml -pH of Initial Solution: pH Bi-distilled Water -Saponin Amount: 0.22 g -DCM Amount: 600 µl -Glycerine Amount: 1 ml -Frit Type: Porosity 3 -Column Size: 130cmX32mmX29.2mm -Total Foaming Time: 0-45 min -Total N_2 Flow Rate: 24-330 ml min^{-1}	Variable Faradiol Esters Total Concentrations [mg l^{-1}] 1. 20 2. 40 3. 60 4. 80 5. 120 6. 160

<table>
<tr><td>C</td><td>-Total Volume of Aqueous Solution: 200 ml
-pH of Initial Solution: pH Bi-distilled Water
- Total Concentration of Initial Solution: 80 mg l^{-1}
FL: 3.9mg l^{-1}; FM: 41.3 mg l^{-1};
FP: 34.5 mg l^{-1}
-Saponin Amount: 0.22 g
-DCM Amount: 600 µl
-Glycerine Amount: 1 ml
-Frit Type: Porosity 3
-Total Foaming Time: 0-45 min
-Total N_2 Flow Rate: 24-330 ml min^{-1}</td><td>Variable Column Heights
[Length X External Ø X Internal Ø]

1. Col. Size: 65cmX32mmX29.2mm
2. Col. Size: 130cmX32mmX29.2mm</td></tr>
<tr><td>D</td><td>-Total Volume of Aqueous Solution: 200 ml
-pH Initial Solution: pH Bi-distilled Water
-Total Concentration of Initial Solution: 80 mg l^{-1}
FL: 3.9mg l^{-1}; FM: 41.3 mg l^{-1}
FP: 34.5 mg l^{-1}
-Saponin Amount: 0.22 g
-DCM Amount: 600 µl
-Glycerine Amount: 1 ml
-Frit Type: Porosity 3
-Column Size: 130cmX32mmX29.2mm
-Total Foaming Time: 0-45 min
-Total N_2 Flow Rate: 24-330 ml min^{-1}</td><td>Variable Foaming Times and Corresponding N_2 Flow Rates
<table><tr><td>Time [min]</td><td>N_2 Flow Rates [ml min^{-1}]</td></tr><tr><td>1. 0-15 ; 15-20
2. 20-25
3. 25-30
4. 30-35
5. 35-40
6. 40-45</td><td>1. 24; 38
2. 54
3. 96
4. 148
5. 213
6. 330</td></tr></table></td></tr>
</table>

Table 3.2 Parameters and Conditions for Faradiol Ester Enrichment

3.3.3.2 Foam Fractionation of Catechins

The enrichment of catechins from green tea commenced by preparing catechins aqueous dilute solutions from green tea extracts at different concentrations and foam fractionating them at glass columns (Table 3.1). The solutions had a good foaming capacity, particularly with the medium size column and to a lesser extent with the large size column. Nevertheless, for both columns the gas flow rate had to be strictly controlled in order to attain favourable bubble size, foam stability and drainage. The foam produced was broken with MeOH and samples of the initial solution, foam and residual solution were filtered, diluted in the proportion 1 sample : 3 MeOH, than quantified by HPLC-UV. The enrichment ratio was calculated and samples corresponding to the extracts as described in 3.3.3.2, gave alone, slight enrichments of some catechins.

The pH value of initial solutions was varied from the acidic to the neutral range and the solutions foamed. Quantification was made as described above and a slight rise in enrichment was observed, corresponding the best values to pH 3.5. In addition, various amounts of NaCl were added to the initial solutions, previous to foaming. Modest enrichments were achieved for concentrations of 5 – 6 g NaCl per 100 ml of solution.

Furthermore, initial solutions were consecutively foamed by the addition of various amounts of saponin, after the first foaming. The amount of saponin that enabled the best foaming conditions was 0.03 g for each consecutive foaming. At last, the gas flow rates were adjusted for each consecutive foaming and samples of the initial solutions, foam collected after each consecutive foaming and residual solutions were filtered and quantified for catechins contents. Enrichment was registered until the fourth consecutive foaming. However, the overall enrichment was only reasonable and more efforts to further optimise the process did not produce improved results. Consequently, the process was considered optimised.

Experimental results denote that the enrichment ratio of catechins from green tea, are mainly dependent of the following parameters and conditions: concentration of catechins in the initial solution, pH value, NaCl addition, consecutive foamings and respective gas flow rates. A specification of the experiments made to study the dependence of the above mentioned parameters and conditions as well as optimised conditions are presented in Table 3.3 A and B.

<table>
<tr><th>Attempt</th><th>Fixed Parameters and Conditions</th><th>Variable Parameters and Conditions</th></tr>
<tr><td rowspan="2">A</td><td rowspan="2">-Total Volume of Aqueous Solution: 200 ml
-Total Concentration of Initial Solution: 455 mg l^{-1}
C: 33.7 mg l^{-1}; Caff:30.9 mg l^{-1}
EC: 6.6 mg l^{-1}; EGC: 54.4 mg l^{-1}
EGCG: 100.5 mg l^{-1}; ECG: 58.6 mg l^{-1}
-Sodium Chloride Amount: 10 g
-Frit Type: Porosity 3
-Column Size: 130cmX32mmX29.2mm
-Total Foaming Time: 50 min
-Total N_2 Flow Rate: 38-73 ml min^{-1}</td><td>Variable pH</td></tr>
<tr><td>1. 2.5
2. 3.5
3. 4.5</td></tr>
<tr><td>B</td><td>-Total Volume of Aqueous Solution :200 ml
-pH of Initial Solution: 3.5
-Total Concentration of Initial Solution: 455 mg l^{-1}
C: 33.7 mg l^{-1}; Caff: 30.9 mg l^{-1}; EC: 6 mg l^{-1} EGC: 54.4 mg l^{-1}; EGCG: 100.5 mg l^{-1} ECG: 58.6 mg l^{-1}
-Sodium Chloride Amount: 10 g
-Frit Type: Porosity 3
-Column Size: 130cmX32mmX29.2mm
-Total Foaming Time: 110 min
-Total N_2 Flow Rate: 38-375 ml min^{-1}</td><td>Consecutive Foamings:
<table>
<tr><th colspan="2">1st Foam</th><th colspan="2">2nd Foam</th><th colspan="2">3rd Foam</th><th colspan="2">4th Foam</th></tr>
<tr><td colspan="2"></td><td colspan="2">Added. Saponin 0.03 g</td><td colspan="2">Added Saponin 0.03 g</td><td colspan="2">Added Saponin 0.03 g</td></tr>
<tr><td>Time [min]</td><td>N_2 Flow [ml min^{-1}]</td><td>Time [min]</td><td>N_2 Flow [ml min^{-1}]</td><td>Time [min]</td><td>N_2 Flow [ml min^{-1}]</td><td>Time [min]</td><td>N_2 Flow [ml min^{-1}]</td></tr>
<tr><td>1.0-22
1.22-50</td><td>1.38
2.73</td><td>1.0-10
2.10-13
3.13-16
4.16-20</td><td>1.73
2.96
3.213
4.375</td><td>1.0-10
2.10-13
3.13-16
4.16-20</td><td>1.73
2.96
3.213
4.375</td><td>1.0-10
2.10-13
3.13-16
4.16-20</td><td>1.73
2.96
3.213
4.375</td></tr>
</table></td></tr>
</table>

Table 3.3 Parameters and Conditions for Catechins Enrichment

3.3.3.3 Foam Fractionation of Tryptanthrin

The enrichment of tryptanthrin from *Isatis tinctoria* began with the preparation of aqueous dilute solutions from *Isatis tinctoria* extracts (3.3.3.3) containing different concentrations of tryptanthrin. Extracts of *Isatis tinctoria* contained only traces of tryptanthrin and large amounts of ballast substances, thus, the amounts of extract used were minimal and the solutions were spiked with tryptanthrin standard.

The solutions were foamed in small and medium size foam fractionation columns (Table 3.1) and it was observed that their foaming capacity was weak. Therefrom, different amounts of saponin were added to the solutions prior to foaming. Foamability was slightly better in the small size column. The foam collected was broken in the foam breaker with moderate vacuum. Samples of the initial solution, foam and residual solution were filtered and quantified using HPLC-UV/DAD. The calculation of the enrichment ratio revealed a small enrichment, the best being obtained at 5 mg l^{-1} of trytanthrin and 0.03 g of saponin in the initial solution.

Afterwards, different amounts of MeOH were added to the latter, prior to foam fractionation. The foaming became difficult and foam stability was poor. Therefore, glycerine was added to the solution, and with 1 ml MeOH and 1 ml glycerine in the initial solution, there was an improvement of the enrichment ratio. Additionally, the pH of the initial solutions was varied in the alkaline range. The enrichment improved once again reaching the highest value at pH 8.

In order to further improve enrichment, the initial solutions were consecutively foam fractionated with the addition of various amounts of saponin. The best foaming conditions were obtained for 0.05 g of saponin for each consecutive foaming. The height of the foam tower and gas flow rates for each consecutive foaming were also adjusted. Samples of the initial solution, foam collected after each consecutive foaming and the residual solution were filtered and quantified as described above using HPLC-UV/DAD. Improvement of enrichment was observed until the third consecutive foaming.

Experimental results indicate that the enrichment of tryptanthrin from *Isatis tinctoria* is mainly dependent on the following parameters and conditions: Concentration of tryptanthrin and pH in initial solution, height of foam tower, consecutive foamings and respective gas flow rates. A detailed account of experiments made to study the dependence on the above men-

tioned parameters and conditions as well as the optimised conditions are presented in Table 3.4 A, B, C and D.

<table>
<tr><th>Attempt</th><th>Fixed Parameters and Conditions</th><th>Variable Parameters and Conditions</th></tr>
<tr><td rowspan="2">A</td><td rowspan="2">-Total Volume of Aqueous Solution: 100 ml
-pH of Initial Solution: pH Bi-distilled Water
-Saponin Amount: 0.03 g
-MeOH Amount: 1ml
-Glycerine Amount: 1 ml
-Frit Type: Porosity 3
-Column Size: 130cmX22.2mmX18.5mm
-Total Foaming Time: 0 – 40 min
-Total N_2 Flow Rate: 13-121 ml min^{-1}</td><td>Variable Tryptantrin Concentrations [mg l^{-1}]</td></tr>
<tr><td>1. 2.5
2. 5
4. 10
5. 15
6. 20</td></tr>
<tr><td rowspan="2">B</td><td rowspan="2">-Total Volume of Aqueous Solution: 100 ml
-Total Concentration of Initial Solution: 5.7 mg l^{-1}
-Saponin Amount: 0.03 g
-MeOH Amount: 1 ml
-Glycerine Amount: 1 ml
-Frit Type: Porosity 3
-Column Size: 130cmX22.2mmX18.5mm
-Total Foaming Time: 40 min
-Total N_2 Flow Rate: 13-121 ml min^{-1}</td><td>Variable pH</td></tr>
<tr><td>1. 5
2. 6
3. 7
4. 8
5. 9</td></tr>
</table>

<table>
<tr><td>C</td><td>-Total Volume of Aqueous Solution: 100 ml
-pH of Initial Solution: pH bi-distilled Water
- Total Concentration of Initial Solution: Try.: 5.7 mg l^{-1} (Spiked)
-Saponin Amount: 0.03 g
-MeOH Amount: 1ml
-Glycerine Amount: 1 ml
-Frit Type: Porosity 3
-Total Foaming Time: 0 – 40 min
-Total N_2 Flow Rate: 13-121 ml min^{-1}</td><td>Variable Column Heights [Length X External Ø X Internal Ø]

1. Col. Size: 65cmX22.2mmX18.5mm
2. Col. Size: 130cmX22.2mmX18.5mm</td></tr>
<tr><td>D</td><td>-Total Volume of Aqueous Solution: 200 ml
-pH of Initial Solution: 8
-Total Concentration in Initial Solution: Try.: 5.7 mg l^{-1}
-MeOH Amount: 1 ml
-Glycerine Amount: 1 ml
-Frit Type: Porosity 3
-Col. Size: 130cmX22.2mmX18.5mm
-Total Foaming Time: 80 min
-Total N_2 Flow Rate: 13-121 ml min^{-1}</td><td>Consecutive Foamings:
1st Foam: Added Saponin 0.03 g
Time [min]: 1. 0-14, 2.14-24, 3.24-34, 4.34-40
N_2 Flow [ml min^{-1}]: 1. 13, 2. 38, 3. 73, 4. 121
2nd Foam: Added Saponin 0.05 g
Time [min]: 1. 0-9, 2. 9-14, 3. 4-20
N_2 Flow [ml min^{-1}]: 1.38, 2. 73, 3. 121
3rd Foam: Added Saponin 0.05 g
Time [min]: 1. 0-9, 2. 9-14, 3. 4-20
N_2 Flow [ml min^{-1}]: 1. 38, 2. 73, 3. 121</td></tr>
</table>

Table 3.4 Parameters and Conditions for Tryptanthrin Enrichment

3.3.3.4 Foam Fractionation of Cannabinoids

The enrichment of cannabinoids from *Cannabis sativa* started by preparing aqueous dilute solutions from cannabis extracts with various concentrations of cannabinoids (3.3.3.4) and foaming them in the small and medium size columns (Table 3.1). The poor foaming capacity of these solutions required the addition of various amounts of saponin, prior to foaming. Foamability was slightly better in the small size column. The foam obtained was broken by the foam breaker with moderate vacuum. Samples of the initial solution, foam and residual solution were filtrated and quantified by HPLC-UV. The calculation of enrichment ratios showed some enrichment, the best corresponding to 62 mg l^{-1} of cannabinoids and 0.05 g of saponin in the initial solution.

In addition, various amounts of MeOH were added to the latter, prior to foaming. The foaming became difficult and the foam stability poor, therefore, glycerine was added to the solution. Initial solutions with 500 μl MeOH and 1 ml glycerine registered an improvement of the enrichment ratio. Moreover, the initial solutions pH value varied in the alkaline range. The enrichment improved once again, corresponding pH 10 to the best enrichments.

At last, the initial solutions were consecutively foamed with the addition of various amounts of saponin. The most favourable conditions corresponded to the addition of 0.05 g of saponin, before each consecutive foaming. The height of the foam tower and gas flow rates for each consecutive foaming, were also adjusted. Samples of the initial solution, foam collected after each consecutive foaming and the residual solution were filtered and quantified as described above, using HPLC-UV. The enrichment augmented until the third consecutive foaming.

Experimental results indicate that the enrichment of cannabinoids from *Cannabis sativa* is mainly dependent of the following parameters and conditions: Concentration of cannabinoids and pH value in initial solution, height of foam tower, consecutive foamings and respective gas flow rates. A comprehensive description of the experiments made to study the dependence on the above mentioned parameters and conditions as well as the optimised conditions are presented in Table 3.5 A, B, C and D.

Attempt	Fixed Parameters and Conditions	Variable Parameters and Conditions
A	-Total Volume of Aqueous Solution: 100 ml -pH of Initial Solution: pH bi-distilled Water -Saponin Amount: 0.05 g -MeOH Amount: 500 µl -Glycerine Amount: 1 ml -Frit Type: Porosity 3 -Column Size: 130cmX22.2mmX18.5mm -Total Foaming Time: 0 – 40min -Total N_2 Flow Rate: 24-249 ml min^{-1}	Variable Total Cannabinoid Concentrations [mg l^{-1}] 1. 30 3. 60 4. 90 6. 120
B	-Total Volume of Aqueous Solution: 100 ml -Total Concentration of Initial Solution: 62 mg l^{-1} (Spiked) CBD: 28.4mg l^{-1}; CBN: 17.8mg l^{-1}; THC: 16.2mg l^{-1} -Saponin Amount: 0.05 g -MeOH Amount: 500µl -Glycerine Amount: 1ml -Frit Type: Porosity 3 -Column Size: 130cmX22.2mmX18.5mm -Total Foaming Time: 40 min -Total N_2 Flow Rate: 24-249 ml min^{-1}	Variable pH 1. 2 2. 4 3. 6 4. 18 5. 12

<table>
<tr><td>C</td><td>-Total Volume of Aqueous Solution: 100 ml
-Total Concentration of Initial Solution: 62 mg l^{-1} (Spiked)
CBD: 28.4 mg l^{-1}; CBN: 17.8 mg l^{-1}
THC: 16.2 mg l^{-1}
-Saponin Amount: 0.05 g
-MeOH Amount: 500 µl
-Glycerine Amount: 1 ml
-Frit Type: Porosity 3
-Column Size: 130cmX22.2mmX18.5mm
-Total Foaming Time: 40 min
-Total N_2 Flow Rate: 24-249 ml min^{-1}</td><td>Variable Column Heights
[Length X External Ø X Internal Ø]

1. Col. Size: 65cmX22.2mmX18.5mm
2. Col. Size: 130cmX22.2mmX18.5mm</td></tr>
<tr><td>D</td><td>- Total Volume of Aqueous Solution:100 ml
-Total Concentration of Initial Solution: 62 mg l^{-1} (Spiked)
CBD: 28.4 mg l^{-1}; CBN: 17.8 mg l^{-1}
THC: 16.2 mg l^{-1}
-MeOH Amount: 500 µl
-Glycerine Amount: 1 ml
-Frit Type: Porosity 3
-Column Size: 130cmX22.2mmX18.5mm
-Total Foaming Time: 90 min
-Total N_2 Flow Rate: 24-249 ml min^{-1}</td><td>Consecutive Foamings
<table><tr><td>1st Foam</td><td colspan="2">2nd Foam</td><td colspan="2">3rd Foam</td></tr><tr><td>Added Saponin 0.05 g</td><td colspan="2">Added Saponin 0.05 g</td><td colspan="2">Added Saponin 0.05 g</td></tr><tr><td>Time [min]</td><td>N_2 Flow [ml min^{-1}]</td><td>Time [min]</td><td>N_2 Flow [ml min^{-1}]</td><td>Time [min]</td><td>N_2 Flow [ml min^{-1}]</td></tr><tr><td>1. 0-10
2.10-20
3.20-30
4.30-35
5.35-40</td><td>1. 24
2. 54
3. 96
4. 148
5. 249</td><td>1. 0-10
2.10-18
3.18-25</td><td>1. 24
2. 54
3. 96</td><td>1. 0-10
2.10-18
3.18-25</td><td>1. 24
2. 54
3. 96</td></tr></table></td></tr>
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Table 3.5 Parameters and Conditions for Cannabinoids Enrichment

4. Results

4.1 Accuracy of Measurements and Quantification of Active Principles by HPLC

The measuring devices used for control of the foam fractionation process and quantification of active principles from the plants under investigation were respectively, the flow-meter and High Performance Liquid Chromatography (HPLC) devices. The accuracy of the measurements achieved by these equipments was kept high by calibrating them. The flow-meter was calibrated under the conditions in which the gas supply system to the foam fractionation column operates. For calibration and quantification purposes, at least three measurements were made for each data point and the resulting data are the average of the measured values. Table 4.1 presents the flow rates calculate using the calibration curve's equation, for the respective flow-meter scale values.

Flow-Meter Scale [1 – 15]	Gas Flow Rate [ml min^{-1}]
1	5
2	13
3	24
4	38
5	54
6	73
7	96
8	121
9	148
10	179
11	213
12	249
13	288
14	330
15	375

Table 4.1 Calibrating Data for the Gas Flow-meter

The HPLC devices utilised for quantification in this work operated the separations in reversed phase HPLC mode. Reversed phase HPLC used non-polar stationary phases and polar mobile phases, being the non-polar stationary phases octadecyl (C_{18}) and octadecylsilyl (ODS). The

polar mobile phases used were HPLC grade water mixed with HPLC grade MeOH or HPLC grade ACN. The active principles, retained by the non-polar stationary phases due to hydrophobic interactions were eluted in order of increasing hydrophobicity. The variable wavelength and DAD-UV detectors used, measured the absorption of the molecules analysed, being the magnitude of the peaks correspondent to their concentrations in the samples.

Faradiol esters quantification was made as per 3.3.1.1, using a Lichrospher RP-18 column as the stationary phase and the mobile being a mixture of aqueous trifluor acetic acid (TFAc) at pH 4, with MeOH. Solvents ratio started with 95% MeOH for 5% aqu. TFAc and ended with the ratio 100% MeOH for 0% aqu. TFAc. Aqueous TFAc was used instead of pure water to reduce baseline noise. Faradiol esters have long retention times, thus, the contents of the organic solvent was high to reduce their retention times in the stationary phase.

Catechins could be quantified by several HPLC separations. The HPLC separation chosen was made as per 3.3.1.2, using a Lichrospher RP-18 column as the stationary phase and as mobile phase, a mixture of 2% aqueous acetic acid (AAc) with ACN. Solvents ratio initiated with the 88% aqu. AAc for 22% ACN and terminated with the ratio 75% aqu. AAc for 25% ACN. Aqueous AAc was used in place of water to reduce baseline noise. Catechins have short retention time, hence, the water contents in mobile phase was high to increase retention time.

Tryptanthrin was quantified in accordance with 3.3.1.3, using a Lichrospher RP-18 column as the stationary phase, consisting the mobile phase of a mixture 2% aqueous AAc with ACN. At the beginning, solvents ratio was 60% aqu. AAc for 40% ACN and finished with 95% ACN for 5%aqu. AAc. Aqueous AAc was used rather than water to reduce baseline noise.

Cannabinoids were quantified as described in 3.3.1.4, using a Spherisorb RP-ODS column as the stationary phase and the mobile phase being a mixture of MeOH, water and acetic acid. Solvents ratio was 85% MeoH, 14.2% water and 0.8% AAc throughout the separation. Acetic acid was used to reduce baseline noise. The high proportion of organic solvent in the mobile phase reduced cannabinoids retention times.

The HPLC devices were calibrated in the range of concentrations and under the analytical HPLC conditions in which the active principles for each plant were quantified. For calibration and quantification purposes, between three and six measurements were made for each data point and the results for data treatment are the average of measured values. Table 4.2 provides the calibration curves and the range of concentrations of the active principles under investiga-

tion for which these curves are valid. Annex I gives experimental values obtained by calibration experiments and respective calibrating curves. The quantification of those principles once enrichment was made could be calculated with Equations (2.5) and (2.6).

Plant	Active Principle	Range of Concentrations	Equation of the Calibration Curve
Calendula officinalis L.	FL	25 mg l^{-1} – 1690 mg l^{-1}	$Y= 0.0003X^2+0.0264X+0.0209$ $R^2= 0.9986$
	FM	390 mg l^{-1} – 8600 mg l^{-1}	$Y= 0.00007X^2+0.0249X+0.2285$ $R^2= 0.9972$
	FP	240 mg l^{-1} – 7500 mg l^{-1}	$Y= 0.00008X^2+0.0258X+0.1547$ $R^2= 0.9978$
Camelia Sinensis L.	C	2 mg l^{-1} – 200 mg l^{-1}	$Y= 0.0013X^2+0.4928X+0.3344$ $R^2= 0.9923$
	EC	2 mg l^{-1} – 200 mg l^{-1}	$Y= 0.0013X^2+0.4928X+0.3344$ $R^2= 0.9923$
	EGC	2 mg l^{-1} – 200 mg l^{-1}	$Y= 0.0013X^2+0.4928X+0.3344$ $R^2= 0.9923$
	EGCG	2 mg l^{-1} – 200 mg l^{-1}	$Y= 0.0013X^2+0.4928X+0.3344$ $R^2= 0.9923$
	ECG	2 mg l^{-1} – 200 mg l^{-1}	$Y= 0.0013X^2+0.4928X+0.3344$ $R^2= 0.9923$
	Caff	2 mg l^{-1} – 200 mg l^{-1}	$Y= 0.0008X^2+0.0038X+1.2349$ $R^2= 0.9976$
Isatis tinctoria L	Try.	1 mg l^{-1} – 500 mg l^{-1}	$Y= 0.0191X^2+1.2983X+1.2193$ $R^2= 0.9967$
Cannabis sativa L.	CBD	10 mg l^{-1} – 500 mg l^{-1}	$Y=-0.0184X^2+7.039X+3.8$ $R^2= 0.9981$
	CBN	10 mg l^{-1} – 500 mg l^{-1}	$Y= 0.0034X^2+1.4189X+5.0302$ $R^2= 0.9996$
	THC	10 mg l^{-1} – 500 mg l^{-1}	$Y= 0.0024X^2+2.1125X+4.5463$ $R^2= 0.998$

Table 4.2 Calibration Data for HPLC Quantification of Active Principles

4.2 *Calendula Officinalis*

4.2.1 Analytical Characterisation of Lupeol Acetate

4.2.1.1 UV-Vis Characterisation

Lupeol acetate synthesized as described in 3.1.2, was the internal standard used for quantification of faradiol esters. The characterisation was made in order to prove the UV-Vis absorption pattern of the synthesized product and the intensity of its absorption at operating conditions utilised for quantification. Therefore, a sample was diluted in MeOH and characterised by UV-Vis spectroscopy (3.2.4) and conditions as described in 3.3.1.1. Figure 4.1 displays the UV-spectrum from lupeol acetate. The spectrum obtained was taken at the wavelengths between 200 nm and 400 nm. The peak maximum at approximately 210 nm corresponds to the lupeol acetate absorption maximum.

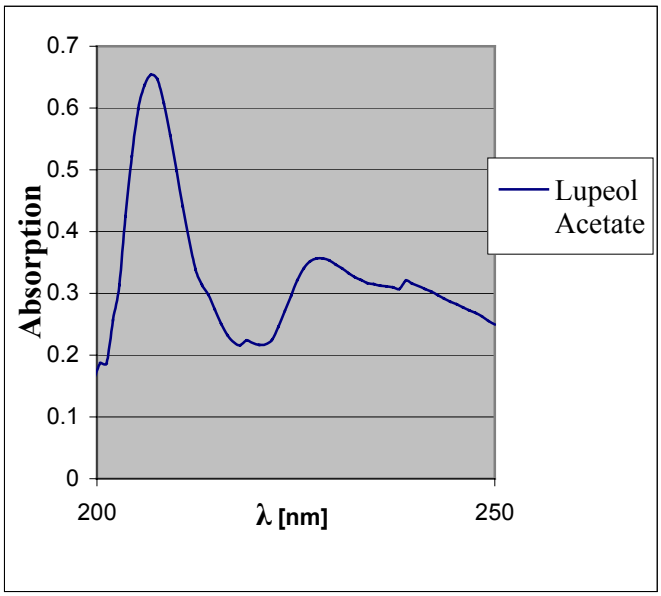

Figure 4.1 UV-Spectrum of Synthesized Lupeol Acetate (λ – 200-250 nm)

4.2.1.2 HRGC-EI/MS Characterisation

The synthesized lupeol acetate standard was diluted in DCM and characterised by HRGC-EI/MS (3.2.3) under the conditions specified in 3.3.1.1. This “hard” ionisation method that

ensues in a positive molecular ion and a large number of positive fragment ions, permitted the identification of the synthesized product. This was made by means of its retention time and fragments corresponding to the molecular ion and main fragment ions in the chromatogram as well as in the spectrum, respectively. The retention time of the peak displayed in the chromatogram was 11:46 min. Table 4.3 depicts fragments of the MS spectrum with high intensity for the *m/z* values from 400 to 470.

Fragment	*m/z*
$[M]^+$	468
$[M-15]^+$	453
$[M-43]^+$	425
$[M-60]^+$	408

Table 4.3 HRGC-EI/MS Main Fragments of Synthesized Lupeol Acetate (*m/z* – 400-470)

4.2.2 Extraction of Faradiol Esters

Faradiol esters extraction from *Calendula* flos was attempted using bi-distilled water with pH's from 2 to 12, with heating, by stirring, ultra-sonic bath extraction and extraction under reflux. All attempts failed, thus, the residues were extracted with DCM and the extracts so obtained contained reasonably high amounts of faradiol esters. Therefore, the samples were extracted with organic solvents, by several methods. Soxhlet extraction with MeOH and ultra-sonic bath extraction with DCM extracted almost all the faradiol esters from the sample. The plant had reasonably high amounts of faradiol esters. Ultra-sonic bath extraction was the method of choice, owing to its simplicity and extraction of low amounts of ballast substances.

The resulting dried extract with a high faradiol esters contents was extracted with bi-distilled water at pH's from 2 to 12 with a heated magnetic stirrer (3.3.2.1). At acidic and alkaline pH's, higher amounts of ballast substances were present in the extract, as compared with the more neutral pH of bi-distilled water. Faradiol esters contents in the aqueous extract were quantified. For the purpose, they were extracted with DCM, the organic phase evaporated to

200 µl and a sample injected in the HPLC-UV. The faradiol esters contents of the extract in DCM is presented in Table 4.4.

Active Principle	Concentration in Extract [mg l^{-1}]
Faradiol Lauritic Acid Ester (FL)	428.13
Faradiol Myristic Acid Ester (FM)	4490.22
Faradiol Palmitic Acid Ester (FP)	3841.40
Total	8759.75

Table 4.4 Concentration of Faradiol Esters in *Calendula Officinalis* Extract

4.2.3 Enrichment of Faradiol Esters

Faradiol esters enrichment by foam fractionation of their aqueous dilute solutions is described in 3.3.2.1. The experiments performed aimed at finding the initial conditions that allowed the foam fractionation to take place. Saponin, a surface active substance was added to increase the foaming capacity of the solution. DCM and glycerine were added to improve quality of the foaming process and the possibility to enrich faradiols by foam fractionation was studied. Faradiol esters could be enriched in the foam and the enrichment showed strong dependence of saponin concentration in the initial solution. Section 4.2.3.1 gives details of saponin, DCM and glycerine addition and a quantification of the effect of saponin amounts on the enrichment of faradiol esters.

The influence of faradiol esters concentration and the pH values of the initial solution on the enrichment was also studied. This influence resulted important for the degree of enrichment achieved. Section 4.2.3.2 provides details and the quantification of the influence of concentration on the enrichment. Contrarily to concentrations, solutions initial pH's were of marginal importance for the foam fractionation. A brief description of the pH influence on enrichment of faradiol esters is also presented in 4.2.3.2. Liquid height above the sparger and the height of the foam tower were also studied to find the effects of these conditions on the enrichment. Liquid height showed no influence on the enrichment, while height of the foam tower showed

to have some influence. Section 4.2.3.3 specifies and gives a quantification of foam tower height effect on enrichment. The evolution of enrichment for each faradiol ester at time intervals at the optimised conditions are detailed in section 4.2.3.4 and the overall enrichments are detailed in section 4.2.3.5

4.2.3.1 Influence of Surfactant and Additives

A condition necessary for the foam fractionation of solutions is their ability to produce foam. The lack of enough foaming capacity of faradiol esters dilute solutions, required the addition of saponin, a surface active substance. Experiments were made to find the right amounts of saponin to be added to enable the solution, to produce foam with the bubble size and stability, convenient for the enrichment. With the addition of 0.1 g of saponin, the solution had good foamability, however, no enrichment was achieved as the faradiol esters deposited at the walls of the glass column. In order to prevent that to happen, reduced amounts of DCM were added to the initial solutions, which lost part of the foaming capacity due to excessive coalescence. To improve foaming capacity, glycerine was added. With the addition of 1 ml o DCM and 1 ml of glycerine no faradiol esters deposited on the column and the solution possessed the ability to foam, however, the bubble size and foam stability were poor.

More experiments were undertaken to find the proportion between saponin, DCM and glycerine that gave the most favourable foam, with larger bubbles, a good stablility and drainage. The proportions were found to be 0.22 g saponin, 600 µl DCM and 1 ml glycerine. Experiments indicated that saponin concentration in the initial solution was crucial for the degree of enrichment obtained. Therefore, and as per Table 3.2 A, the process parameters and variables were kept constant and saponin amounts varied to study the influence on enrichment. Figure 4.2 below illustrates the enrichment drop as function of an excess in saponin concentration.

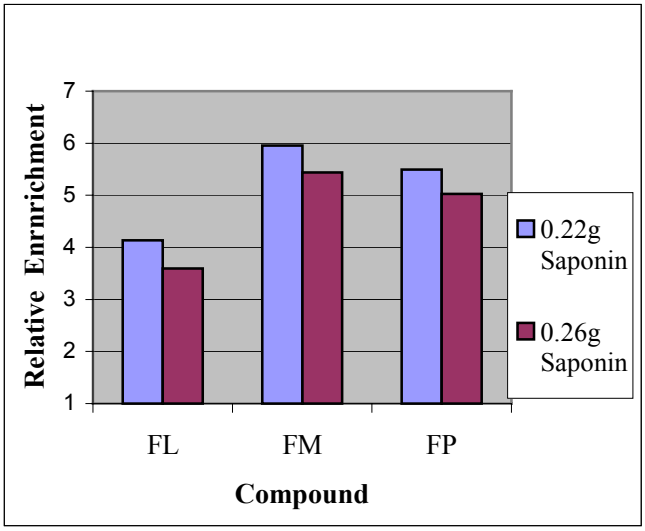

Figure 4.2 Enrichment of Fradiol Esters by Foam Fractionation, in Dependence of Saponin Concentration in the Initial Solution

Above the optimal value, an increase in saponin amount of 0.04 g in the initial solution, produced the drop of enrichment as illustrated graphically. Faradiol lauritic acid ester enrichment droped 13%, faradiol myristic acid ester and faradiol palmitic acid esters droped 8%. Saponin amounts above the optimal amount produced a dry foam with smaller bubbles while below the optimal amount, foam humidity was high, the bubbles were excessively large and no foam exited the column.

4.2.3.2 Influence of Solute Concentration and pH

The influence of faradiol esters concentration in the initial solution on enrichment was studied in the range of total concentrations between 20 mg l^{-1} and 160 mg l^{-1} (Table 3.2 B). Enrichment raised with the rise of faradiol esters concentration in the initial solution, to reach a peak at 80 mg l^{-1} and than declined gradually as portrayed in Figure 4.3. The rise on enrichment from 20 mg l^{-1} to 80 mg l^{-1} was about 54.91% for faradiol lauritic acid ester, 37.70% for faradiol myristic acid ester and 42.10% for faradiol palmitic acid ester. The decline from 80 mg l^{-1} to 160 mg l^{-1} was about 57.64% for faradiol lauritic acid ester, 34.97% for faradiol myristic acid ester and 44.21% for faradiol palmitic acid ester.

In the whole, the foam fractionation process varied to some extent with the variation of concentration in the initial solution. It was observed that at lower concentration, the coalescence was somewhat lower, the bubbles somewhat smaller and the foam somewhat dryer. The opposite was observed at higher concentrations. However, the foam was stable and with large bubbles in the range of concentrations studied.

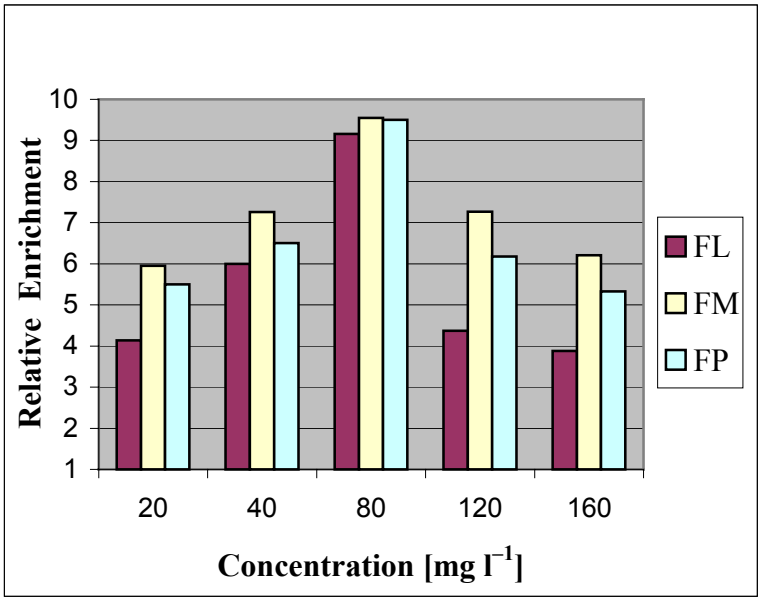

Figure 4.3 Enrichment of Faradiol Esters by Foam Fractionation, in Dependence of their Concentration in the Initial Solution

The pattern of flow fractionation also varied with the concentration of faradiols esters in the initial solution. There was no major change in foam stability along the foam fractionation process for initial solutions with concentrations of 20 mg l^{-1} and with 120 mg l^{-1} and more. Initial solutions with concentrations from 40 mg l^{-1} to 80 mg l^{-1} registered an increase in foam stability, towards the end of the process. The variation of initial solutions pH values produced some effect on the foam fractionation. At the most acidic and most alkaline pH's the initial solutions contained reduced amounts of precipitates that interfered with the process. The effect of pH was studied for solutions with initial concentration of 80 mg l^{-1} and only a very small improvement of enrichment was registered for the alkaline pH range.

4.2.3.3 Influence of Height of Foam Tower and Height of Liquid Pool

A variation of the height of foam tower influenced the enrichment and type of foam that was collected for analysis. The height of foam tower was varied by foam fractionating the solutions with a column with the length of 130 cm and a column with the length of 65 cm (Figure 3.1). Other foam fractionation conditions were kept constant in accordance with Table 3.2 C. The longer column had a foam tower about 65 cm higher than the shorter column. The foam characteristic along both columns was different. For the shorter column, the bubble size at the bottom of the foam tower was only slightly smaller than the bubble size of the foam exiting the column. For the longer column, the bubble size at the bottom of the foam tower was noticeable smaller than the bubble size of the foam exiting the column.

The volume of foam collected with the shorter column was higher and contained appreciably more saponin than that collected with the longer column. The foam carried more liquid then the foam deriving from the longer column. That could be seen after the destruction of the foam with the same amounts of DCM. Comparing the amounts of liquid in the flask containing the foam collected from the shorter column, with that containing the foam collected from the longer column, the amount of liquid in the latter was relatively low. The analysis of the enrichment showed that the longer column provided for a higher enrichment. Figure 4.4 depicts the graphical representation of the decrease of the enrichment with the decrease of the height of the foam tower.

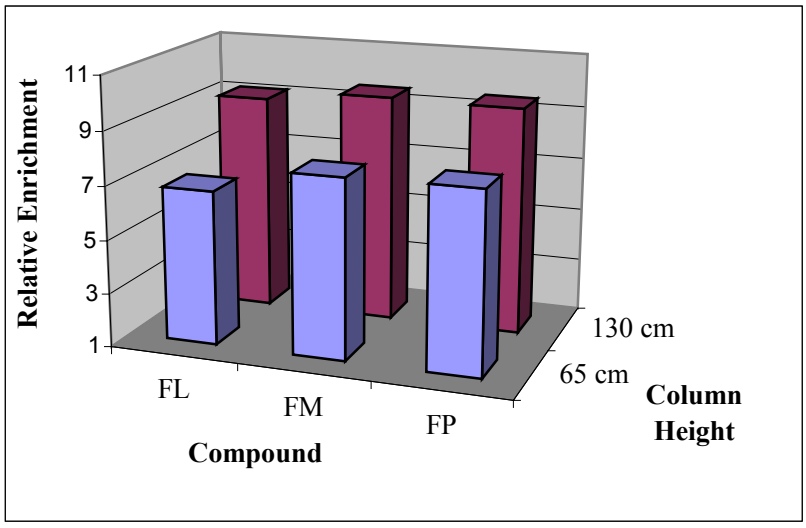

Figure 4.4 Enrichment of Faradiol Esters by Foam Fractionation, in Dependence of Column Height

A decrease of about 65 cm of the foam tower caused a decrease in enrichment of 26.20% for faradiol lauritic acid ester, 19.16% for faradiol myristic acid ester and 18.32% for faradiol palmitic acid ester. The influence of liquid pool above the sparger was studied by foam fractionating in the longer column with the heights of liquid pool of 7.50 cm and 5.5 cm, keeping other parameters and conditions unchanged. The decrease of enrichment was negligible.

4.2.3.4 Influence of Time of Foaming and Gas Flow Rates

The time of foaming and respective gas flow rates showed a strong correlation with the enrichment achieved by the foam fractionation of faradiol esters. Studies were performed to find out how the time of foaming and respective gas flow rates influence the enrichment. Therefore, other parameters and conditions were kept constant as described in Table 3.2. D and the foam was withdrawn at 5 min intervals. The flow of gas supplied to the column was strictly controlled for each time interval, since the quality of the foam was strongly dependent of finding the right gas flow rates for the given time intervals. At the commencement of the process, the flow rates were the lowest possible to produce a foam with large bubbles and low liquid contents. Figure 4.5 illustrates the evolution of enrichment with the time of foaming and corresponding gas flow rates.

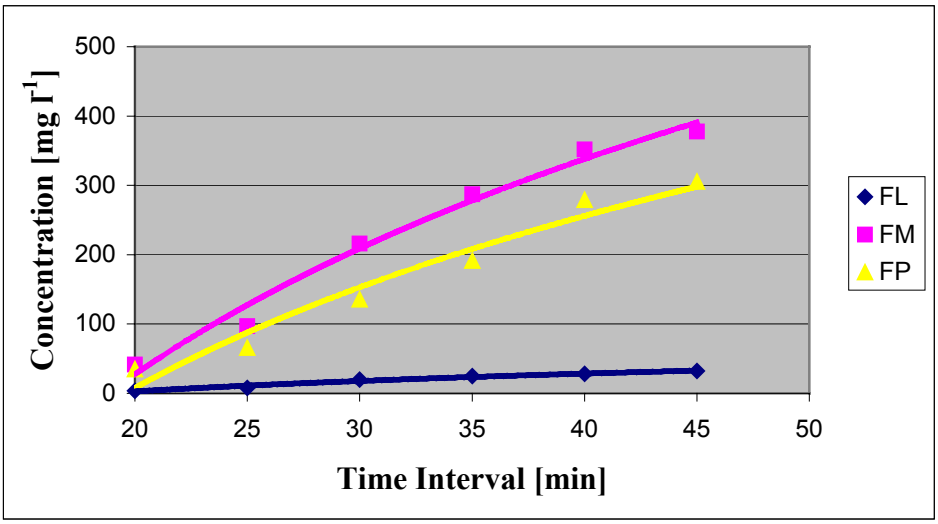

Figure 4.5 Increase in Faradiol Esters Concentration from Initial Solution, to Foam Collected Along the Foam Fractionation Process

Quantification of the foam samples withdrawn at each time interval revealed that during the foam fractionation of faradiol esters, the enrichment varied with the time. At the beginning of the process, the enrichment was reduced, gradually increased at the middle of the process. The largest enrichment occurred towards the end of the process, namely the last two time intervals. This pattern of enrichment was more pronounced for faradiol myristic acid ester and faradiol palmitic acid ester. Further foaming of the solution beyond the time set for the termination of the process, produced no increase in enrichment. After that time, the foam fractionation process became easier, the foam was more stable and a strict control of gas flow rate supplied to the column was no longer needed.

4.2.3.5 Optimised Overall Enrichment Process

Faradiol esters optimised overall enrichment was achieved when the parameters and conditions that provided for the highest enrichments were attained, as described in the previous sections. Table 4.5 presents the initial solution concentrations, concentrations in the foam and concentration in the residual solution, for each faradiol ester. The overall relative enrichment and percent enrichment are also supplied. The enrichment of faradiol lauritic acid ester is about 4 % and 3.5% lower than the enrichments of faradiol myristic acid ester and faradiol palmitic acid ester respectively.

Faradiol Ester	Initial Solution Total Volume : 200 ml	Foam Total Volume: 20 ml	Residual Solution Total Volume: 178.5 ml	E_R	%E
	Concentration [mg l^{-1}]	Concentration [mg l^{-1}]	Concentration [mg l^{-1}]		
FL	3.97	36.33	Not detectable	9.16	91.63
FM	39.99	381.87	Not detectable	9.55	95.50
FP	33.06	314.03	Not detectable	9.50	94.99
Total	77.01	732.24			

Table 4.5 Concentration of Faradiol Esters in Initial Solution, Overall Foam and Residual Solution, for the Foam Fractionation Process. Relative Enrichment and Percent Enrichment

A graphical representation of the concentrations of faradiol esters in the initial solution, in the foam collected for the overall process and in residual solution is presented at Figure 4.6. In general, the increase of concentration from the initial solution to the final foam is above nine fold. Faradiol esters contents in the residual solution was so low that was below the range of detection. Therefore, their concentration in the residual solution was assumed to be non existent.

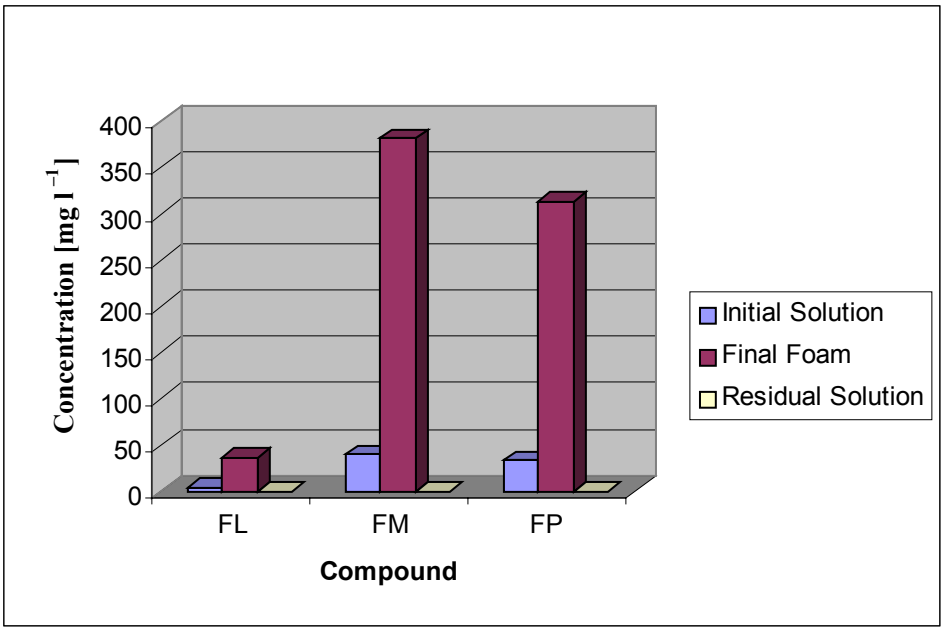

Figure 4.6 Concentration of Faradiol Esters in Initial Solution, Overall Process Foam and Residual Solution , for the Foam Fractionation Process

The overall enrichment of faradiol esters is displayed in Figure 4.7 by a comparison of the chromatograms corresponding to the initial solution, foam collected for the overall process and the residual solution. There is an increase of faradiol esters peak areas from the chromatogram corresponding to the initial solution, to the chromatogram corresponding to the foam collected for the overall process. These peaks are not discernible in the chromatogram corresponding to the residual solution. Xanthophille peak, not seen in any chromatogram since elution occurs after 75 min, outside of the range of the chromatogram presented, also increases from chromatograms for initial solution to overall foam.

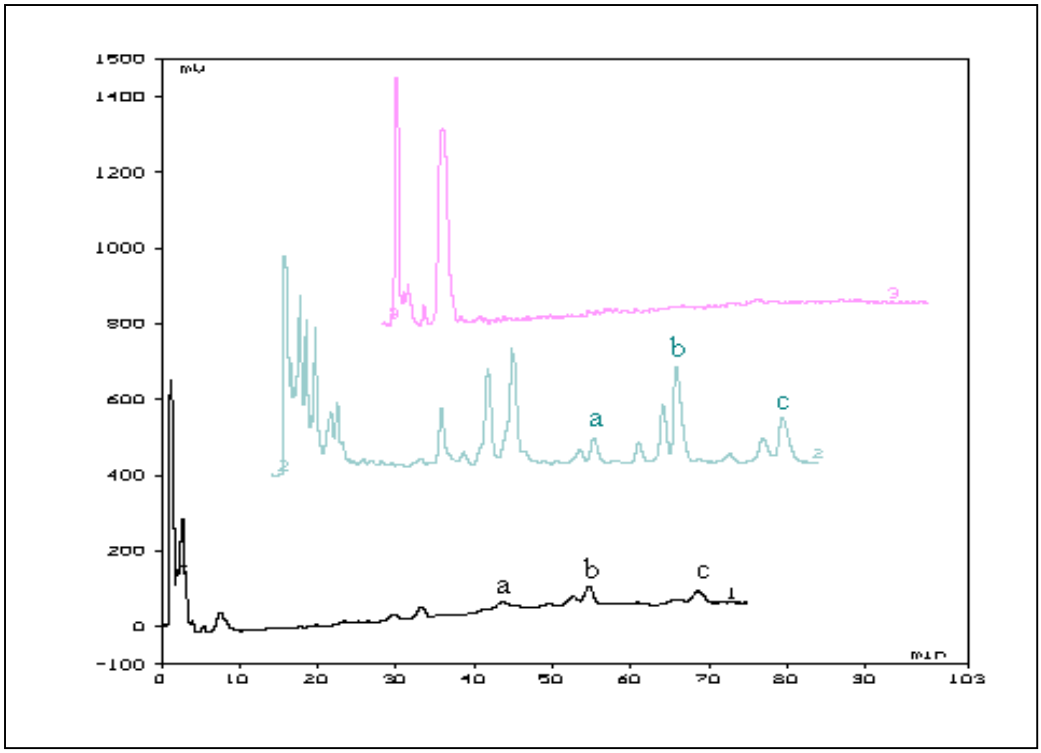

Figure 4.7 HPLC-UV Chromatograms of the Overall Faradiol Esters Foam Fractionation Process

Chromatogram 1 – Initial Solution
Chromatogram 2 – Overall Foam
Chromatogram 3 – Residual Solution

a – Faradiol Lauritic Acid Ester
b – Faradiol Myristic Acid Ester
c – Faradiol Palmitic Acid Ester

4.3 *Camellia Sinensis*

4.3.1 Analytical Characterisation of Catechins

4.3.1.1 UV-Vis Characterisation

Catechin standard used for quantification of catechin, together with Epigallocatechin gallate standard were analysed for their absorption patterns in the UV-Vis region, to check the intensity of their absorptions at operating conditions used for quantification. Samples of the standards were diluted in MeOH and characterised by UV-Vis spectroscopy (3.2.4) at conditions decribed in 3.3.1.1. Figure 4.8 presents the UV-spectrums from catechin and epigallocatechin

gallate. The spectrums were taken at wavelengths between 200 nm and 400 nm. The peak maximums are at about 280 nm for catechin and 275 nm for epigallocatechin gallate.

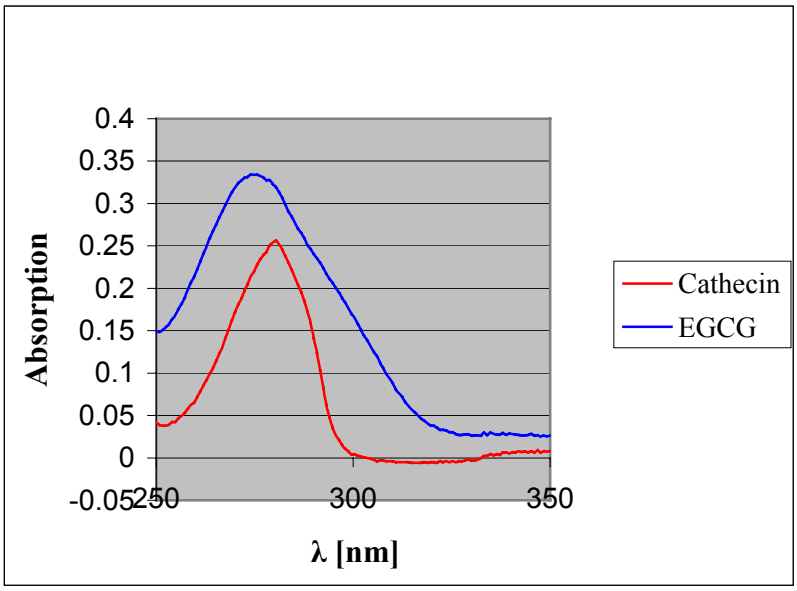

Figure 4.8 UV-Spectrum of Catechin Caffeine and Epigallocatechin Gallate (λ – 250-350 nm)

4.3.1.2 Nuclear Magnetic Resonance Characterisation

Epigallocatechin gallate standard was analysed by nuclear magnetic resonance spectroscopy for the characteristic ^{1}H and ^{13}C spectrum, to check for possible degradation during storage. A sample of the standard was diluted in MeOH-d_4 and analysed by NMR (3.2.5) and conditions as per 3.3.1.2. The chemical shifts (δ) corresponding to the resonances of ^{1}H and ^{13}C atoms in the epigallocatechin gallate molecule obtained, were in accordance with the data widely published in the literature.

4.3.2 Extraction of Catechins

Catechins extraction from green tea leaves with water in a heated magnetic stirrer was an effective method of extraction. Catechins contents in the plant was high and the extraction removed almost all cathecins from the sample. The concentration of catechins in the extract was high. Caffeine, an undesirable substance, was also co-extracted. The pH of the water was var-

ied from 2 to 5.4 (pH of di-ionised water) and the extraction temperature from 65 °C to 100 °C. Higher pH's and temperatures were avoided since catechins degrade at pH values higher than 6.5 and too high temperatures (2.3.2.3).The pH of di-ionised water and the temperature of 100 °C resulted better for the extraction and catechins from green tea were extracted in accordance with 3.3.2.2. The extracts prepared were refrigerated prior to use.

For control purposes, the sample was also extracted with organic solvents and mixtures of organic solvents and water. These methods resulted less effective as the extraction method used in this work. The extraction method developed by Todd (1994), in which green tea is extracted with MeOH and water, followed by extraction with hexan : water and finally the extraction with ethyl acetate : water at pH 3.5, to which NaCl was added, resulted the most effective method for catechins extraction. Caffeine co-extracted by aqueous extraction, could be considerably reduced by further extraction with DCM. Cathechins and caffeine contents in the aqueous extract is presented in Table 4.6.

Active Principle	Concentration in Extract [mg l^{-1}]
Catechin (C)	33.68
Caffeine (Caff.)	30.92
Epicatechin (EC)	176.64
Epigallocatechin (EGC)	54.44
Epigallocatechin Gallate (EGCG)	100.48
Epicatechin Gallate (ECG)	58.64
Total	458.8

Table 4.6 Concentration of Catechins and Caffeine in *Camellia Sinensis* Extract

4.3.3 Enrichment of Catechins

The description of catechins enrichment from their aqueous dilute solutions using foam fractionation is presented in 3.3.2.2. A set of experiments was conducted to investigate the possibility to enrich catechins contained in green tea. Aqueous dilute solutions with various con-

centrations of catechins were prepared with the green tea extracts and foamed. The quality of the foam was good, however somewhat too humid. Solutions prepared as per 3.3.3.2 gave slight catechins enrichments, however a strict control of gas flow rates to the column was important. The achievement of enrichment was only possible for solutions with catechins concentrations as the latter. Caffeine was also co-enriched. When caffeine was extracted from the initial solutions with DCM prior to foaming, no enrichment was registered. Further studies demonstrated that the pH values and additives could improve the enrichment. Therefore, the above mentioned solutions were foam fractionated from acidic to neutral pH's and with the addition of electrolytes. Section 4.3.3.1 details the foam fractionation of such solutions and the quantification of pH effect and NaCl addition on enrichment.

In trying to improve enrichment, the solutions were consecutively foam fractionated. For further foam fractionation of the solutions, addition of saponin was required, as the solutions no longer possessed foam capability. The control of the gas flow rates supplied to the column during the consecutive foam fractionations required even stricter control as that of the first foam fractionation. Section 4.3.3.2 particularises details of the consecutive foamings, respective gas flow rates and evolution of enrichment of each catechin with consecutive foaming. Section 4.3.3.3 presents the overall enrichments achieved at optimised conditions.

4.3.3.1 Influence of Solute Concentration, Additives and pH

Foam fractionation of catechins aqueous dilute solutions were realised on solutions with initial concentrations varying from 115 mg l^{-1} to 720 mg l^{-1}. The solutions corresponded to extracts prepared with amounts varying from 0.125 g to 2 g of green tea leaves per 100 ml di-ionised water, the extraction method being similar as the described in 3.3.3.2. The solutions possessed foaming capability, the quality of the foam was good, with stable foam and large bubbles, however, humidity was somewhat high. Addition of organic solvents to reduce humidity failed and lowered the quality of the foam. Therefore, the gas flow rate had to be strictly controlled to reduce the consequences of excessive foam humidity.

The foam fractionation of such solutions produced no enrichments, with exception to the solutions prepared with 1 g green tea leaves per 100 ml di-ionazed water. The foam fractionation of the latter provided for very small enrichments of the catechins with higher molecular

masses, epicatechin gallate and epigallocatechin gallate. Caffeine, an undesired compound was also co-enriched. In trying to prevent this to happen, the solutions were extracted with 1/3 of their volumes with DCM, which substantially diminished the amount of caffeine in the solutions. These solutions with low caffeine contents were foam fractionated and no enrichment was achieved.

Solutions with catechins concentrations varying from 115 mg l^{-1} to 720 mg l^{-1} were foamed in the acidic an neutral pH's. Once again, solutions with about 450 mg l^{-1} gave small catechin enrichments in acidic pH values, being the best values correspondent to the pH 3.5. Caffeine was also co-enriched. In trying to improve enrichment for solutions with 450 mg l^{-1}, NaCl in amounts varying from 1 g to 8 g NaCl per 100 ml was added to the solutions and the foaming was conducted at pH 3.5. The solutions containing between 1 g and 4 g of NaCl per 100 ml solutions gave no enrichment and there was more catechins in the initial solution as in the foam. Further enrichment was registered with the addition of 5 g and 6 g of NaCl per 100 ml of the initial solutions. Higher NaCl concentrations produced no enrichment.

The influence of initial solutions pH on enrichment was studied for solutions with concentrations of 450 mg l^{-1}, 5 g of NaCl per 100ml of solution and pH's varying from 2.5 to 4.5 as described in Figure 3.3 A. The highest enrichment was once more obtained for the pH 3.5. Figure 4.9 displays the graphical enrichment of catechins and caffeine as function of the pH's of the initial solutions.

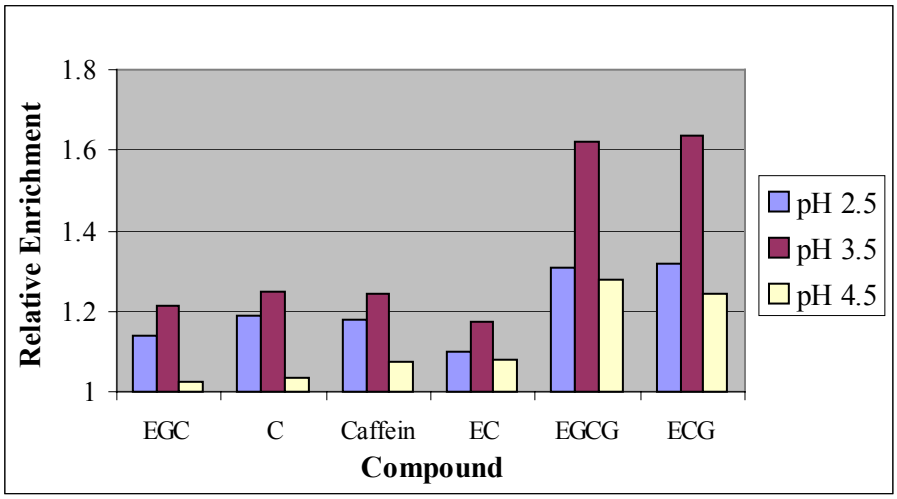

Figure 4.9 Enrichment of Catechins and Caffeine by Foam Fractionation, in Dependence of the Initial Solution pH

For the initial solutions, an increase or decrease of pH's to values below and above the optimal pH produced notable changes of the enrichments for some catechins. The improvement of enrichment for epigallocatechin from pH 2.5 to pH 3.5 was 5.79%, the drop from pH 3.5 to pH 4.5 was 15.7%. For catechin enrichment raised 4.8%, from pH 2.5 to pH 3.5 and declined 16.8% from pH 3.5 to 4.5. The rise registered for Caffeine from pH 2.5 to pH 3.5 was 4.85% and the decline from pH 3.5 to pH 4.5 was 13.71%. From pH 2.5 to 3.5 epicatechin's enrichment rose 5.95% and the declined 7.69%, from pH 3.5 to 4.5. Epigallocatechin gallate's enrichment raised 19.1% from pH 2.5 to pH 3.5 and the declined 20.99% from pH 3.5 to pH 4.5. Finally for epicatechin gallate the increase from pH 2.5 to 3.5 was 19.02% and the decrease from pH 3.5 to pH 4.5 was 23.93%.

4.3.3.2 Influence of Consecutive Foaming and Gas Flow Rate

A single foam fractionation of catechins solutions alone, provided for relatively lower enrichment ratios. In order to try to improve enrichment, the solutions were consecutively foam fractionated maintaining the other parameters and conditions unchanged in accordance with Table 3.3 B. Addition of 0.03 g of saponin prior to foaming was required, while the solutions no longer possessed the foaming capability. Enrichments were obtained until the fourth consecutive foaming. The foam characteristic changed from the beginning to the end of each foam fractionation and from the first to the last foam fractionation. The main change that occurred in each fractionation process was the increase in bubble size, in a way that towards the end of each foam fractionation the bubbles were excessively large. The main change that occurred from each consecutive foaming to other, was a little reduction in the humidity of the foam. At the commencement of each foam fractionation, the gas flow rate was set somewhat high to counter-balance the "heavy" foam. Throughout each foam fractionation, the controlled supply of the gas flow to the column was of key importance to obtain a foam with the lowest liquid content possible. Therefore, a strictly controlled supply of gas to the column, permitted to obtain a dryer foam with the most favourable bubble size.

To study the evolution of enrichment with each consecutive foaming and at strictly controlled flow rates, foam was withdrawn at the end of each foam fractionation. Figure 4.10 illustrates the evolution of consecutive foam fractionation. An analysis of the graphic shows that enrichment of catechins and caffeine is reduced at the beginning of the process and that the

curves corresponding to the higher molecular weight catechins evolution along the consecutive foam fractionations, are still ascending.

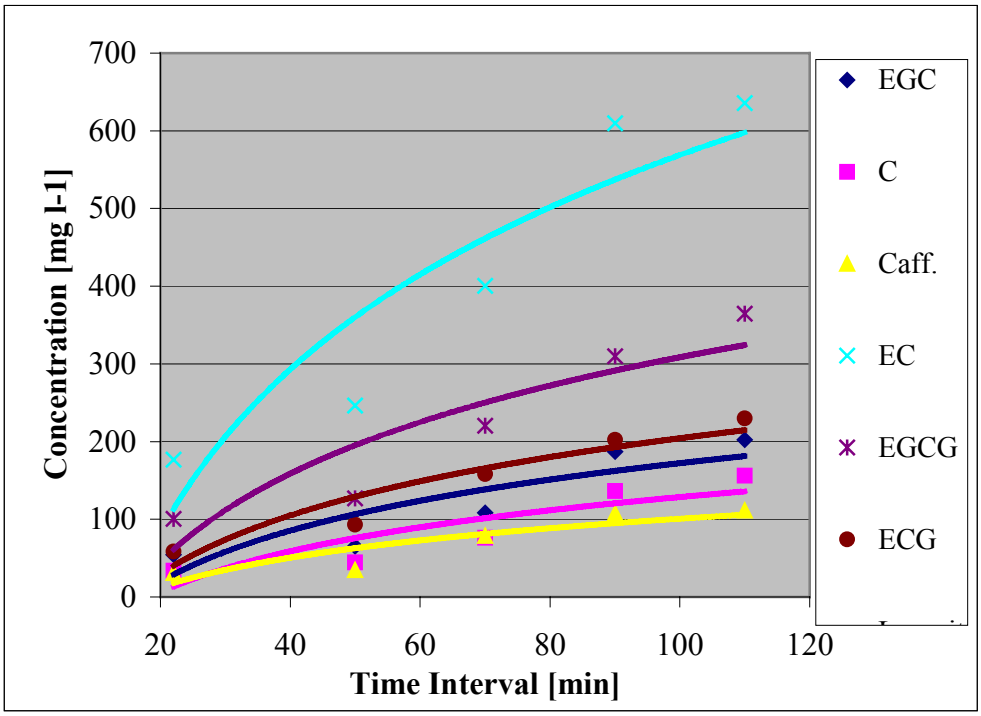

Figure 4.10 Increase in Catechins and Caffeine Concentration from the Initial Solution, to the Foam Collected Along the Consecutive Foam Fractionation Process

4.3.3.3 Optimised Overall Enrichment Process

The achievement of catechins optimised overall enrichment was possible when the parameters and conditions that provided for higher enrichments were met, as described in the previous sections. Table 4.7 depict the concentrations of catechins in the initial solutions, in the foam collected for the overall process and the residual solution. The relative and percent enrichments for catechins and caffeine are also given. The relative enrichment of catechin is comparatively higher than the other catechins or caffeine. Relative catechin enrichment is about 17.03% higher than the average for the other catechins and caffeine enrichments. The percent enrichment is 16.95% higher.

Catechins and Caffeine	Initial Solution Total Volume: 200 ml	Foam Total Volume: 28 ml	Residual Solution Total Volume: 170 ml	E_R	%E
	Concentration [mg l^{-1}]	Concentration [mg l^{-1}]	Concentration [mg l^{-1}]		
C	33.67	156.12	16.48	4.64	64.91
Caff.	30.93	111.22	15.16	3.60	50.35
EC	176.66	635.78	86.56	3.60	50.38
EGC	54.43	202.44	26.68	3.72	52.07
EGCG	100.47	364.42	49.24	3.63	50.78
ECG	58.62	230.03	28.72	3.92	54.94
Total	454.78	1700.01	222.84		

Table 4.7 Concentration of Catechins and Caffeine in the Initial Solution, Overall Foam and Residual Solution, for the Foam Fractionation Process. Relative Enrichment and Percent Enrichment

The differences in concentrations of catechins and caffeine in the initial solution, the foam collected for the overall process and the residual solution are presented graphically at Figure 4.11. The overall increase of catechins and caffeine concentration from the initial solution to the foam is above three fold. The residual solution still contains fairly high amounts of catechins and caffeine.

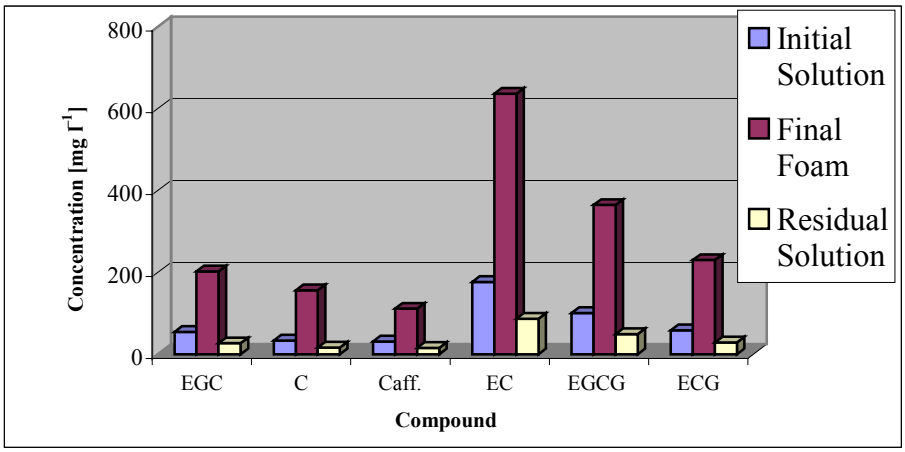

Figure 4.11 Concentration of Catechins and Caffeine in the Initial Solution, Overall Foam and Residual Solution, for the Foam Fractionation Process

Figure 4.12 presents the chromatogram comparison corresponding to catechins and caffeine initial solutions, foam collected for the overall process and the residual solution. There is a noticeable increase in peak areas of catechins and caffeine from the chromatogram corresponding to the initial solution to the chromatogram corresponding to foam collected for the overall process. Catechins and caffeine presence in fairly high quantities in the residual solution, are illustrated in the chromatogram corresponding to the residual solution.

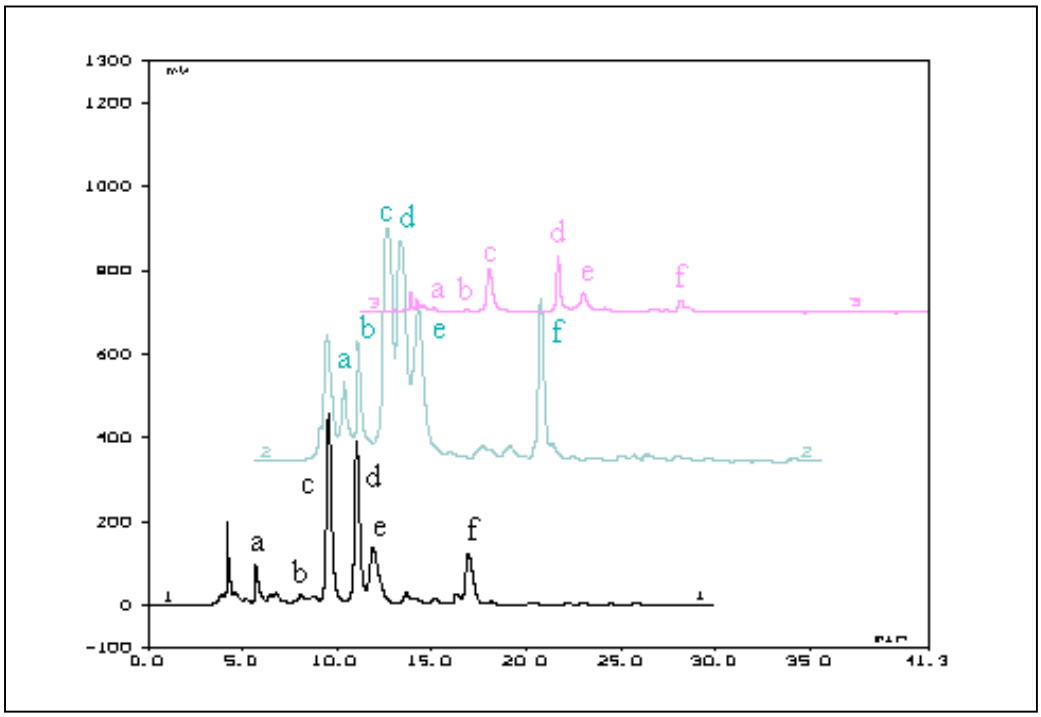

Figure 4. 12 HPLC-UV Chromatograms of the Overall Catechins and Caffeine Foam Fractionation Process

Chromatogram 1 – Initial Solution	a – EGC	d - EC
Chromatogram 2 – Overall Foam	b – C	e - EGCG
Chromatogram – 3 Residual Solution	c – Caffeine	f - ECG

4.4 *Isatis Tinctoria*

4.4.1 Analytical Characterisation of Tryptanthrin

4.4.1.1 UV-Vis Characterisation

Tryptanthrin standard used for quantification was analysed for the absorption pattern in the UV-Vis region, to check the intensity of its absorption at the operating conditions selected for quantification. A sample of the standard was diluted in MeOH and characterised by UV-Vis spectroscopy (3.2.4) and conditions specified in 3.3.1.1. Figure 4.13 presents the UV-spectrum of tryptanthrin. This spectrum was taken at the wavelengths between 200 nm and 400 nm. The peak maximum at about 251 nm corresponds to the absorption maximum for tryptanthrin.

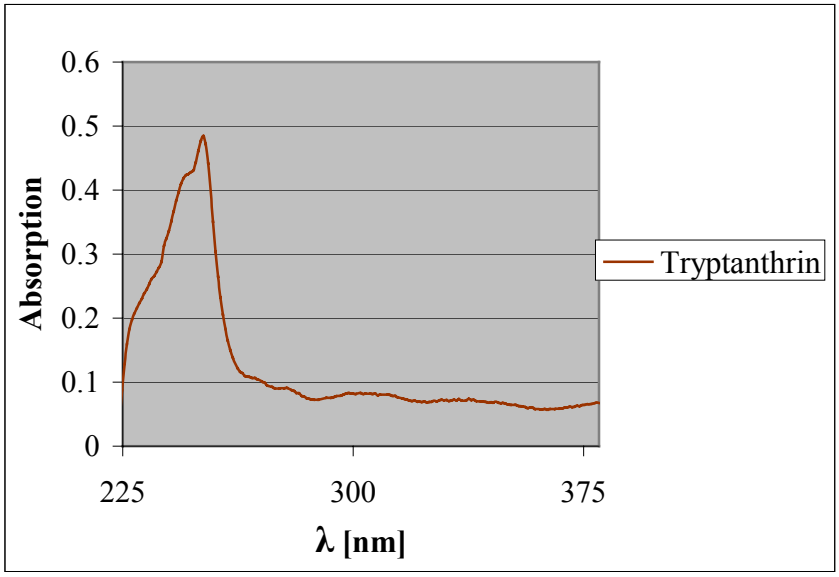

Figure 4.13 UV-Spectrum of Tryptanthrin (λ – 225-375 nm)

4.4.1.2 Fourier Transform Infrared Characterisation

Tryptanthrin was analysed by Fourier Transform Infrared Microspectroscopy for the characteristic absorptions, correspondent to the vibrations of tryptanthrin molecule most characteristic functional groups. The analysis aimed at checking tryptanthrin stability during storage and to access its structure, since tryptanthrin is a new class of active principle. A sample of the

standard was diluted in chloroform and analysed with NMR (3.2.6) at the conditions as per 3.3.1.2. Figure 4.14 gives the FTIR of the tryptanthrin molecule. The absorptions with higher wavenumbers: 1727.73 cm^{-1}, 1685.42 cm^{-1} and 1594.79 cm^{-1}, refer to those of the most characteristic functional groups. The latter is identified in the literature as corresponding to the absorption of C═C group in the aromatic ring.

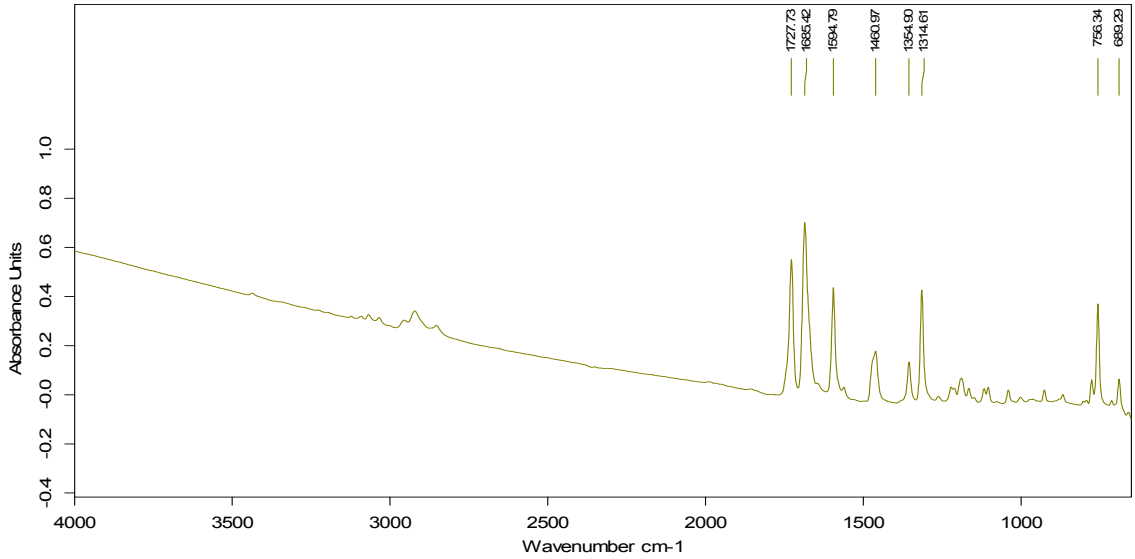

Figure 4.14 Solid Phase Mid-FTIR Spectrum of Tryptanthrin

4.4.2 Extraction of Tryptanthrin

Tryptanthrin extraction from *Isatis tinctoria* was tried with water at pH's varying from 2 to 12, with heating, by stirring, ultra-sonic bath extraction and extraction under reflux. All tries were unsuccessful. The residue from aqueous extraction was extracted with various organic solvents but no tryptanthrin was present in those extracts. Therefore, samples were than extracted with organic solvents using various methods. Extraction had some success with MeOH (70% v/v) and by reflux as described in 3.3.2.3. The concentration of tryptanthrin in the plant was extremely low. The amounts of tryptanthrin extracted varied considerably between the various extracts, and the extracts contained high amounts of ballast substances. The dried extract with relatively low tryptanthrin concentration was extracted with water at pH's varying from 2 to 12, with a heated magnetic stirrer. The neutral extracts, particularly with water at pH 6.5, contained very low concentrations of tryptanthrin and relatively high amounts o ballast substances, while the acidic and alkaline extracts also contained very low concentrations

of tryptanthrin, but even higher amounts of ballast substances. Tryptanthrin contents of the extracts at pH 6.5 is presented in Table 4.8.

Active Principle	Concentration in Extract [mg l^{-1}]
Tryptanthrin (Try.)	0-1.5

Table 4.8 Concentration of Tryptanthrin in *Isatis Tinctoria* Extract

4.4.3 Enrichment of Tryptanthrin

Tryptanthrin enrichment from the aqueous dilute solutions by foam fractionation is detailed in section 3.3.2.3. Various experiments were realised to find out the possibility to enrich tryptanthrin from these solutions. To start with, there was a need to improve the foaming capacity of the solutions and obtain a stable foam, with large bubbles. Addition of saponin gave the solution a good foaming capability. MeOH and glycerine addition provided for better foam quality. The analysis of the foam resulting from the process showed that tryptanthrin can be enriched by foam fractionation. The effect of tryptanthrin concentrations in initial solutions on the enrichment, was studied. Enrichment denoted a dependence of tryptanthrin concentration in the initial solution. Section 4.4.3.1 briefly lays out saponin, MeOH and glycerine addition, and gives a description and quantification of the effect of initial solution concentrations on the enrichment. The effect of pH of initial solutions on the enrichment was also studied. It was observed that tryptanthrin enrichment depended on pH values of the initial solutions. This dependence, together with the quantification of that dependence is described in section 4.4.3.2.

The effects of the variation of height of liquid pool above the sparger and the height of foam tower on enrichment were investigated. A variation of height of liquid pool produced no effect on enrichment while the variation of the height of foam tower caused a noticeable effect. A description of these variations and the quantification of the effect of height of foam tower are presented in section 4.4.3.3. In trying to improve enrichment, the initial solutions were consecutively foamed. An analysis of the overall foam reveals that consecutive foaming en-

ables an increment of tryptanthrin enrichment. A description of the evolution of enrichment is given in 4.4.3.4. Section 4.4.3.5 describes the overall optimised enrichment process.

4.4.3.1 Influence of Solute Concentration, Surfactants and Additives

The foam fractionation of tryptanthrin aqueous dilute solutions required addition of saponin to improve the foaming capacity of these solutions. Experiments made to define the right amount of saponin indicated that saponin alone tended to produce a foam that was too dry and with small bubbles. The stability of the foam was also poor and the initial solution was turbid. Glycerine and MeOH were added to the solutions, which became less turbid, the bubbles large, the foam more stable and no longer too dry. With the addition of 0.03 g of saponin, 1 ml of MeOH and 1 ml of glycerine the turbidity was at a minimum and the quality of the foam was good. To study the influence of the initial solution's concentration on enrichment, the latter conditions remained unchanged and the concentrations of the initial solutions varied in accordance with Table 3.4 A. The enrichment increased to reach the highest value at a concentration of 5 mg l^{-1} and decreased again. Figure 4.15 presents a graphical illustration of concentration influence on enrichment.

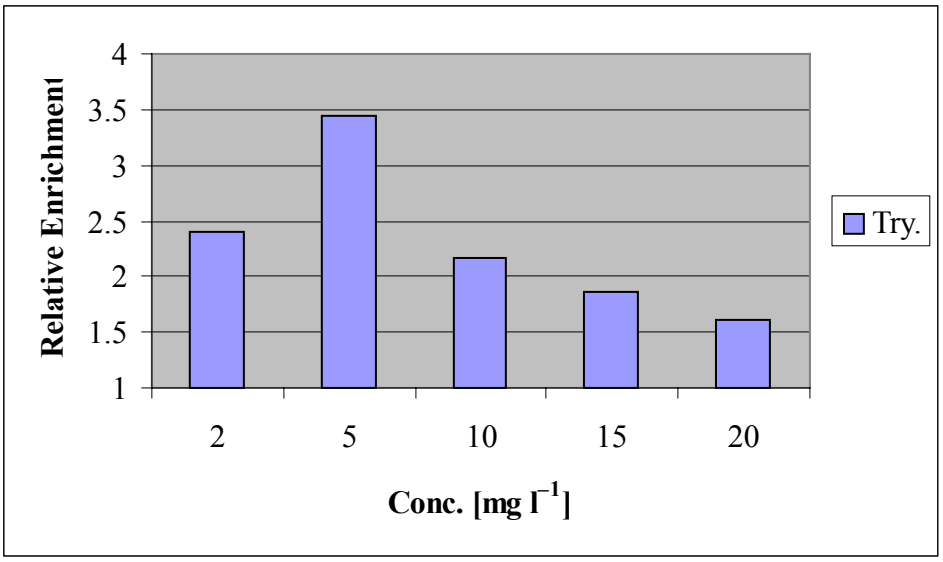

Figure 4.15 Enrichment of Tryptanthrin by Foam Fractionation, in Dependence of its Concentration in the Initial Solution

The rise in concentration from 2.5 mg l^{-1} to 5 mg l^{-1} produced an increase in enrichment of 30.52%, the rise to 10 mg l^{-1} caused an increase of 36.92%. The rise of 15 mg l^{-1} from 5 mg l^{-1} to 20 mg l^{-1} resulted in a decrease in enrichment of 52.9%. The stability of the foam during foam fractionation remained almost unchanged within the range of concentrations studied.

4.2.3.2 Influence of pH

Initial solutions pH values influence on the enrichment of tryptanthrin was studied by varying the pH of the initial solutions, while maintaining other parameters and conditions constant as per Table 3.4 B. The results obtained, revealed that the enrichment of tryptanthrin is dependent on the pH of the initial solution, increasing with the increase of pH, from the acidic to the alkaline range. Enrichment reached a maximum at pH 8 and decreased gradually at more alkaline pH's. Figure 4. 16 illustrates tryptanthrin enrichment in dependence of initial solutions pH. With the increase of the alkalinity up to pH 9, the solutions became somewhat less turbid, the foam fractionation became easier to control and the foam produced in the process was more stable.

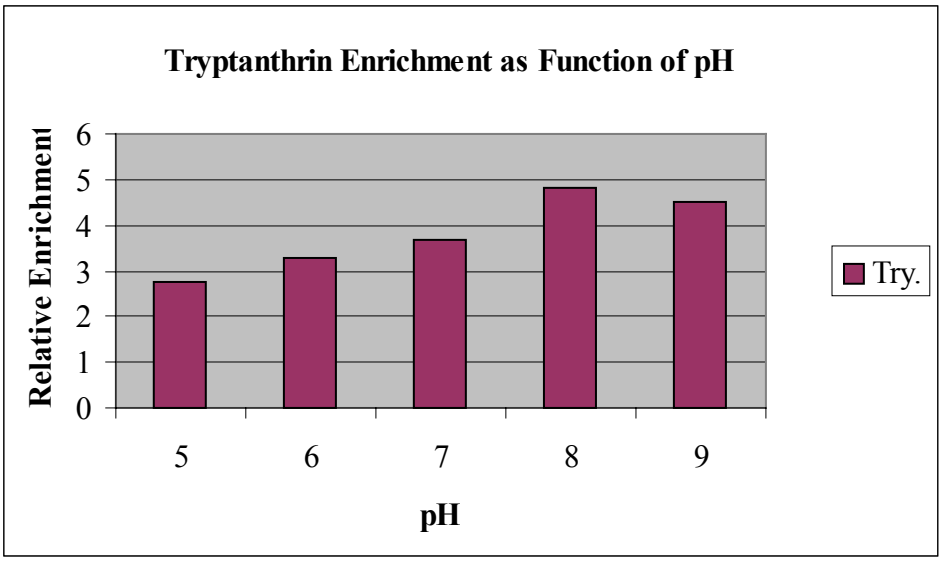

Figure 4.16 Enrichment of Tryptanthrin by Foam Fractionation , in Dependence of Initial Solution pH

Considering the variation of tryptanthrin enrichment registered in dependence of initial solution's pH variation, the improvement of enrichment from pH 5 to 8 was 42.64% and the diminution of enrichment with the decrease of pH from pH 8 to 9 was 6.83%.

4.4.3.3 Influence of Height of Foam Tower and Height of Liquid Pool

Experimental results indicated that the variation of height of foam tower influenced the enrichment of tryptanthrin. These experiments were carried out by foam fractionating the solutions in one column with the length of 130 cm and other column with the length of 65 cm (Table 3.1). Other parameters and variables were maintained constant as per Table 3.4 C. The difference in the foam height between the two columns was 65 cm. There was a difference of bubble size along the column, between the shorter column and the longer column. For both columns, the bubble size was small at the bottom of the foam tower. However foam exiting the shorter column had smaller bubbles and carried more liquid than foam exiting the top of the longer column. The presence of more liquid in the foam originating from the shorter column could be noticed after the breakage of the foam exiting both columns, with vacuum. Liquid amount collected from the shorter column was higher than that of the long column. The analysis of the foam showed that tryptanthrin enrichment increased with the increase of the foam tower. Figure 4.17 illustrates the decrease in enrichment with decrease of the height of the foam tower.

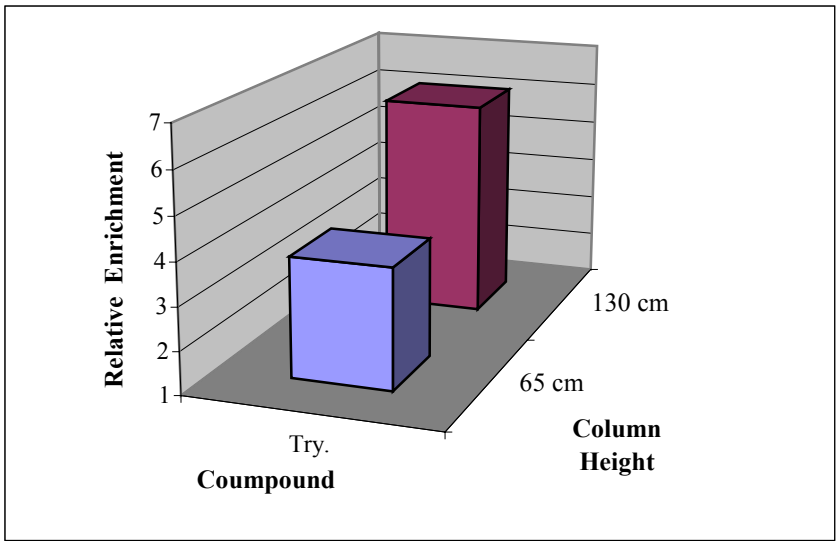

Figure 4.17 Enrichment of Tryptanthrin by Foam Fractionation, in Dependence of Column Height

A change in foam height of 65 cm caused a decrease on tryptantryn enrichment of 26.97%. The influence of height of liquid pool was studied by foam fractionating the solutions with liquid heights of 9.30 cm and 7.00 cm, with other parameters and conditions kept constant. There was no noticeable decrease in enrichment.

4.4.3.4. Influence of Consecutive Foaming and Gas Flow Rates

Consecutive foaming and control of the gas flow rates supplied to the foam fractionation column were explored as ways to improve the enrichment of tryptanthrin. For the purpose, other parameters and conditions were kept constant as specified in Table 3.4 D and the initial solution was consecutively foamed. Consecutive foaming required the addition of saponin, thus, 0.05 g of saponin were added to the solutions prior to each consecutive foaming. The flow of gas supplied to the column was controlled for each foaming. Foam was withdrawn for each consecutive foaming and the quantification showed that there was an enrichment of tryptanthrin until the third consecutive foaming. Figure 4.18 illustrates the evolution of enrichment with consecutive foaming. An analysis of the evolution of tryptanthrin enrichment with consecutive foaming revealed that enrichment occurs gradually with each consecutive foaming.

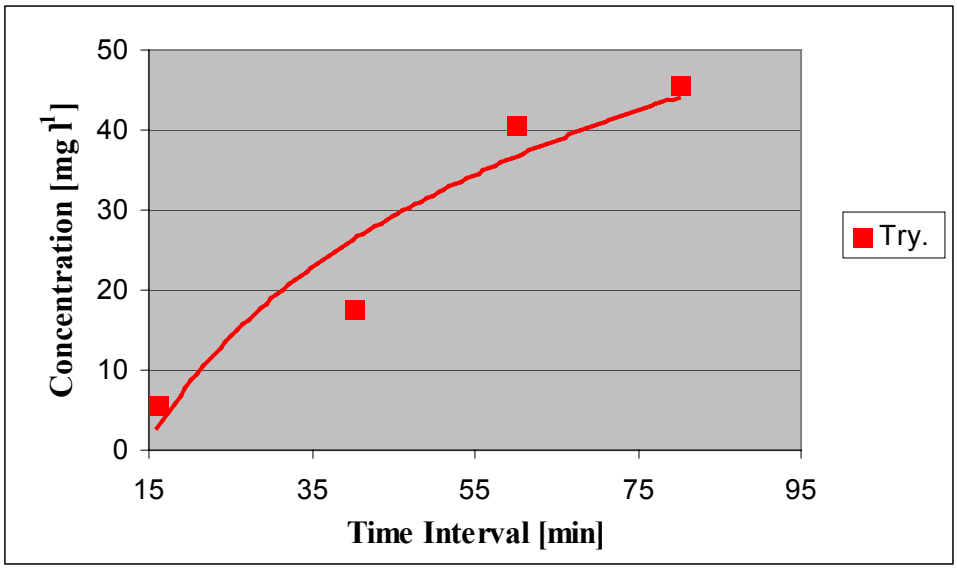

Figure 4.18 Increase of Tryptanthrin Concentration from Initial Solution, to Foam Collected Along the Consecutive Foam Fractionation Process

4.4.3.5 Optimised Overall Enrichment Process

The optimised overall enrichment of tryptanthrin was attained when the parameters and conditions that provided for higher enrichments were met as described in the previous sections. Table 4.9 provides the concentration of tryptanthrin present in the initial solution, foam collected for the overall process and the residual solution. Relative and percent enrichments for the overall process are also given.

	Initial Solution Total Volume: 100 ml	Foam Total Volume: 9 ml	Residual Solution Total Volume: 90ml	E_R	%E
	Concentration [mg l^{-1}]	Concentration [mg l^{-1}]	Concentration [mg l^{-1}]		
Try.	5.65	45.70	1.58	8.08	72.76

Table 4.9 Concentration of Tryptantrin in Initial Solution, Overall Foam and Residual Solution, for the Foam Fractionation Process. Relative Enrichment and Percent Enrichment

The difference between tryptanthrin concentrations in the initial solution, in the foam collected for the overall process and in the residual solution is illustrated graphically in Figure 4.19. The increase of tryptanthrin concentration from the initial solution to the foam collected for the overall process is above eight fold. As per the graphic, the residual solution contains reduced amounts of tryptanthrin.

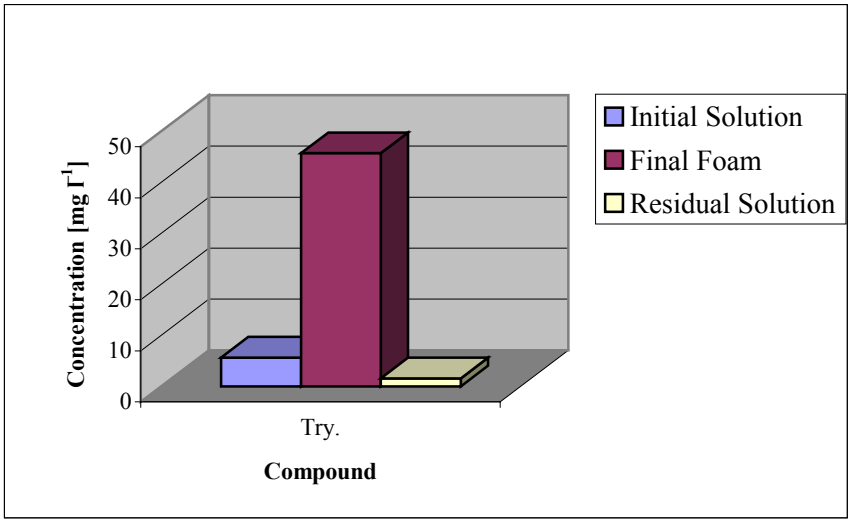

Figure 4.19 Concentration of Tryptanthrin in Initial Solution, Overall Foam and Residual Solution, for the Foam Fractionation Process

Figure 4.20 illustrates the increase of tryptanthrin concentration for the overall process by a comparison of the chromatograms corresponding to the initial solution, the foam for the overall process and the residual solution. Tryptanthrin peak area in the chromatogram for the overall process foam is larger than the area of the tryptanthrin peak for the chromatogram corresponding to the initial solution. The residual solution's chromatogram displays a small tryptanthrin peak, corresponding to the amount of tryptanthrin that was not transferred from the solution to the foam. Peaks corresponding to isatan b and other indigo precursors are relatively high for the overall foam, when comparing that chromatogram with that of the initial solution. In the chromatogram for residual solution, indigo peak is relatively higher than that from the initial solution's chromatogram.

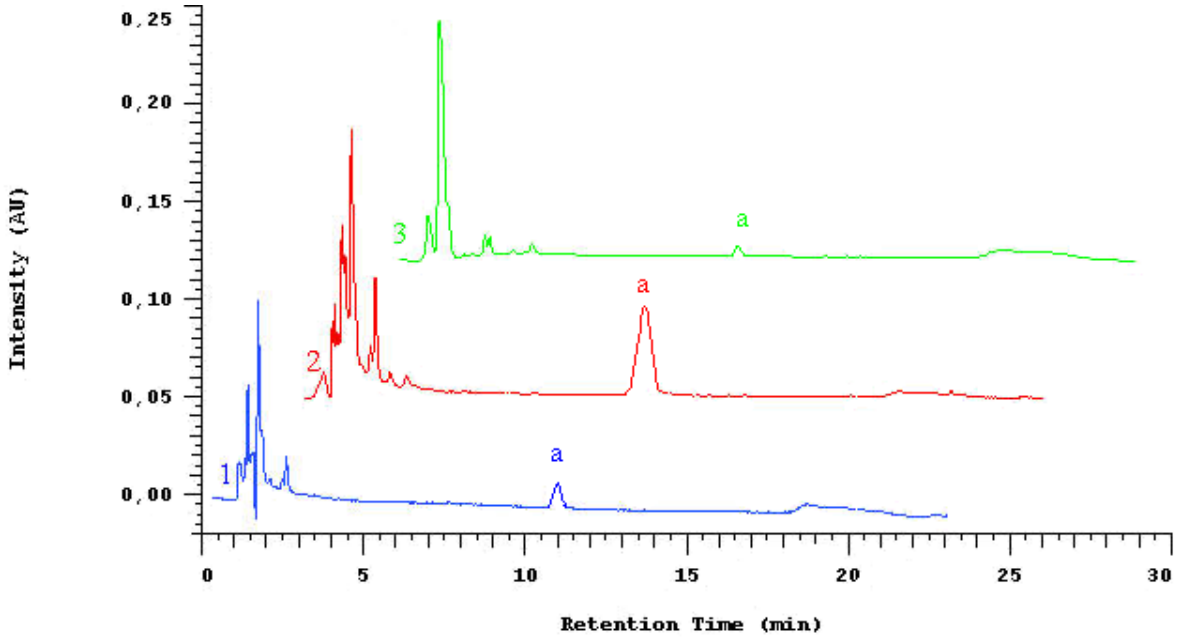

Figure 4.20 HPLC-UV/DAD Chromatograms of the Overall Tryptanthrin Foam Fractionation Process

Chromatogram 1 – Initial Solution a – Try.

Chromatogram 2 – Overall Foam

Chromatogram 3 – Residual Solution

4.5 *Cannabis Sativa*

4.5.1 Analytical Characterisation of Cannabinoids

4.5.1.1 UV-Vis Characterisation

Cannabinoid standards used for quantification purposes, were analysed in the UV-Vis region for their absorption patterns, to check their absorption at the operating conditions chose for quantification. Samples of the standards were diluted in MeOH and characterised by UV-Vis spectroscopy (3.2.4) and conditions in accordance with 3.3.1.1. Figure 4.21 presents the UV-spectrums from cannabidiol, cannabinol and Δ^9 – tetrahydrocannabinol. The spectrums were taken at the wavelengths between 200 nm and 400 nm. The peak maximums are approximately at 231 nm for cannabidiol and cannabinol and 227 nm for tetrahydrocannabinol.

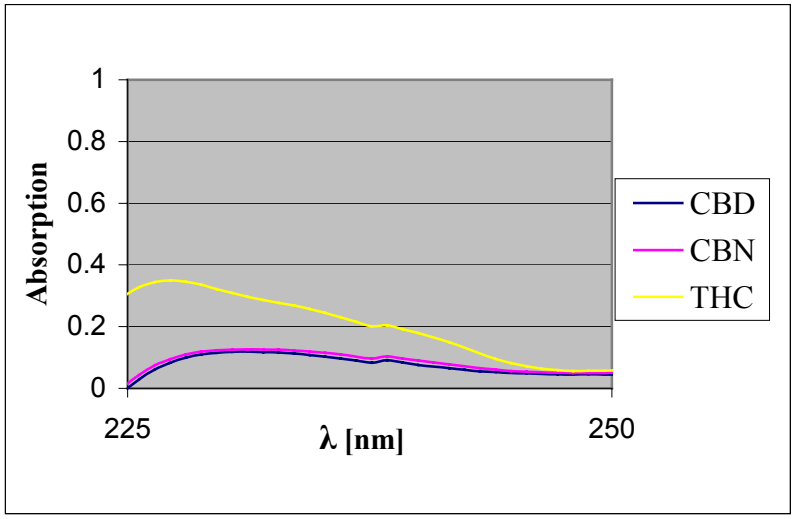

Figure 4.21 UV-Spectrum of Cannabidiol, Cannabinol and Δ^9 –Tetrahydrocannabinol (λ – 225-250 nm)

4.5.2.2. HRGC-EI/MS Characterisation

The cannabinoid standard Δ^9 –Tetrahydrocannabinol was characterised by HRGC-EI/MS (3.2.3) and conditions as per 3.3.1.4. The characterisation of Δ^9 –Tetrahydrocannabinol, realised with HRGC-EI/MS, allowed accessing stability of Δ^9 –Tetrahydrocannabinol during storage. Even more important was the assessment of the cause of its high instability during processing. The retention time of the peak displayed in the chromatogram was 38.12 min. Table 4.10 gives the main fragments of the MS spectrum corresponding to the molecular ion and fragment ions, for the *m/z* values from 240 to 320 .

Fragment	*m/z*
$[M]^+$	314
$[M-15]^+$	299
$[M-43]^+$	271
$[M-56]^+$	258
$[M-71]^+$	243

Table 4.10 HRGC-EI/MS Main Fragments of Δ^9 –Tetrahydrocannabinol (*m/z* – 240-320)

4.5.2 Extraction of Cannabinoids

Cannabinoids extraction from *Cannabis sativa* was carried out using water as a solvent, with heating, by stirring, ultra-sonic bath and extraction under reflux. The pH's of the water were varied from 2 to 12. Extraction by heated magnetic stirrer resulted the most effective extraction method. The amounts of cannabinoids extracted at the acidic range were higher than those extracted at the neutral and alkaline ranges. Higher extraction was achieved with water at pH 4. The aqueous extract obtained at this pH contained low amounts of cannabidiol and no cannabinol or tetrahydrocannabinol. The residue from aqueous extraction was extracted with various organic solvents and almost no cannabinoids were present in the concentrated extract. Samples of plant materials were than extracted with various organic solvents using various methods. The method that had some success was the extraction of MeOH by ultra-sonic bath (3.3.2.4). The latter extracted almost all cannabinoids from the plant. The cannabis plant contained reduced amounts of cannabidiol, cannabivarin and only very low amounts of cannabinol and terahydrocannabinol. After drying with vacuum, with MeOH extract contained relatively high contents of cannabidiol, cannabivarin and low contents of cannabinol and terahydrocannabinol. This extracted was then extracted with water at pH 4, in a heated magnetic stirrer. The amount of cannabinoids contained in the aqueous extract is presented in Figure 4.11.

Active Principle	Concentration in Extract [mg l^{-1}]
Cannabidiol (CBD)	5
Cannabinol (CBN)	0-1
Δ^9 – Tetrahydrocannabinol (THC)	0-0.5

Table 4.11 Concentration of Cannabinoids in *Cannabis Sativa* Extract

4.5.3 Enrichment of Cannabinoids

Cannabinoids enrichment from aqueous dilute solutions by means of foam fractionation is described in 3.3.2.4. The likelihood of the enrichment of cannabinoids by foam fractionation

was tested by a number of experiments. The foaming capability of cannabinoids aqueous dilute solutions was not enough for the foam fractionation to take place. Accordingly, saponin was added to increase the foaming capacity of the solutions. MeOH and glycerine were also added to improve foam properties. Preliminary results indicated that cannabinoids can be enriched by foam fractionation. However, tetrahydrocannabinol was very unstable in aqueous solution, degrading to cannabinol, within 10 to 12 hours, stored in dark at –20°C.

Further experiments proved that concentration of cannabinoids in the initial solution was important for the improvement of enrichments obtained. Section 4.5.3.1 details the influence of saponin, MeOH, glycerine and concentration on enrichment and the quantification of the influence of concentration on that enrichment.

The influence of initial solution's pH on enrichments achieved by foam fractionation was also studied. Initial solutions pH values resulted of key importance for the enrichment of cannabinoids. Section 4.5.3.2 provides details and a quantification of initial solution's pH influence on enrichment. Studies on the influence of liquid pool heights and height of foam tower on enrichment, showed that the height of foam tower variation markedly affects the enrichment, while the variation of liquid pool heights, has a marginal influence. Section 4.5.3.3 describes both influences on enrichment and presents the quantification of height of foam tower influence. Cannabinoids optimised enrichment was achieved with consecutive foaming at strictly controlled gas flow rates. Section 4.5.3.4 specifies the importance of consecutive foaming and strict control of gas flow rate for the enrichment. Section 4.5.3.5 provides a description of cannabinoids enrichment at optimised conditions.

4.5.3.1 Influence of Solute Concentration, Surfactants and Additives

Aqueous dilute solutions of cannabinoids only possessed a weak foaming capacity and addition of saponin was necessary to render the solutions a required foaming capacity. The right amount of saponin was found by experimentation. Addition of 0.05 g of saponin or more, enabled the foam fractionation to take place, however the coalescence was poor. To improve the properties of the foam, MeOH and glycerine were added. With the addition of 0.05 g of saponin, 500 μl of MeOH and 1 ml of glycerine, the foam possessed good properties.

In order to investigate the influence of concentration on enrichment, saponin, MeOH and glycerine amounts were kept unchanged in accordance with Table 3.4 A and the concentration of cannabinoids in the initial solution was varied between 30 mg l^{-1} and 120 mg l^{-1}. With the increase of cannabinoids concentration in the initial solution the stability of the foam decreased. At higher concentrations, only a strict control of the gas flow supplied to the foam fractionation column granted the achievement of good foam stability along the process, for the whole range of concentrations studied. The enrichment of cannabinoids increased with concentration to reach a peak at the concentration of 60 mg l^{-1} and decreased again. Figure 4.22. illustrates the enrichment obtained at different cannabinoids concentration.

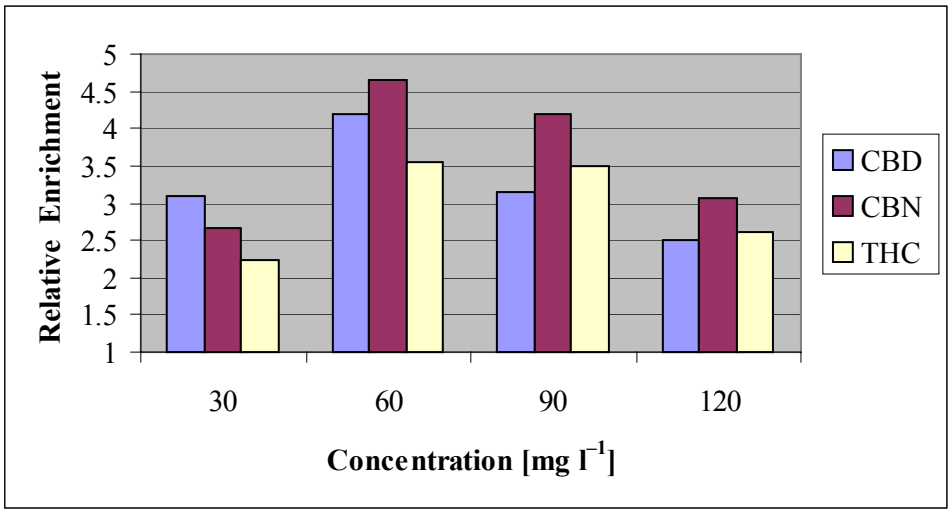

Figure 4.22 Enrichment of Cannabinoids by Foam Fractionation, in Dependence of their Concentration in Initial Solution

An increase of concentration from 30 mg l^{-1} to 60 mg l^{-1} resulted in an improvement in enrichment of 26.01% for cannabidiol, 42.58% for cannabinol and 37.64% for tetrahydrocannabinol. An increase in concentration from 60 mg l^{-1} to 120 mg l^{-1} caused a decline in enrichment of 40.1% for cannabidiol, 34.19% for cannabinol and 26.40% for tetrahydrocannabinol.

4.5.3.2 Influence of pH

The enrichment obtained by the foam fractionation of cannabinoids dilute aqueous solutions was influenced by the pH of those solutions. This influence was studied by varying the pH values of the solutions from 2 to 12, while maintaining the other parameters constant as per

Table 3.4 B. Foam fractionation in the acidic range was easier than foam fractionation in the neutral range. In the alkaline range and up to pH 10 the foam fractionation was difficult. The stability of the foam decreased from the acidic range to the alkaline range up to pH 10. At pH higher than pH 10, the stability of the foam was similar to that of the acidic range. To improve the quality of the foam in the alkaline range, the flow of gas supplied to the foam fractionation column had to be strictly controlled. Quantification of the foam collected at the various pH's showed that the enrichment raised with the rise of pH, reaching a peak at pH 10 and declined again with the rise of pH. Figure 4.23 gives an illustration of the enrichments obtained at the range of pH's studied.

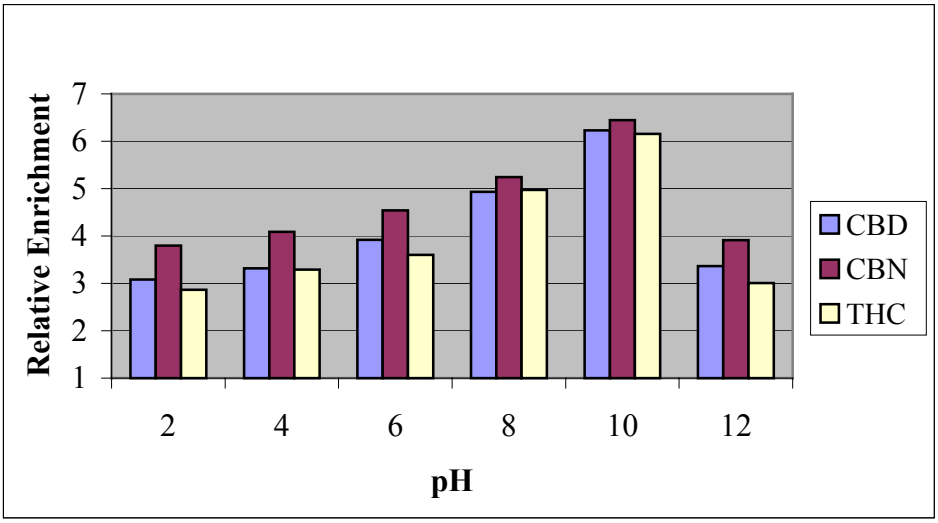

Figure 4.23 Enrichment of Cannabinoids by Foam Fractionation, in Dependence of Initial Solution pH

The increase of initial solutions pH's from 2 to 10, produced an increase in enrichment of 50.56% for cannabidiol, 41.00% for cannabinol and 53.33% for tetrahydrocannabinol. The increase of pH from 10 to 12 produced a decrease in enrichment of 45.91% for cannabidiol, 39.13% for cannabinol and 51.06% for tetrahydrocannabinol.

4.5.3.3 Influence of Height of Foam Tower and Height of Liquid Pool

The variation of height of foam tower influenced the enrichment achieved by cannabinoids foam fractionation. To study the influence of height of foam tower on enrichment, foam fractionation columns with the length of 130 cm and 65 cm (Table 3.1) were used. Other parameters and conditions were kept constant as per Table 3.4 C. The difference of foam tower height between both columns was 65 cm. For the short column, the bubble size of the foam exiting the column was only a little bigger than the bubble size of the foam at the bottom of the foam tower. For the long column, the bubble size of the foam exiting the column was visibly bigger than the bubble size of the foam at the bottom of the foam tower. The foam from the shorter column also contained more liquid. The quantification showed that the enrichment of cannabinoids was higher with the long column. Figure 4.24 displays the graphical enrichment as function of column height.

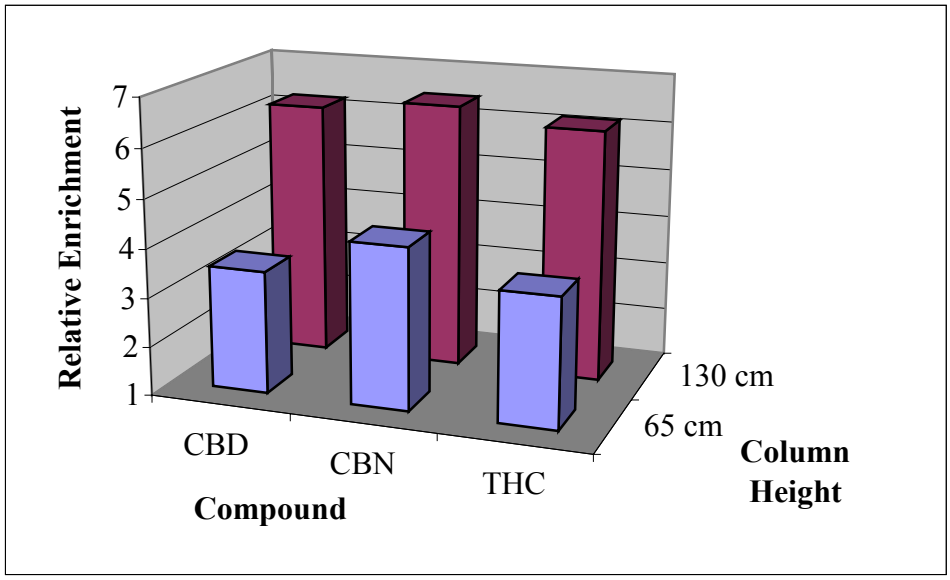

Figure 4.24 Enrichment of Cannabinoids by Foam Fractionation, in Dependence of Column Height

An augment in foam height of 65 cm caused a decline in enrichment of 43.66% for cannabidiol, 33.22% for cannabinol and 40.81% for tetrahydrocannabinol. The influence of the height of liquid pool was studied for heights of liquid pool of 9.3 cm to 7 cm, maintaining the other parameters and conditions unchanged. The change in enrichment was negligible.

4.5.3.4. Influence of Consecutive Foaming and Gas Flow Rates

Consecutive foaming and gas flow rates supplied to the foam fractionation column resulted important for the enrichment of cannabinoids. Experiments were made to study what influence consecutive foaming and the respective gas flow rates had on enrichment. For the purpose, 0.05 g of saponin had to be added to the solutions prior to each consecutive foaming. Other parameters and conditions were maintained constant as detailed in Table 3.4 D and the solutions consecutively foam fractionated at strictly controlled gas flow rates. The amount of liquid carried by the foam and foam stability, strongly depended on the gas flow rates. Therefore, a strict control of the gas flow rate supplied to the foam fractionation column was required to obtain a foam with low liquid contents and good stability. The consecutive foaming provided for the further enrichment of cannabinoids up to the third consecutive foaming. Figure 4.25 illustrates the evolution of the enrichment with the consecutive foaming.

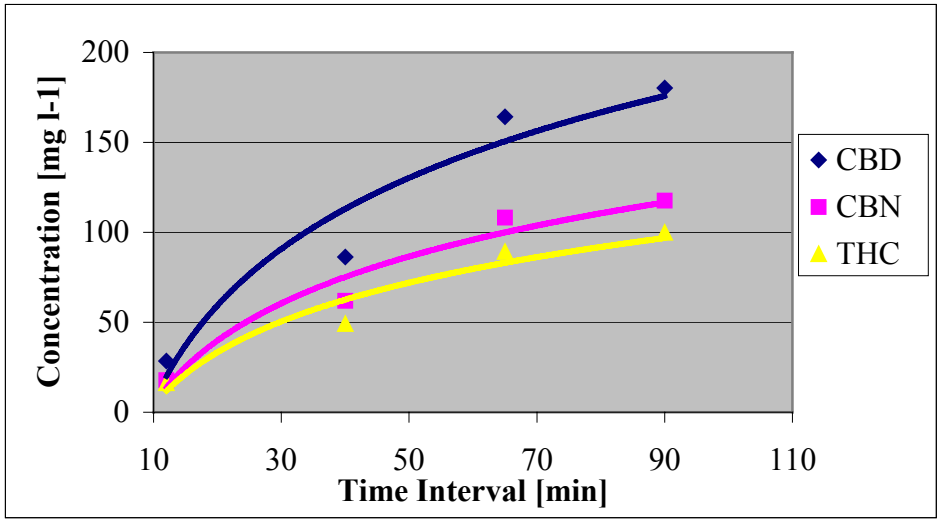

Figure 4.25 Increase of Cannabinoids Concentration from Initial Solution, to Foam Collected Along the Consecutive Foam Fractionation Process

The analysis of enrichment with consecutive foaming indicates that the increase of enrichment occurred gradually. Therefore, up to the third consecutive foaming, almost the same enrichment was registered for each consecutive foaming. Further foam fractionation beyond the third consecutive foaming produced no enrichment and an increase in foam stability was observed. Therefrom, the strict control of gas flow rate to the column was no longer necessary.

4.2.3.5 Optimised Overall Enrichment Process

Cannabinoids optimised overall enrichment was achieved when all parameters and conditions that allowed for the highest enrichment were found, as described in the previous sections. Table 4.11 presents the initial concentrations, concentrations in the foam collected for the overall process and concentration in residual solution, for each cannabinoid. The overall relative and percent enrichments are also presented. Cannabinoids enrichment are very close to each other. The difference in enrichment from tetrahydrocannabinol to cannabidiol is only 2.29% and from tetrahydrocannabinol to cannabinol is 5.74%.

Cannabinoid	Initial Solution Total Volume: 100 ml	Foam Total Volume: 12 ml	Residual Solution Total Volume: 86.5 ml	R_E	%R
	Concentration [mg l^{-1}]	Concentration [mg l^{-1}]	Concentration [mg l^{-1}]		
CBD	28.43	180.32	7.11	6.34	76.10
CBN	17.75	117.67	3.82	6.63	79.55
THC	16.22	99.80	4.40	6.15	73.81
Total	62.41	397.79	15.32		

Table 4.12 Concentration of Cannabinoids in the Initial Solution, Overall Foam and Residual Solution, for the Foam Fractionation Process. Relative and Percent Enrichment

The contrast of cannabinoid concentrations in the initial solution, in the foam collected for the overall process and the residual solution is graphically illustrated in Figure 4.26. The increase in cannabinoid concentration from the initial solution to the foam is above six fold. The residual solutions still contain small quantities of cannabinoids which could not be transfered to the foam.

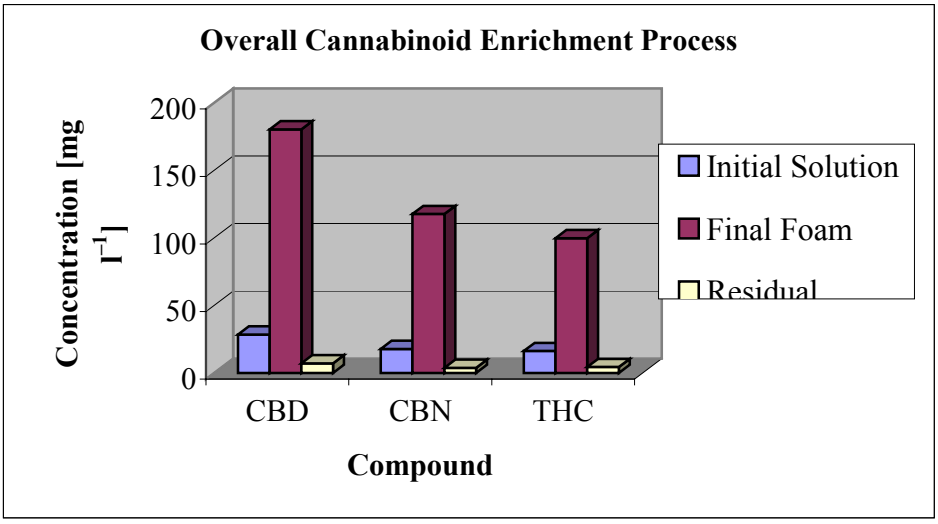

Figure 4.26 Concentration of Cannabinoids in the Initial Solution, Overall Foam and Residual Solution, for the Foam Fractionation Process

The overall increase in cannabinoids concentrations is illustrated in Figure2.27 by the comparison of chromatograms from cannabinoid initial solution, foam collected for the overall process and residual solution. There is an evident increase of cannabinoid peak areas, from chromatogram corresponding to the initial solution to chromatiogram corresponding to the foam for the overall process. The chromatogram corresponding to the residual solution presents only very small peaks.

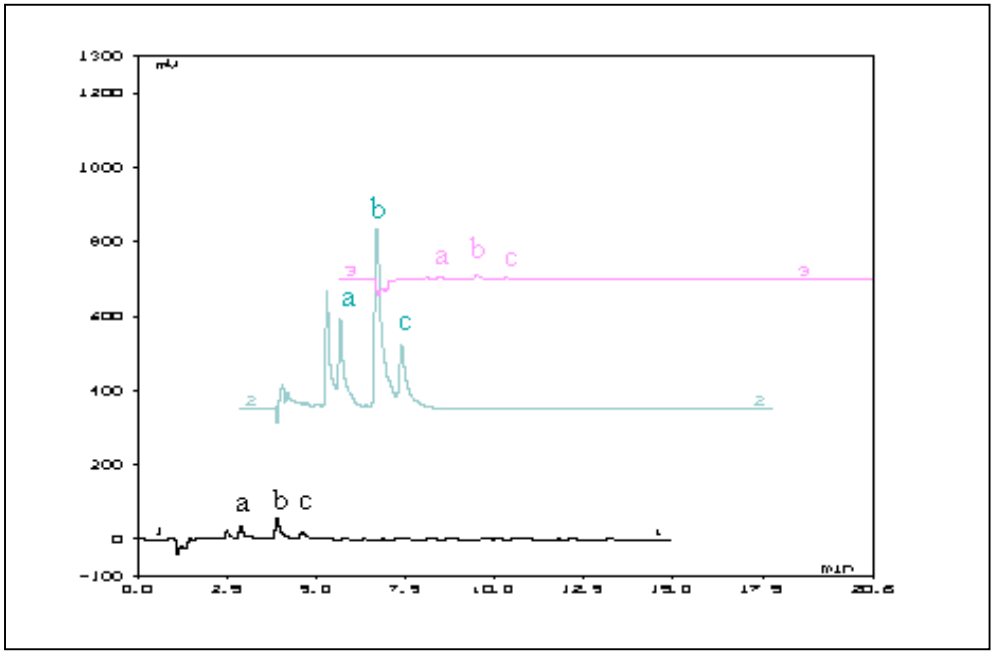

Figure 4.27 HPLC-UV Chromatograms of the Overall Cannabinoid Foam Fractionation Process

Chromatogram 1 – Initial Solution
Chromatogram 2 – Overall Foam
Chromatogram 3 – Residual solution

a – Cannabidiol
b – Cannabinol
c – Δ^9 – Tetrahydrocannabinol

5. Discussion

5.1 *Calendula Officinalis*

5.1.1 Characterisation of Lupeol Acetate

Lupeol acetate, the internal standard synthesized and utilised to record the calibration curve for faradiol esters quantification, was characterised. The aim was to check UV absorption intensity at operating conditions for quantification and purity, by UV-Vis spectroscopy and HRGC-EI/MS. The UV-spectrum (Figure 4.1) displays the characteristic absorption pattern of lupeol acetate for the given wavelength interval. This molecule has a poor UV absorption, because it is mostly saturated, contains only few double bonds and due to the scarcity of oxygen atoms. Therefore, the concentration in the sample for analysis should be high. The absorption maximum at 210 nm is typical from lupeol acetate absorption maximum at this interval. This is the highest absorption displayed by the molecule in the UV region. Lupeol acetate absorption pattern is similar to those of faradiol esters, in accordance to Frauen (2001).

The chromatogram resulting from the HRGC-EI/MS characterization depicts a large peak corresponding to the retention time of lupeol acetate. The MS spectrum for the *m/z* between 400 and 470 resulting from the same analysis (Table 4.3) shows the characteristic fragments $[M]^+$, $[M-15]^+$, $[M-43]^+$ $[M-60]^+$. Such fragmentation is in conformity with the spectrum for lupeol acetate from the MS database NIST-98. These fragments correspond to the molecular ion and the fragment ions originated from the successive cleavage of the acetyl group, which leads to the loss of acetic acid (Pyrek, 1977a; 1977b; Boguslaw, 1985). The UV-Vis and HRGC-EI/MS results denote that the synthesized lupeol acetate had a high purity.

5.1.2 Extraction of Faradiol Esters

Faradiol esters extraction from *Calendula* flos samples available for this work failed when water was used as a solvent. Faradiol esters have low solubility in water due to their high hydrophobicity (2.3.1.3). Another motive was the modest faradiol contents of the samples. Organic extraction with DCM and evaporation of the extract to dryness allowed to obtain a highly concentrated faradiol esters extract. These faradiols could then be extracted from that extract, using hot water as a solvent. The variation of the water's pH value had no influence

on the amount of faradiol esters extracted. At acidic and alkaline pH's, more insoluble ballast substances were extracted.

Some ballast substances were pH dependent precipitated flavonoids and pectins, surface active saponins, insoluble chlorophyll, insoluble celluloses and relatively high amounts of insoluble carotenoids as xanthophylle (2.3.1.4). The presence of precipitated and insoluble ballast substances should be reduced to the lowest possible, because elimination of such low amounts of those substances was difficult and their presence in solution negatively influenced the foam fractionation of aqueous extracts.

Since the use of hot water and prolonged extraction time could extract significant amounts of insoluble substances present in large amounts at the plant, like chlorophyll and carotenoides, extraction time was kept short. Moreover, pH of water used for the extraction was neutral to prevent extraction of significant amounts of flavonoids and pectins. The aqueous extracts so obtained, contained large amounts of the faradiols esters faradiol lauritic acid ester, faradiol myristic acid ester and faradiol palmitic acid ester (Table 4.4). Other faradiol esters were also present in significant quantities as well as reduced amounts of the surface active saponins, chlorophyll and significant amounts of xanthophylls.

5.1.3 Enrichment of Faradiol Esters

The faradiol esters faradiol lauritic acid ester, faradiol myristic acid ester and the faradiol palmitic acid ester are highly hydrophobic molecules (2.3.1.3). This hydrophobicity stems from the existence in the molecule of a long hydrocarbon chain, the bulkiness of the molecule and the scarce existence of oxygen atoms and double bonds (2.2.1). The presence of faradiol esters molecules in the aqueous solution is hence not favourable, since the faradiol ester molecules interfere to a great extent with the water molecules and for that reason their presence in the gas-liquid interface is more favourable as in the bulk of the liquid.

This condition means that the enrichment of faradiols esters from their aqueous dilute solutions by foam fractionation is possible, but nevertheless, requires that the solution is able to produce foam when aerated by a gas. It also requires that the faradiol ester molecules can be adsorbed at the gas-liquid interface of the rising foam bubbles. Upon reaching the top of the column and when the process is optimised, the foam collected ought to be rich in faradiol es-

ters. As per 4.2.3, optimised conditions for enrichment of faradiol esters by foam fractionation are strongly dependent on surfactant and other additives addition, concentration of faradiol esters in the aqueous dilute solutions, height of foam tower, time of foaming and respective gas flow rates. Therefore, further sections will discuss the influence of the above mentioned parameters and conditions on enrichment. For their importance, influence of height of liquid pool above the sparger, pH value of initial solutions and optimised conditions will also be discussed.

5.1.3.1 Influence of Surfactants and Additives

In accordance with the preceding section, the foam fractionation of faradiol esters aqueous dilute solutions requires the generation of foam that provides the gas-liquid interface for the adsorption of faradiol esters molecules. These molecules are hydrophobic in nature and thus, lack the foaming capacity, which is property of surface active molecules. Saponins, which are surface active substances are present in the aqueous dilute solutions of faradiol esters (5.1.2). However experiments as described in 3.3.2.1 showed that the amount of saponin was not enough to give the solution the required foaming capacity.

Addition of saponin was required and with the foaming of the solutions, faradiol esters adhered to the walls of the glass column, since faradiol molecules were present as solid particles and not diluted, which is detrimental for the foam fractionation (2.2.5.2). A small amount of DCM was added. This lowered the ionic strength of the solution and solubilized the faradiol esters present in solution. Addition of DCM, an organic solvent with a low polarity index, disrupted the foam by excessive coalescence, which entailed too much thinning and drainage (2.2.2.2). To prevent excessive coalescence glycerine was added to raise viscosity and thus, retard drainage and decrease coalescence to a favourable degree. The foam with a favourable degree of drainage and coalescence, possessed large bubbles and good stability (2.2.2.3), conditions convenient for the enrichment by foam fractionation .

Experimental results evinces that the amounts of saponin added to the solutions had a strong influence on the enrichments achieved (4.2.3.1). A graphical representation of that influence is shown in Figure 4.2. The amount of saponin required for the foaming was high and an increase from 0.22 g of saponin as the optimal amount, to 26 g of saponin, caused a decrease of

13% in faradiol lauritic acid ester, and 8% in faradiol myristic acid ester and faradiol palmitic acid ester. This results indicate that the optimal amount of saponin was close to the c.m.c and further increase had a negative influence on enrichment, since miccelles were formed (2.2.5.1), with detrimental effects for the enrichment.

5.1.3.2. Influence of Solute Concentration and pH

Enrichment by foam fractionation is successful using aqueous dilute solutions of the molecules to be separated. Therefore, enrichment of faradiol esters was performed by foam fractionating aqueous dilute solutions of faradiol esters. The range of concentrations for which the foam fractionation was performed was between 20 mg l^{-1} and 160 mg l^{-1}. The illustration of the results of those experiments is presented in Figure 4.3.

The analysis of those results shows that the enrichment increased gradually with the concentration of faradiol esters in the initial solution, from 20 mg l^{-1}, the lowest concentration, to reach a peak at 80 mg l^{-1}, the optimal concentration. Then, the concentration decreased gradually until 160 mg l^{-1}. For faradiol lauritic acid ester, faradiol myristic acid ester and faradiol palmitic acid ester, there was an increase of 54.91%, 37.70% and 42.10% respectively, from the lowest concentration to the optimised concentration. For the same faradiols, a decrease of 57.64%, 34.97% and 44.21% from the optimal concentration to the highest concentration was registered. This denotes the strong influence of faradiol esters concentration on the enrichment by foam fractionation. The influence of initial concentration on enrichment is more pronounced for faradiol lauritic acid ester, which presents the largest increase and decrease in enrichment as a function of concentration.

For initial concentrations of 40 mg l^{-1} and 80 mg l^{-1} of faradiol esters, the pattern of flow fractionation varied from the initiation of the process to the end (4.2.3.2). The increase of foam stability towards the end of the process means that the faradiol ester molecules interfered with the foam. When there was a substantially complete removal of the molecules due to enrichment, the stability of the foam increased (2.2.3.2). For solutions with initial concentrations of 20 mg l^{-1}, there was no change in foam stability, the coalescence was lower, the bubbles smaller and the foam dryer. This means that the concentration of faradiol esters in solution was too low, causing no interference to the foam. For solutions with 120 mg l^{-1} and more, the

opposite was observed. This signifies that the concentration of the faradiol esters in solution was too high and close to c.m.c, the interference with the foam was too high, which was detrimental to enrichment.

The variation of the pH of initial solutions produced no noticeable changes in the enrichments of faradiol esters. This indicates that the charges of the faradiol esters molecules changed only slightly, or even remained unchanged with the change of pH. Accordingly, there was no significant change in the hydrophobicity of the faradiol molecules.

5.1.3.3 Influence of Height of Foam Tower and Height of Liquid Pool

During foam fractionation of faradiol esters, the quality of the foam was strongly influenced by the foam tower's height. As described in 4.2.3.3, initial solutions were foamed in a long column, which permitted a high foam tower and a short column with approximately the half of the longer column's foam tower. The foam produced in the longer column possessed large bubbles, the coalescence and diffusion were favourable, which resulted in an internal reflux that permitted the rise of a foam with low liquid contents and rich in faradiol esters. These conditions are favourable for the increment of enrichment (2.2.2.3). Coalescence provided for the disappearance of small bubbles and the growth of large bubbles with a large surface area for adsorption. The drainage that arouse from that coalescence constituted an internal reflux. Faradiol esters enrichment is favoured for columns with high foam towers, thus, high residence times in which large bubbles can be formed due to coalescence and were the internal reflux is maximised to such an extent that the foam reaching the top of the column has high faradiol esters contents but low liquid contents.

Shorter columns as that used in the present work, produced a wet foam with small bubbles, since the short height of foam tower, hence, short residence time of the foam in the column, minimised the growth of the rising bubbles. Coalescence, drainage and internal reflux were also minimised, which resulted in wet foams with small bubbles as described above. The influence of height of foam tower (Figure 4.4), shows that a decrease of 65 cm in foam height resulted in a significant enrichment decline. Faradiol lauritic acid ester, faradiol myristic acid ester and faradiol palmitic acid ester registered the decrease of 26.20%, 19.16% and 18.32%,

respectively. The influence of the height of foam tower is therefore, higher for faradiol lauritic acid ester, which is present in solution at accentually lower quantities.

The variation of liquid pool's height (4.2.3.3) had no influence in the foam fractionation. Primarily adsorption of faradiol esters takes place in the liquid pool, during the rise of the bubbles from the bottom till the top of the liquid pool. Diffusion of faradiol ester molecules from the bulk of the liquid to the gas-liquid interface is a slow process. Therefore, the residence times of the bubbles should be sufficient to permit adsorption to take place (2.2.5.8). A modification of the height of liquid pool changes the contact time between bubbles and solution influencing adsorption, what made a change in enrichment predictable. In this work, a change from 7.5 cm to 5.5 cm produced no noticeable changes in enrichment. This suggests that the height of liquid pools chosen to study the effect on enrichment were above the minimum required to accomplish improved enrichments.

5.1.3.4 Influence of Time of Foaming and Gas Flow Rates

Foaming time and the respective gas flow rates supplied to the foam fractionation have a marked influence on the enrichment of faradiol esters. Figure 4.5, illustrates the enrichment of faradiol esters at 5 min time intervals with the strict control of the gas flow rates supplied to the column.

The enrichment increased with each time interval however, there were differences in enrichments between the various intervals. Comparatively, the enrichment at the first two time intervals was lower than the enrichment at the last ones. The enrichment of faradiol esters occured towards the end of the process, because faradiol ester molecules are large, which means that their diffusion and adsorption was retarded (2.2.1). Other factor is that at the beginning of the process, faradiol esters molecules had to compete with the more hydrophobic carotenoid molecules, which were present in the solution in reduced quantities. A relatively high amount of surface active saponin molecules that should be added to the solution to improve the foaming capacity, also posed a competition to the faradiol molecules. Towards the end of the process, the curves corresponding to the enrichment of each faradiol ester are close to reach a plateau, denoting that the maximum enrichment possible is close to be achieved.

During the foam fractionation of faradiol esters, gas flow rates supplied to the glass column were of key importance to obtain a foam with low liquid content and an improved enrichment. The gas flow was the lowest possible and controlled in such a way that the gas contained in the bubbles rising along the foam tower, could diffuse from smaller bubbles to larger bubbles (2.2.5.7), causing the disappearance of the smaller bubbles (2.2.2.3). The result was a foam with large bubbles, a favourable drainage and reflux ratio and saturated with faradiol esters molecules and poor in liquid content.

5.1.3.5 Optimised Overall Enrichment

When the optimal conditions were attained, foam fractionation of faradiol esters provided for a remarkable enrichment of faradiol lauritic acid ester, faradiol myristic acid ester and faradiol palmitic acid ester. Table 4.5 and Figure 4.6 provide the enrichments for each faradiol ester. The enrichment of faradiol lauritic acid ester is 4.00% and 3.50% lower than the enrichments of faradiol myristic acid ester and faradiol palmitic acid ester. This signifies the effect of two combined factors: Lower hydrophobicity and lower concentration of faradiol lauritic acid ester in initial solution, when confronted with that of faradiol myristic acid ester and faradiol palmitic acid ester.

Figure 4.7 illustrates a chromatogram comparison of the initial solution, overall foam and residual solution, for the foam fractionation of faradiol esters. The increase of peak areas corresponding to faradiol lauritic acid ester, faradiol myristic acid ester and faradiol palmitic acid ester, from the initial solution to the foam, represent the enrichment. Other peaks corresponding to other faradiol esters also enriched by foam fractionation are visible in the chromatograms. Saponin peaks in both chromatograms are small, however the chromatogram corresponding to residual solutions displays a large saponin peak, which means that large amounts of saponin remained in the solution. The inexistence of faradiol esters peaks in the residual solution means that almost all faradiol esters were enriched by foaming. The undesired xantophille is also co-enriched. This enrichment is noticeable in Annex 9, by comparing xantophille typical deep yellow colour displayed by the overall foam in contrast with the pale yellow colour displayed by the initial solution.

5.2 *Camellia Sinensis*

5.2.1 Characterisation of Catechins

Catechin and epigallocatechin gallate were the standards used for the recording of the calibration curves for catechins quantification as well as for control purposes. These standards were analysed by UV-Vis spectroscopy and ^{1}H and ^{13}C-NMR to check for their UV absorption at operating conditions utilised for quantification and possible degradation during storage. The UV-Vis spectrum (Figure4.8) shows the characteristic absorption patterns for the given absorption interval. The absorption maximum at 280 nm for catechine, and 275 nm for epigallocatechin gallate, is in conformity with Frauen (2001). In the UV region, the absorption maximum for both catechin and epigallocatechin gallate is outside the interval between the wavelengths 250-350 nm. However, the absorption intensity of these molecules is high, due to the presence of numerous double bounds and oxygen atoms. Therefore, analysis of samples with low concentrations of both catechins is possible, even at about 275 nm.

The ^{1}H-NMR and ^{13}C-NMR spectrums obtained are well in accordance with the findings of numerous authors and in the extensive work on the subject made by Ninomiya *et al.* (1997). These results allow the conclusion that epigallocatechin gallate was not degraded during storage.

5.2.2 Extraction of Catechins

Catechins extraction from green tea samples available for this work was effective when using hot water as the extracting solvent. The effectiveness of the extraction with hot water derives from the hydrophilic character of catechins (2.3.2.3), which makes them soluble in water. Another factor contributing for the effectiveness of the extraction, was the high concentration of catechins in the samples available for this study. The use of boiling water for extraction caused no degradation of catechins, which are heat sensible, since the temperature reached by boiling water do no cause degradation of catechins. Instead, extraction with hot water permitted to obtain higher amounts of catechins, as the solubility of catechins improves with the increase in temperature of the water. The pH value of the extracting water was 5.4, that of deionised water. The use of lower pH's caused no improvement in extraction and higher pH's were avoided since catechins degrade at pH's higher than 6.5.

Ballast substances were co-extracted with catechins when hot water was used as the extracting solvent. Some ballast substances are the alkaloid caffeine, the polysaccharide starch that when heated polymerise, forming colloid dispersions in water, saponin and the amino acid theanine (2.3.2.4). The presence of these components in green tea aqueous extracts is due to their solubility in water, particularly hot water and/or their large quantities in green tea samples. Catechins, as catechin, epicatechin, epigallocatechin, epigallocatechin gallate, epicatechin gallate and caffeine were by far the major components of the aqueous green tea extract (Table 4.6). Other catechins were also present in some quantities.

Caffeine was undesired in the extract due to the high quantities and negative properties. The extraction of caffeine from the aqueous extract with DCM was possible, due to caffeine's affinity to DCM. This property enables its extraction from aqueous extract of green tea. Other compounds of the aqueous extract were starches, which came in low quantities, saponins and theanine that came even in lower quantities.

5.2.3 Enrichment of Catechins

Catechins molecules are hydrophilic in nature and degrade at pH's higher than 6.5 (2.3.2.3). The hydrophilic character of catechins molecules derives from the presence in their structures, of several double bonds, phenol groups and oxygen atoms, which increases the polarity of a molecule (2.2.1). The hydrophillicity of catechins diminish with the increment in the size of the molecules. The hydrophilic character of catechins molecules signifies that when in aqueous solution, they interact with the water molecules, so that their existence is more favourable in the bulk of the liquid, than at the gas-liquid interface.

The enrichment of molecules by foam fractionation requires that these molecules are able to diffuse from the bulk of the liquid to the gas-liquid interface and then adsorb in that interface. This phenomena is not characteristic from catechins molecules, which makes them unable to be separated by foam fractionation. In order for such phenomena to take place, the nature of catechins molecules should modify in such a way that made it possible. Catechins are highly reactive molecules, forming complexes with a number of different molecules (2.3.2.5). Caffeine and starch are some of those molecules that react with catechins forming complexes. Both, caffeine and starch are undesired components of the green tea extract, caffeine present in large quantities and starch in low quantities. During the studies made while trying to enrich

catechins from aqueous dilute solutions, the importance of the association of catechins with caffeine and starch became evident for this work. Therefore, the reactivity of catechins with caffeine and starch will be discussed further on, in the light of the foam fractionation process.

In aqueous solutions, catechins associate with caffeine forming hydrophobic complexes (2.3.2.6). These hydrophobic complexes are termed “tea cream” and can be observed in practice as a turbidity that builds in green tea aqueous extracts. Low temperatures favour the formation of these complexes. The stoicheiometry of the complexes is determinant for their hydrophobicity and it reaches a maximum in case the catechins hydroxyl groups are hydrogen bounded to caffeine. An insufficiency or excess of caffeine reduces the hydrophobicity of the complexes. The esterified and bulkier catechins, epigallocatechin gallate and epicatechin gallate, form more hydrophobic complexes as those of the smaller and non-esterified catechins epicatechin, catechin and epigallocatechin, due to more availability of hydroxyl groups and to their bulkiness.

In aqueous media, catechins also forms complexes with polysaccharides as starch (2.3.2.7), which are termed “clathrate compounds”. Catechins present in solution, partition between caffeine and starch (2.3.3.8). The hydrophobicity of the complex starch – catechins occurs when their concentrations in solution are close to saturation. At concentrations lower than saturation, the solubility of both catechins and starch is 5 to 7 times higher than each molecule alone.

The significance of what is described in the preceding paragraphs for the foam fractionation of catechins aqueous dilute solutions is ample. Caffeine and starch regarded as undesired substances present in the extracts, became important in light of the foam fractionation of catechins. That importance pertains to their capacity to modify the nature and interaction of catechins molecules with water molecules, during foam fractionation. The presence of caffeine in green tea extracts, implies that hydrophobic complexes are formed between both molecules and these complexes can be enriched by foam fractionation. The extraction of caffeine from the solution with DCM, resulted in no enrichment of catechins and the residual solution contained more catechins than the foam. The presence of low amounts of starch in solutions, implies that excess catechins can form highly soluble complexes, which in solution have stronger interaction with water molecules. Therefore, water molecules show more preference

for the most soluble starch – catechins complexes, making the presence of caffeine – catechins complexes, less favourable in solution.

Preliminary studies of the possibility to enrich catechins from green tea by foam fractionation asserted that the possibility exists. Under the conditions of the present work (4.3.3), a reasonable enrichment of catechins by foam fractionation could be achieved when the optimised parameters and conditions were attained. Such enrichment strongly depends on the concentration of catechins and caffeine in the initial solutions, the addition of electrolytes, the pH values of initial solutions, consecutive foaming and respective gas flow rates supplied to the column. Further sections will examine the influence of the above parameters and conditions. Moreover, aspects related to the enrichment at optimised conditions will also be discussed.

5.2.3.1 Influence of Solute Concentration, Additives and pH

Enrichment of catechins from aqueous dilute solutions requires that catechins and caffeine form hydrophobic complexes that can diffuse from the bulk of the liquid and adsorb at the gas-liquid interface of the bubbles, rising within the foam tower. For optimised conditions, the foam collected at the top of the column should be rich in catechins molecules. As per 4.3.3.1, catechins can be enriched by foam fractionation, only when the concentration and proportions of catechins and caffeine in the extract correspond to that of the extraction described in 3.3.3.2. This indicates that the concentrations and proportions of catechins and caffeine obtained for this extract permit the formation of caffeine – catechins hydrophobic complexes and starch – catechins hydrophillic complexes.

Catechins aqueous dilute solutions bore the foaming capacity required for the foam fractionation. The foam was generally stable and the bubbles large, however that stability was somewhat excessive and the foam's liquid contents too high. This signifies that the viscosity of the solutions was too high, due to the presence in solution of polimerised starch, which was released during the extraction by hot water. This starch with high viscosity, together with reduced saponin amounts present in the extracts, rendered the solutions a good foaming capacity, yet, a too high viscosity. Consequently, the flow of gas supplied to the column was strictly controlled, in order to obtain a foam, with the lowest liquid contents possible.

As described in the previous sections, in aqueous solutions, catechins and starch form complexes that are highly hydrophilic, when the concentration of those molecules is below saturation, which was the case under the conditions of this work. The ensuing effect is that the solubility of caffeine – catechins complexes decreases due to the presence of starch – catechins complexes. The addition of NaCl, augmented the hydrophobicity of catechins complexes (2.2.5.3). The amount of NaCl was such that the ionic strength and activity of the solvent decreased, with consequent decrease of solubility of the catechins complexes. These complex molecules with lower solubility tended to more readily adsorb at the gas liquid-interface. Further addition of NaCl above the optimal amount provided no enrichment and disturbed the foam stability.

The pH also affected the enrichment of the caffeine – catechins complex. The enrichment increased with pH value, to reach a maximum at pH 3.5 and diminished again (4.3.3.1). At acidic pH's, the difference of one pH unit to the pH corresponding to the maximum enrichment already influenced the enrichment. This means that caffeine – catechins complexes net charge varies with the pH. There is a defined pH, known as the isoletric point (pI), in which the net charge of this complex is zero, which means that hydrophobicity of the molecule is at its maximum (4.3.3.1). Figure 4.9 shows that enrichment of caffeine – catechins complexes reaches a maximum at pH 3.5 and decreases again. Therefore, this pH is the pI for this catechins hydrophobic complex and the most convenient pH for enrichment, due to their maximum hydrophobicity.

5.2.3.2 Influence of Consecutive Foaming and Gas Flow Rates

In order to obtain a reasonable enrichment of catechins, the initial solutions should be consecutively foamed at controlled gas flow rates (4.3.3.2). Figure 4.10 illustrates the evolution of enrichment with consecutive foaming. In general, the enrichment of catechins was poor at the beginning of the process and increased towards the end, being the last two intervals, those that provided for higher enrichments. Additionally, the curves corresponding to the enrichment of epigallocatechin gallate and epicatechin gallate were still ascending.

The poor enrichment of catechins at the beginning of the process derives from the slow diffusion and adsorption of these molecules at the gas-liquid interface, due to their bulkiness. The

presence in solution of reduced quantities of surface active saponins, posed a competition for the catechin molecules, causing even more delay of their adsorption at the interface. The fact that the curves corresponding to the enrichments of epigallocatechin gallate and epicatechin gallate are still ascending, indicating that contrary to the other catechins, these two could be further enriched by foam fractionation. The higher hydrophobicity of epigallocatechin gallate and epicatechin gallate molecules when compared with the other catechins is the reason for higher enrichment that can be achieved for these molecules.

Gas flow rates supplied to the column were of major importance to accomplish the enrichment of catechins by foam fractionation. As mentioned in 4.3.3.2, at the start of the foam fractionation, the foam was too “heavy” and the foam characteristics altered from the beginning to the end of each process. The main alteration was the increase of bubble size in such a way that towards the end of the process the bubbles were excessively large. Changes that occurred from one consecutive foaming to another, was the reduction of the humidity of the foam.

The obtainment of an “heavy” foam at the start of the process, indicates that the foam had a high liquid content. The excessive increase of bubble size towards the end of the foam fractionation means that the gas diffusion between bubbles occurred (2.2.2.3), but nevertheless, the rupture of the larger bubbles was retarded. The origin of both, high liquid contents and the existence of large and stable bubbles is that, the viscosity of the solution was beyond the appropriate level, due to the polymerised starch present in the solution. The reduction of foam’s humidity from the first to the last consecutive foaming, evinces that a small part of the starch was carried away with the liquid contained in the foam. To counteract retarded drainage resulting from excessive viscosity, and consequent high liquid contents in the foam, the flow of gas supplied to the column was strictly controlled. This was realised, firstly by setting the flow rates somewhat high to counterbalance the “heavy” foam and then keep it to a minimum, to allow for a longer residence time of the foam in the column and thus, increase drainage and internal reflux.

5.2.3.3 Optimised Overall Enrichment

Enrichment of catechins by foam fractionation, when the optimised conditions were met, was laborious and could only accomplish modest results. Table 4.7 and Figure 4.11 depict differ-

ent expressions of the enrichments obtained for each catechin. The enrichment of catechin was the most favoured, followed by the enrichment of epicatechin gallate. As described in the previous sections, the most hydrophobic catechins are epigallocatechin gallate and epicatechin gallate. Therefore, the higher enrichments should pertain to these two catechins. Catechin registered an increased enrichment of 17.03% for relative enrichment and 16.95% for percent enrichment, when compared with the enrichment obtained for epigallocatechin gallate. A close examination of catechins concentrations and proportions in the initial solutions, leads to conclude that they played a role on the enrichment.

Fig 4.12 displays a chromatogram comparison of the initial solution, foam for the overall process and residual solution. The increase of catechins and caffeine peaks from the chromatogram for the initial solution to that of the foam, translates the magnitude of the enrichment. Catechins that could not be enriched and stayed in solution, are visible in the chromatogram corresponding to the residual solution. The chromatograms contain peaks corresponding to other catechins that were co-enriched with the catechins under investigation.

5.3 *Isatis Tinctoria*

5.3.1 Charaterisation of Tryptanthrin

Tryptanthrin standard necessary to record the calibration curve for quantification purposes was tested by UV-Vis and FTIR spectroscopy. The objective was to gain knowledge on the intensity of its absorption at operating conditions for quantification and storage stability. Tryptanthrin is a new class of active component and the knowledge of the molecule is scarce. The UV spectrum (Figure 4.13) shows the characteristic tryptanthrin absorption pattern in the given wavelength interval. The absorption maximum at 251 nm is the highest registered for the molecule in the UV region. Tryptanthrin has a fairly high absorption intensity that stems from the presence in the molecule of several double bounds and oxygen atoms, particularly the carbonyl group.

Characterisation of tryptanthrin by FTIR (Figure 4.14) supplied the vibrations of the tryptanthrin molecule functional groups. The vibrations of the most characteristic functional groups are at the wavenumbers 1727.73 cm^{-1}, 1685.42 cm^{-1} and 1594.79 cm^{-1}. The latter is accord-

ing with Honda *et al.* (1980), typical to the vibrations of tryptanthrin C═C (aromatic ring) group. The above first and second vibrations correspond to the C═O (ketone carbonyl) and RNC═O (amide) groups, respectively. Such results indicate that tryptanthrin remained stabile during storage.

5.3.2 Extraction of Tryptanthrin

Tryptanthrins extraction from *Isatis tinctoria* samples was unsuccessful when water was used as a solvent. The extraction with MeOH was only somewhat better than with water, because reduced amounts of tryptanthrin could be extracted. The reasons for the reduced or no tryptanthrin extraction from both samples entailed two reasons. One was the extremely low tryptanthrin contents in the samples available for this work. The other was the poor solubility of tryptanthrin in water despite being a polar substance and its better solubility in organic solvents of medium polarity (2.3.3.3). Dried extracts obtained from extraction with MeOH, contained low amounts of tryptanthrin that could be once again extracted with hot water. Tryptanthrin is sensitive to heat, however, still stable at the temperature of boiling water. The aqueous extracts so obtained contained reduced amounts of tryptanthrin and extremely high amounts of ballast substances. At pH 6.5, the extraction of ballast substances was somewhat disfavoured.

Ballast substances present in the aqueous solution were mainly indigo and indigo precursors, particularly isatan b (2.3.3.5). The extraction of tryptanthrin with hot water at pH 6.5, somewhat hindered indigo and isatan b extraction. The extremely high indigo and isatan b contents of the samples available for this work, resulted that every extract obtained with water or organic solvents, contained extremely high amounts of indigo and isatan b. In contrast, tryptanthrin contents was reduced (Table 4.8). Considerable amounts of other indigo precursors, chlorophyll and saponins were also present in the extract.

5.3.3 Enrichment of Tryptanthrin

The tryptanthrin molecule is slightly basic, polar and do not solubilize in water. This is due to the existence in the molecule of the cyclic groups amine, amide and ketone. The poor solubil-

ity means that in aqueous solutions, the presence of tryptanthrin molecules is more favoured at the gas-liquid interface, than at the bulk of the liquid.

The preference of tryptanthrin molecules for the gas-liquid interface instead of the bulk of the liquid, makes their enrichment by foam fractionation possible. This enrichment occurs when gas is sparged trough the solution to produce foam. Tryptanthrin molecules can then diffuse from the bulk of the liquid to the surface of the gas bubbles, which are rising to the top of the column. The foam collected at the top of the column should be rich in tryptanthrin molecules. The optimised conditions for tryptanthrin enrichment are dependent on their concentration of in the initial solution, surfactants, additives and the pH value of the initial solution, height of foam tower, consecutive foaming and respective gas flow rates (4.4.3). Further sections will describe the influence of the above mentioned parameters. Due to their importance for foam fractionation, the influence of height of liquid pool above the sparger and optimised conditions will also be discussed.

5.3.3.1 Influence of Solute Concentration, Surfactants and Additives

Tryptanthrin molecules are polar, poorly soluble in water and possess no foaming capacity. Saponins, which are surface active, are present in tryptanthrin aqueous extract (5.3.2), however, not in enough quantity to produce the foam required for the enrichment by foam fractionation (3.3.2.3). Therefore, saponin was added to the aqueous dilute. To improve tryptanthrin and other substances dissolution in the dilute aqueous solutions, MeOH an organic solvent of medium polarity was added. Glycerine was also added to decrease excessive coalescence and drainage caused by the addition of MeOH.

Enrichment by foam fractionation is achieved for aqueous dilute solutions of the molecules to be separated. Accordingly, dilute aqueous solutions of tryptanthrin were prepared with concentrations between 2.5 mg l^{-1} and 20 mg l^{-1}. The enrichments obtained for these initial concentrations are presented in Figure 4.15. Examination of the graphic shows that enrichment raised from solutions with initial concentration of 2.5 mg l^{-1} to reach a peak for solutions of 5 mg l^{-1} and then declined gradually, reaching the minimum enrichment for the concentration of 20 mg l^{-1}. Therefore, the optimal concentration of tryptanthrin in the initial solution was 5 mg l^{-1}.

Increase in enrichment from initial solutions with the concentration of 2.5 mg l^{-1} to solutions with the optimal concentration of 5 mg l^{-1} was 30.5%. The decrease of enrichment for the solutions with the optimal initial concentrations to the solutions with 10 mg l^{-1} was 36.92% and to solutions with the concentration of 20 mg l^{-1} was 52.9%. Such results mean that at concentrations higher than the optimal, the decrease in enrichment is more pronounced. In terms of foam stability, no noticeable modification with the change in concentrations of the initial solutions was observed.

5.3.3.2. Influence of pH

Enrichment of tryptanthrins by foam fractionation requires that the molecules are able to adsorb at the gas-liquid interface. For this to happen, the solution should not contain high amounts of ballast substances that as tryptanthrin, are poorly soluble in water. When such substances are present, they compete with tryptanthrin, reducing the adsorption of these molecules at the surface of the rising bubbles (2.2.1). The ballast substances present in *Isatis tinctoria* aqueous extract are mainly indigo and indigo precursors as isatan b (5.3.2).

Indigo and its precursors solubility in water, range from poor to fairly soluble and the pH may influence that solubility. The influence of pH on the solubility of indigo and its precursors, signifies that a change in pH of the initial solution may influence the enrichment of tryptanthrin. Their presence in a soluble form decreases the competition these molecules pose to tryptanthrin adsorption at the gas-liquid interface. As described in 4.2.3.2, the pH of the solutions influences tryptanthrin enrichment by foam fractionation. Figure 4.16 attests that tryptanthrin enrichment increased with the rise in pH, from the acidic to the alkaline range reaching a peak at pH 8 and decrease again. The solubility of indigo is influenced by pH and at pH's of about 8.5 – 9, indigo in the form of indigo salt, is soluble in water (2.3.3.5). Indoxyl is soluble at the whole pH range while isatan b solubility is somewhat favoured at acidic pH values.

The achievement of an optimal enrichment at pH 8 indicates that this is the pH in which higher amounts of ballast substances exist in solution in their soluble form. An increase in enrichment of tryptanthrin from pH 5 to 8, the optimal pH, is 42.44%. The increase of enrichment from pH 7 to 8 is 24.02% and the decrease in enrichment from pH 8 to 9 is 6.83%.

The difference in enrichment from pH 8 to 7 and to 9, demonstrates that indigo is the major competitor for tryptanthrin during enrichment by foam fractionation. At pH 8, the total amounts of insoluble indigo and isatan b are at a minimum. This is consubstantiated by the fact that at pH 8 and 9 the foam quality improves which pertains to the fact that most indigo and isatan b are at the soluble form.

5.3.3.3 Influence of Height of Foam Tower and Height of the Liquid Pool

Height of foam tower influenced the enrichment achieved by tryptanthrin foam fractionation. Foam fractionation was performed in a longer column, which allowed for a high foam tower and in a short column that allowed for half the height of that foam tower (4.4.3.3). The foam reaching the top of the longer column possessed large bubbles, more favoured coalescence and diffusion, resulting in an internal reflux provided the rise of a foam with low liquid contents. These conditions are favourable for the increment of enrichment since larger bubbles possess a larger surface area for adsorption and drainage resulting from coalescence of small bubbles constitutes an internal reflux. Tryptanthrin enrichment is favoured for columns, which realise high foam towers. This contributes to provide high residence times, large bubbles, were the internal reflux is such that the foam reaching the top of the column has low liquid and high tryptanthrin contents.

The shorter columns as that used in the present work, produced a wet foam with small bubbles and had short foam residence times in the column. The influence of the height of foam tower (Figure 4.17), shows that a decrease of 65 cm of foam height, resulted in a considerable decline on tryptanthrin enrichment of 26.97%.

The height of the liquid pool variation (4.4.3.3), caused no significant change in the foam fractionation. This means that the residence times of the bubbles in the liquid pool was sufficient to allow for the adsorption to take place. Changes in the height of liquid pool, vary the contact time between bubbles and solution influencing adsorption. Therefore, an alteration in enrichment is expected. In this work, a reduction of liquid pool's height from 9.30 cm to 7.00 cm produced no difference in enrichment. On that account, it is concluded that the heights of liquid pool chosen to study this influence, were above the minimum necessary for the achievement of improved enrichments.

5.3.3.4 Influence of Consecutive Foaming and Gas Flow Rates

Consecutive foaming and the respective gas flow rates supplied to the foam fractionation, influenced the enrichment of tryptanthrin. Figure 4.18, depict the enrichment of tryptanthrin up to the third consecutive foaming, at controlled gas flow rates supplied to the column.

The enrichment of tryptanthrin increased gradually with each consecutive foaming up to the third one. This evinces that the adsorption of tryptanthrin at the gas-liquid interface suffered no major delays by competing molecules as indigo and isatan b. Both molecules are larger and at the operating conditions, more soluble in the aqueous solutions than tryptanthrin. This factor provided that the retard caused by their co-adsorption with tryptanthrin was minimized. Towards the end of the process of tryptanthrin foam fractionation, the curve approximates a plateau, which indicates that the maximum enrichment possible is to be achieved.

The gas flow rates supplied to the column during tryptanthrin foam fractionation were important to produce a foam with low liquid content and attain an improved enrichment. The gas flow supplied to the column was controlled to produce a foam with large bubbles, favourable drainage and reflux ratio.

5.3.3.5 Overall Optimised Enrichment

Foam fractionation of tryptanthrin at the optimised conditions provides for relatively high enrichment. Table 4.9 and Figure 4.19 illustrate the quantitative and graphical tryptanthrin enrichment. Figure 4.20 shows a comparison of the chromatograms corresponding to the initial solution, foam for the overall process and residual solution. The increase of the tryptanthrin peak in the initial solution to that in the foam, represents the enrichment. Other peaks correspond to isatan b and indigo precursors also enriched by foam fractionation. The large peak in the final solution corresponds to indigo in the soluble form, which preferably remained in the solution.

5.4 *Cannabis Sativa*

5.4.1 Characterisation of Cannabinoids

Cannabinoid standards required to record the calibration curves for the quantification were checked for their absorption intensity at the operating conditions used for quantification by UV-Vis spectroscopy. Δ^9- tetrahydrocannabinol stability during process and storage was accessed by HRGC-EI/MS. The UV spectrum (Figure 4.21) shows the characteristic absorption patterns for cannabinoids at the given wavelength interval. The absorption maximum of 231 nm for cannabidiol and cannabinol as well as 227 nm for Δ^9- tetrahydrocannabinol, are the highest for the UV region, in conformity with Sigma Product Data Sheet. The intensity of cannabinoids absorption is fairly high, since the molecules possess some double bonds and oxygen atoms. The concentration of the samples for analysis can be realised at relatively low concentrations.

The chromatogram resulting from the HRGC-EI/MS characterization of Δ^9- tetrahydrocannabinol displays a large peak that corresponds to its retention time. The MS spectrum for the *m/z* between 240 and 320 for this analysis (Table 4.10) shows the characteristic fragments $[M]^+$, $[M-15]^+$, $[M-43]^+$ $[M-71]^+$. Such fragmentation is in accordance with the spectrum for Δ^9- tetrahydrocannabinol, from the Sigma Product Data Sheet. These fragments correspond to the molecular ion and the fragment ions originated from the sucessive cleavage of the Δ^9- tetrahydrocannabinol hydrocarbon side chain, which results in the loss of C_5H_{11} chain (Harvey, 1976; Knaus *et al.*, 1976). The HRGC-EI/MS results demonstrate that Δ^9- tetrahydrocannabinol is stable during storage for a reasonable period, as long as the required storage conditions are met. The rapid degradation that occurs 10-12 hours after processing, originates from the high alkalinity of the solution in which Δ^9- tetrahydrocannabinol is dissolved. The measure taken to conteract that degradation is the decrease of the solution pH.

5.4.2 Extraction of Cannabinoids

Cannabinoids extraction from *Cannabis sativa* samples available was ineffective, when both water or organic solvents were used. The cause of such ineffectiveness, was the reduced amount of cannabinoids in the samples. Cannabidiol contents in the samples was low, while there were only traces of cannabinol and tetrahycannabidiol. Cannabinoids are poorly soluble

in water and soluble in solvents as MeOH (2.3.4.3). Nevertheless, in hot water at pH 4, cannabinoids possess a good solubility. Their extraction using hot water at pH 4 or MeOH, extracted reasonable amounts of cannabinoids.

Extraction with MeOH was preferred as the first step, for being somewhat more effective and having the advantage that MeOH could be fully evaporated. A dried extract with reasonable amounts of cannabidiol, reduced amounts of cannabinol and tetrahydrocannabinol, could then be obtained. The extraction of cannabinoids from the dried extract with hot water at pH 4, permitted the extraction of a reasonable amount of cannabidiol, reduced amounts of cannabinol and tetrahydrocannabinol. Canabivarin was also present in the extract, being its contents as high as that of cannabidiol.

Some ballast substances present in *Cannabis sativa* extract were monoterpenes, sesquiterpenes, chlorophyll and saponins (2.3.4.4). The use of hot water and prolonged extraction time could extract significant amounts of the insoluble monoterpenes and sesquiterpenes that are present in the plant in large amounts and thus, extraction time was kept short. The aqueous extract so obtained had a reasonable contents of cannabidiol, cannabivarin and reduced amounts of cannabinol as well as tetrahydrocannabinol (Tabble 4.11). Some other cannabinoids were present in the extract in small quantities. Reduced amounts of monoterpenes, sesquiterpenes and chlorophyll were also present.

5.4.3 Enrichment of Cannabinoids

Cannabinoid molecules are hydrophobic and unstable at strong acid and alkaline media (2.3.4.3). The hydrophobicity of cannabinoids is due to the existence in the molecules of an hydrocarbon chain and the scarce existence of phenol groups and oxygen atoms. This hydrophobicity implies that when in aqueous solutions, the presence of cannabinoid molecules is more favourable at the gas-liquid interface, than at the bulk of the liquid.

The preference of cannabinoid molecules to the gas-liquid interface instead of the bulk of the liquid, enables the enrichment of these molecules by foam fractionation. Enrichment of cannabinoids takes place when gas is sparged trough the solution to produce foam. Therefrom, cannabinoid molecules can diffuse from the bulk of the liquid to the surface of the gas bub-

bles, rising to the top of the column. The foam exiting the column should be rich in cannabinoid molecules. At optimised conditions, cannabinoids enrichment by foam fractionation depend on their concentration in the initial solution, surfactants and additives present, pH values, height of the foam tower, consecutive foaming and respective gas flow rates (4.5.3). The following sections will describe the influence of the above mentioned parameters and their importance for foam fractionation. The influence of height of liquid pool above the sparger and optimised conditions will also be discussed.

5.4.3.1 Influence of Solute Concentration, Surfactants and Additives

Since cannabinoid molecules are hydrophobic, they have no foaming capacity. The aqueous extracts from *Cannabis sativa* contain saponins which are surface active substances (5.4.2) However, the amount is not enough to produce the foam required for the enrichment of cannabinoids by foam fractionation. Therefore, saponin was added to the aqueous dilute solutions to produce the required foam. In order to improve cannabinoids dissolution, MeOH was added. To decrease excessive coalescence caused by MeOH, glycerine was also added to the solutions.

Enrichment by foam fractionation is achieved for aqueous dilute solutions of the molecules to be separated. Therefore, cannabinoids aqueous dilute solutions were prepared, with concentration varying from 30 mg l^{-1} and 120 mg l^{-1}. The enrichments obtained for the solutions with the initial concentrations as the latter are presented in Fig 4.22. The graphical representation of enrichment in dependence of concentration, shows that the enrichment augmented from solutions with initial concentrations of 30 mg l^{-1} to reach a peak for solutions with concentration of 60 mg l^{-1}. A gradual reduction was observed to concentrations higher than 60 mg l^{-1}, reaching the minimum for concentrations of 120 mg l^{-1}.

The increase in enrichment from initial solutions with the concentrations of 30 mg l^{-1} to solutions with the optimal concentration of 60 mg l^{-1} was 26.01% for cannabidiol, 42.58%for cannabinol and 37.64% for tetrahydrocannabinol. The decline in enrichment for the solutions with the optimal initial concentrations of 60 mg l^{-1} to the solutions with minimum concentration of 120 mg l^{-1} was 40.10% for cannabidiol, 34.19% for cannabinol and 26.40% for tetrahydrocannabinol. These results indicate that at concentrations lower than the optimal concen-

tration, cannabidiol enrichments are higher, compared with other cannabinoids. At concentrations higher than the optimal concentration, cannabidiol enrichment is lower than for other cannabinoids. This suggests that cannabidiol molecules interaction with other molecules is higher at higher concentrations and that this interaction influences its enrichment ratio. The variation of concentrations of the initial solutions produced no noticeable changes in the foam stability.

5.4.3.2 Influence of pH

The solubility of cannabinoids in aqueous solutions is influenced by pH (4.5.3.2). This influence implies that the enrichment obtained by foam fractionation is also pH dependent. The most favourable pH for enrichment should be the one in which, the solubility of cannabinoids reaches a minimum. At such pH value, also defined as the pI, cannabinoids enrichment is maximised. Figure 4.23 displays the enrichment obtained at different pH's studied. The enrichment rises with the rise of pH, from the acidic range to the alkaline range, to reach a peak at pH 10 and sharply disminishes at pH 12.

The improvement in enrichment registered from pH 2 to 10, the optimal pH, was 50.56% for cannabidiol, 41.00% for cannabinol and 53.33% for tetrahydrocannabinol. The decrease in enrichment from pH 10 to 12 was 45.91% for cannabidiol, 39.13% for cannabinol and 51.06% for tetrahydrocannabinol. This proves that cannabinoids hydrophobicity is strongly influenced by pH and hydrophobicity increase with pH to reach a maximum for pH 10, the pI for cannabinoids and than decreases sharply for pH 12. This sharp decrease to levels as those of pH 2, indicates that cannabinoid molecules are no longer stable both at pH 12 and 2, well in accordance with what was described in previous sections of this work. Foam stability is also strongly influenced by pH and declines with the rise of pH value up to pH 10 and than declines once again at pH 12 (4.5.3.2). This effect results from the increase of hydrophobicity of the cannabinoid molecules which when more hydrophobic, interfere with the foam reducing its stability. At highly acidic and alkaline pH's, cannabinoids are degraded and the resulting molecules have a much lower interference with the foam, which results in an improved foam stability.

The foam fractionation of cannabinoids at pH 10 provided for optimised enrichments and cannabinoid molecules remained stable during the whole foam fractionation process. Nevertheless, the stability of cannabinoids in the foam collected for analysis was limited between 10 and 12 hours, even when stored at –20 °C and in the dark. To prevent rapid degradation of the foam samples, the pH value should be adjusted to 7.

5.4.3.3 Influence of Height of Foam Tower and Height of Liquid Pool

Height of foam tower influenced the enrichment achieved by the foam fractionation of cannabinoids. As per 4.5.3.3, cannabinoid initial solutions were foam fractionated in a long column which allowed for high foam tower and a short column. The latter, realised a foam tower about half the height of the longer column. For the longer column, the foam collected at the top of the column possessed large bubbles, fairly good stability, drainage and internal reflux ratio. This means that the longer column permitted the growth of bubble size by gas diffusion from smaller bubbles to larger bubbles. The resulting improved drainage constituted the internal reflux. Such reflux enabled to obtain a foam with low liquid contents and high cannabinoids contents. The enrichment of cannabinoids is thus, favoured for columns with high foam tower.

The short column used in the present work produced a wet foam with small bubbles and the foam had a short residence time in the column. A reduction of foam tower's height of 65 cm, produced a obvious decrease in cannabinoids enrichment of 43.66% for cannabidiol, 33.22% for cannabinol and 40.81% for tetrahydrocannabinol (Figure 4.24). Once more, cannabidiol was more sensitive, this time to foam tower's height.

The height of the liquid pool was varied and caused no noticeable change in the enrichment (4.5.3.3). This indicates that the residence times of the bubbles in the liquid pool was enough to enable for the adsorption in the surface of the gas bubbles rising trough the liquid pool. The change in height of liquid pool causes a variation of the contact time between rising bubbles and solution influencing adsorption, which produces a change in enrichment. The height of liquid pools of 9.30 cm and 7.00 cm, produced no noticeable changes in enrichment. This proves that these liquid pool heights were above the minimum necessary for the achievement of improved enrichments.

5.4.3.4 Influence of Consecutive Foaming and Gas Flow Rates

Consecutive foaming and respective gas flow rates supplied to the foam fractionation column influenced cannabinoids enrichment. Figure 4.25, illustrates cannabinoids enrichment until the third consecutive foaming, at strictly controlled gas flow rates supplied to the column.

Cannabinoids enrichment increased with each consecutive foaming until the third and the increase was gradual. This denotes that cannabinoids adsorption at the gas-liquid interface was not subject to hindrance by competing molecules. Cannabidiol was the single molecule for which there was a slight delay of enrichment at the first consecutive foaming. This suggests that cannabidiol adsorption may suffer from interaction with other cannabinoids. Towards the end of the process of foam fractionation the curves concerning the three cannabinoids reach a plateau, which indicates that the maximum enrichment possible was about to be achieved.

The gas flow rates supplied to the foam fractionation column were important for cannabinoids enrichment. As mentioned in previous sections, at more alkaline pH's and until pH 10, cannabinoid molecules interfered to a greater extent with the foam. One of the measures taken to counterbalance the interference posed by these molecules, was the strict control of gas flow rates supplied to the column, in order that the foam living the column could carry the minimum amount of liquid possible.

5.4.3.5 Optimised Overall Enrichment

Cannabinoids foam fractionation at the optimised conditions provides for fairly high enrichment. Table 4.12 and Figure 4.26 display the quantitative and graphical enrichment of cannabinoids. Figure 4.27 illustrates a chromatogram comparison of the initial solution, foam for the overall process and residual solution, for the foam fractionation of cannabinoids. The increase registered for the peaks corresponding to each cannabinoid from the initial solution to that in the foam, represents the enrichment. Other peaks concerning other cannabinoids co-enriched by foam fractionation can also be discerned.

6. Conclusions and Recommendations

6.1 Conclusions

Foam fractionation is a separation method suitable for the enrichment of active principles contained in plant materials. The goodness of the method was proven by the application of the theoretical foundations of foam fractionation, to enrich active principles contained in the following plants: *Calendula officinalis*, *Camellia sinensis*, *Isatis tinctoria* and *Cannabis sativa*. From the results and discussion presented in this work, the following conclusions are drawn:

1. The active principles contained in plants can be successfully extracted with hot water and when the concentration of the active principles in plants is too low, organic solvents with affinity to the active principles may be used for extraction. The resulting organic extract shall be evaporated to obtain a dried extract rich in the active principles, which is then extracted with hot water. Organic solvents used for the extraction are recovered and recycled with virtually no losses.

2. A pre-requisite for the enrichment of active principles is that the molecules are surface active, hydrophobic or able to associate with other molecules to form hydrophobic complexes. For multi-component mixtures as plant extracts, it is an advantage when hydrophobicity can be varied with pH value, addition of organic solvents or electrolytes.

3. For multi-component mixtures, the presence of high quantities of undesired surface active, hydrophobic molecules or complexes should be avoided – in particularly if their properties cannot be modified by means as derivatisation, complexation or degradation. Such modification should lead to the reduction of surface activity or hydrophobicity of undesired molecules or complexes.

4. Surfactants and viscosity enhancers can be added to solutions with poor foaming capacity or foam stability, in order to render or improve these properties during foam fractionation.

5. Efficiency of the enrichment implies that during foam fractionation, height of foam tower, height of liquid pool and gas flow rates should permit long residence times, molecular diffusion and adsorption, proper degree of coalescence, drainage and internal reflux.

6.2 Recommendations

The efficiency of foam fractionation to enrich active principles from plants is unquestionable. However, improvements can be made with a view to foster the use of the method by making it more attractive to the industry. Therefore, recommendations for further work are as follows:

1. Plant samples used in the development of a foam fractionation method for the enrichment of a particular active principle, should originate from plant cultivars. The desired characteristics of such cultivars should be the high contents of active principles and a uniform quantity of their constituents.

2. Components of the foam fractionation unit should be standardised in terms of geometry, dimensions, construction materials and operation principles, to ascertain an easy scale-up.

3. Foam fractionation methods should be developed for the enrichment of active principles in a continuous mode of operation.

7. Summary

Foam fractionation is a method suitable to selectively enrich or separate molecules dissolved in aqueous dilute solutions by virtue of their surface activity, hydrophobicity or their readiness to associate with other molecules to form hydrophobic complexes. Such an enrichment or separation is ascribed to the adsorption of these molecules or complexes at the gas – liquid interface of foam bubbles produced by the aeration of a solution with foaming capacity. The main features of the method are low investment and running costs derived from the simplicity of equipments used and low energy consumption, as well as the environmental friendliness of the method. Although foam fractionation has been overlooked as a method for enrichment or separation, there is a great potential to use the method in laboratory, pilot and industrial scale, when small quantities of molecules are dispersed in solution, conditions in which other cost effective methods usually fail.

For the advantages and main features of foam fractionation, the method is well suited to the enrichment of active principles contained in plants. Plants are known sources of active principles with pharmacological and biological activities. The traditional or folk medicine has made available such active principles in the form of aqueous extracts, alcoholic extracts, powders, creams and essential oils. To date, the modern pharmaceutical industry heavily relies on active principles to produce medicines, some from plant origin and the majority from synthetic substitutes. Plant's active principles are inexpensive when compared with synthetic active principles and the possibility to inexpensively, efficaciously and selectively enrich these principles by foam fractionation, would make them an economically advantageous replacement for synthetic active principles.

The core of the present work was the study of possibilities to selectively enrich the active principle from the plants *Calendula officinalis*, *Camellia sinensis*, *Isatis tinctoria* and *Cannabis sativa*. When this possibility was confirmed, a method was developed to attain an optimised process that ultimately allowed for the maximisation of enrichment. Method development took in consideration the basic principles of foam fractionation, the physicochemical properties of the active principles and the parameters and conditions governing the process of foam fractionation.

Of particular interest for the design and optimisation of the foam fractionation methods were the following parameters: Molecular diffusion and adsorption in the turbulent flow, rheology and quality of foams, hydrophobicity of the molecules or complexes to be separated, influence of surfactants, organic solvents and viscosity enhancers addition, pH value, height of foam tower and liquid pool, gas flow rates and time of foam fractionation. The systematic of method development for each plant proceeded in the following order: Analytical characterisation of standards for the active principles, solvent extraction of active principles from plant samples, tentative foam fractionation in the batch mode of operation and optimisation of the foam fractionation method. Quantification of enrichment obtained with foam fractionation were measured with HPLC devices. The following paragraphs provide a brief description of foam fractionation methods developed for the enrichment of active principles contained in *Calendula officinalis*, *Camellia sinensis*, *Isatis tinctoria* and *Cannabis sativa*.

Calendula officinalis active principles are the faradiol esters, which are highly hydrophobic molecules. Owing to their hydrophobicity, they could be enriched by foam fractionation. Foam fractionation was performed for faradiol esters aqueous dilute solutions, with the addition of a surfactant, organic solvent and a viscosity enhancer, to attain favourable bubble size and foam stability. Height of the foam tower and liquid pool, control of gas flow rates and time for the foam fractionation enabled improved coalescence, drainage and internal reflux. The enrichment of faradiol esters at optimised parameters and conditions was remarkably high, however, xantophylle, an unsought substance present in solution, was also co-extracted.

Camellia sinensis active principles are the relatively hydrophilic catechin molecules. Catechins alone could not be enriched by foam fractionation due to their hydrophilic character. On account of the hydrophobic complex formed between catechins and caffeine, which is an undesired substance present in the extract, the catechins could be enriched by foam fractionation. Foam fractionation was performed for catechins aqueous dilute solutions at an acidic pH, with the addition of an electrolyte. Height of the foam tower and liquid pool, strict control of gas flow rates and time for the foam fractionation permitted to achieve the most convenient coalescence, drainage and internal reflux. The enrichment of catechins at optimised parameters and conditions was modest, caffeine, an unwanted substance, was also co-extracted.

Isatis tinctoria active principle is the tryptanthrin molecule, a polar hydrophobic molecule. Tryptanthrin molecule's hydrophobicity enables its enrichment by foam fractionation. Foam

fractionation was performed for tryptanthrin aqueous dilute solutions at an alkaline pH, with the addition of a surfactant, organic solvent and viscosity enhancer, to render the foam a favourable bubble size and stability. Height of the foam tower and liquid pool, gas flow rates and time for the foam fractionation provided a proper coalescence, drainage and internal reflux. Tryptanthrin enrichment obtained at optimised parameters and conditions was relatively high. Despite the alkaline pH used for foam fractionation, undesired substances as reduced quantities of indigo and fairly high quantities of indigo precursors present in solution were also co-enriched.

Cannabis sativa active principles are the cannabinoid molecules. These molecules are hydrophobic, which makes their enrichment by foam fractionation possible. Foam fractionation was performed for cannabinoid molecules aqueous dilute solution at an acidic pH, with addition of a surfactant, organic solvent and a viscosity enhancer, to obtain conditions propitious for the desired bubble size and foam stability. Height of the foam tower and liquid pool, control of gas flow rates and time for the foam fractionation provided a favourable coalescence, drainage and internal reflux. Cannabinoids enrichment at optimised parameters and conditions were fairly high. Immediately after foam fractionation, the pH value of the enriched foam should be reduced to prevent degradation of the cannabinoid Δ^9 – tetrahydrocannabinol.

In general, the active principles contained in the plants under investigation could be enriched by foam fractionation. Elimination of undesired hydrophobic species was not possible during the enrichment of faradiol esters from *Calendula officinalis* and tryptanthrin from *Isatis tinctoria*. Exception was made for *Camellia sinensis*, where caffeine, an undesired substance present in the solution, formed a complex with catechins, which permitted catechins enrichment. Effects of key importance for the success of enrichment in this work were the pH value of the solutions, the height of foam tower, the gas flow rates and time of foaming. Additives as surfactants, organic solvents, electrolytes and viscosity enhancers were important, because of the improving effect they operated on the flow conditions.

8. Literature

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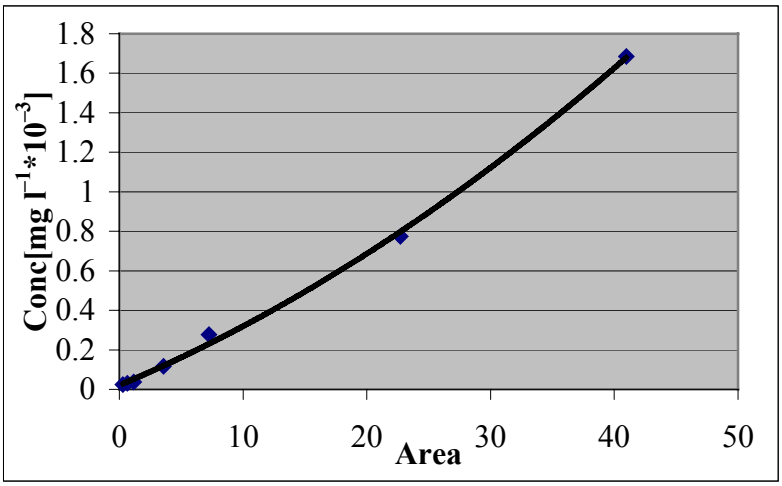
9. Annexes

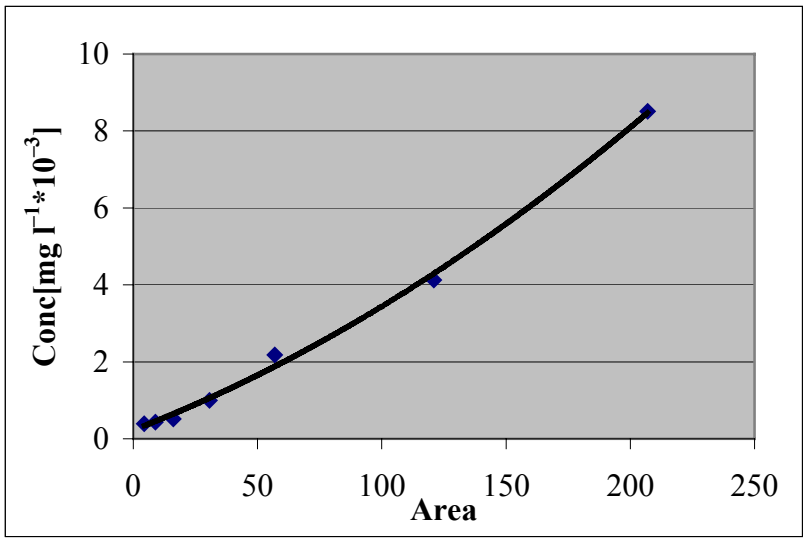
Annex I. Calibration Curves for the Active Principles

Faradiol Esters Standards Experimental Data:

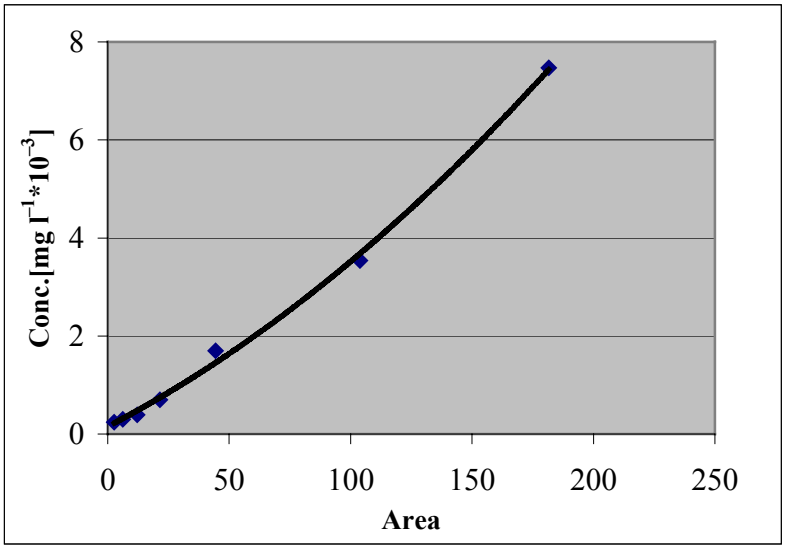
Faradiol Lauritic Acid Ester Calibration Curve



Faradiol Myristic Acid Ester Calibration Curve

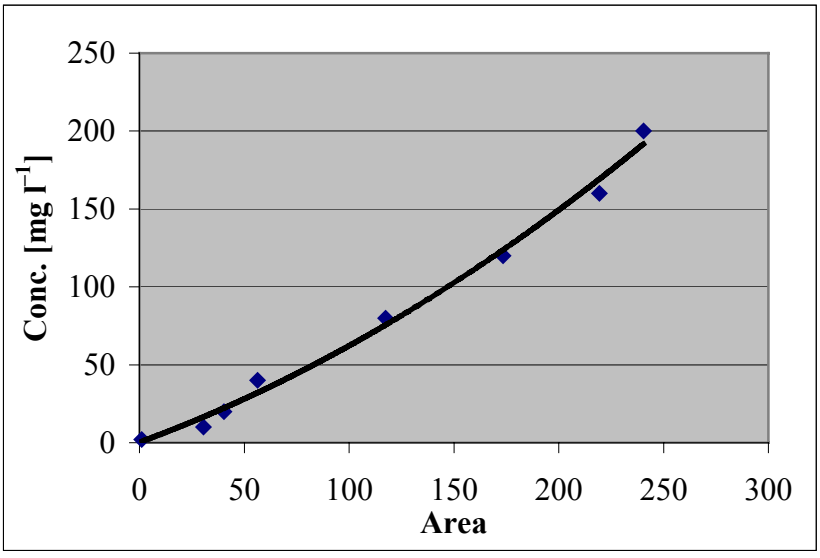


Faradiol Palmitic Acid Ester Calibration Curve

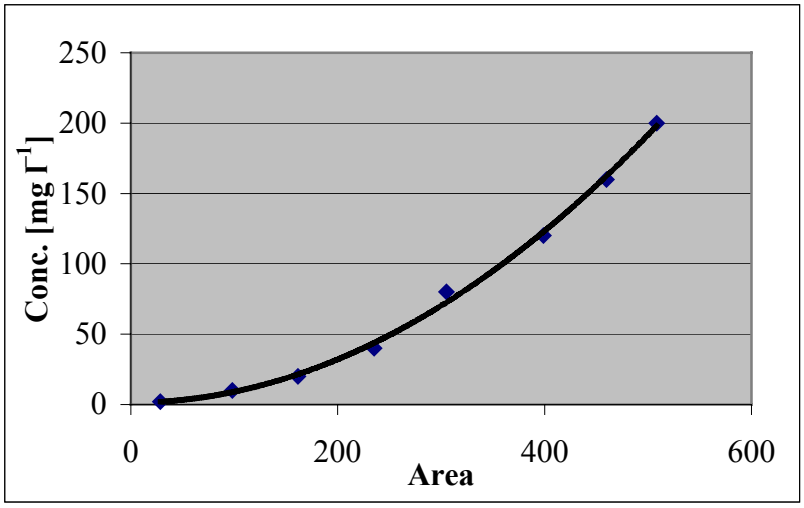


Catechins and Caffeine Standards Experimental Data:

Cathecins Calibration Curve

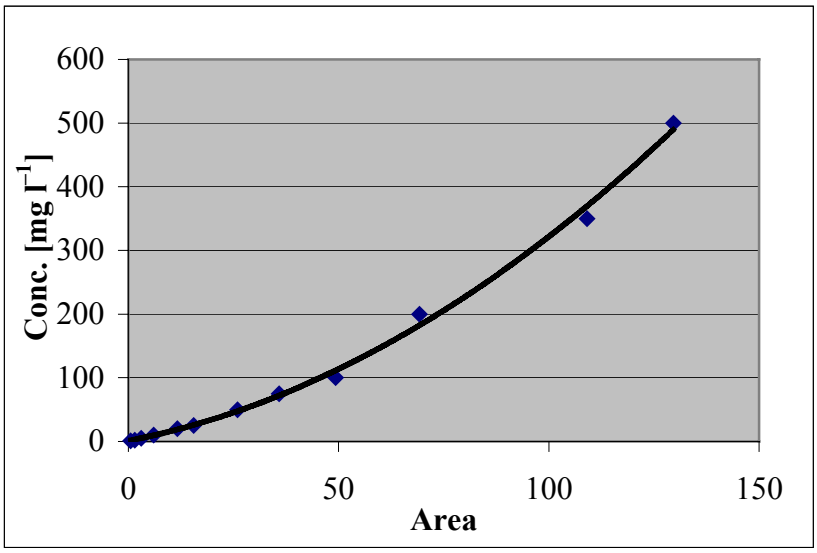


Caffeine Calibration Curve



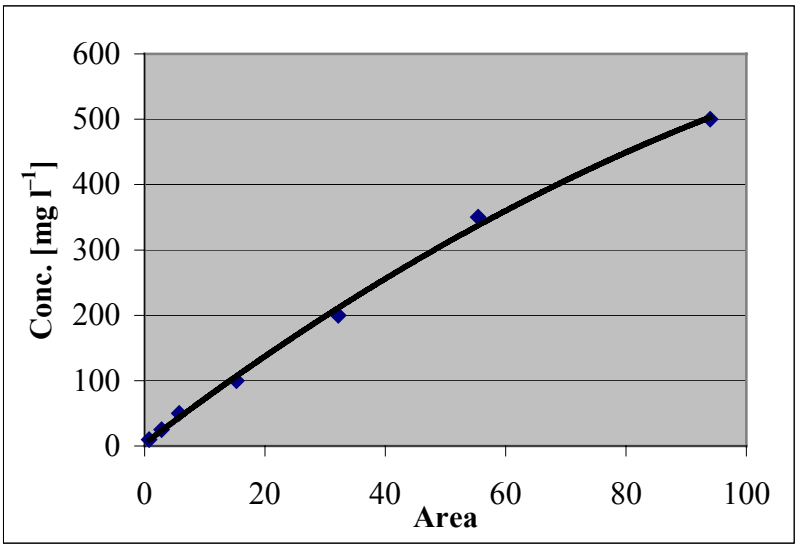
Tryptanthrin Standard Experimental Data:

Tryptanthrin Calibration Curve

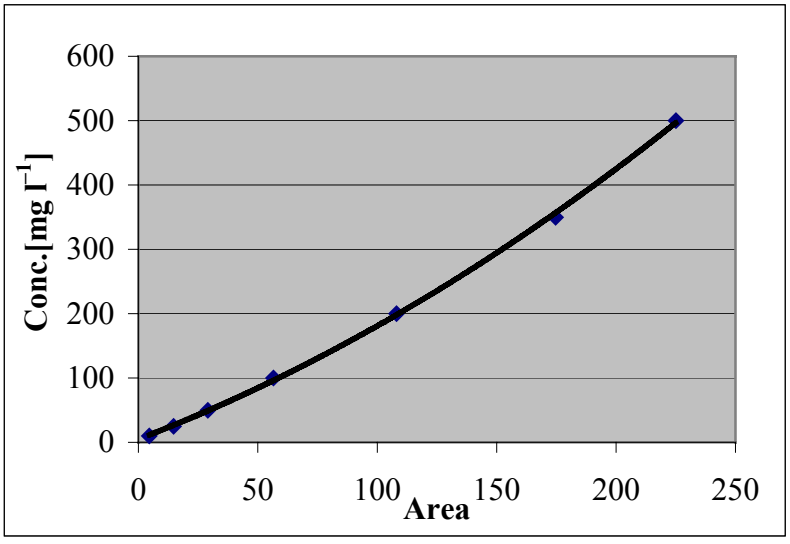


Cannabinoids Standards Experimental Data:

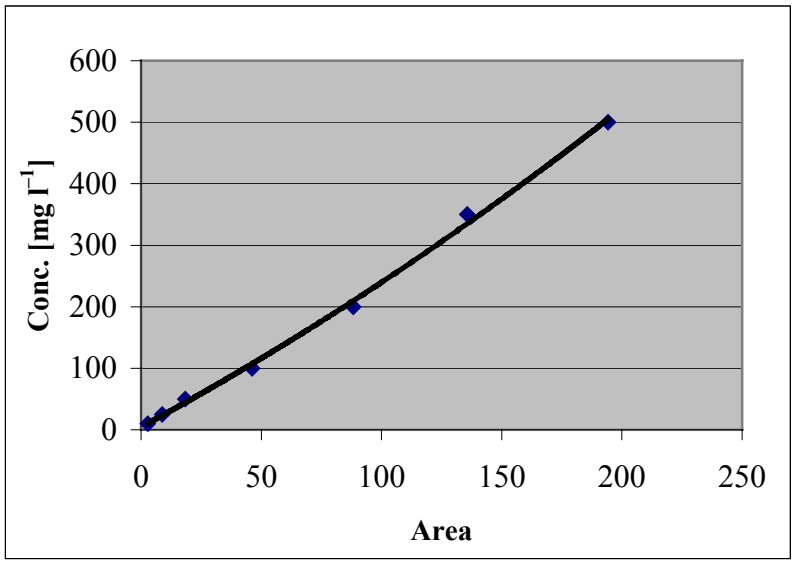
Cannabidiol Calibration Curve



Cannabinol Calibration Curve



Δ^9 – Tetrahydrocannabinol Calibration Curve



Annex II Enrichment of Faradiol Esters by Foam Fractionation

Enrichment in Dependence of Saponin Concentration in Initial Solution

Initial Solution [mg l^{-1}]	Saponin Concentration [g]	FL Relative Enrichment	FM Relative Enrichment	FP Relative Enrichment
20	0.22	4.13	5.95	5.50
20	0.26	3.59	5.44	5.03

Enrichment in Dependence of Concentration in Initial Solution

Initial Solution [mg l^{-1}]	FL Relative Enrichment	FM Relative Enrichment	FP Relative Enrichment
20	4.13	5.95	5.50
40	6.00	7.26	6.51
80	9.16	9.55	9.50
120	4.37	7.27	6.18
160	3.88	6.21	5.33

Enrichment in Dependence of Column Height

Initial Solution [mg l^{-1}]	Column Heigth [cm]	FL Relative Enrichment	FM Relative Enrichment	FP Relative Enrichment
80	65	6.76	7.72	7.76
80	130	9.16	9.55	9.50

Increase of Concentration from Initial Solution to Foam Collected in the Process

Time Interval [min]	FL [mg l^{-1}]	FM [mg l^{-1}]	FP [mg l^{-1}]
Initial Solution (0-20 min)	3.88	41.33	34.50
1st Foam (20-25 min)	7.78	97.04	65.78
2nd Foam (25-30 min)	20.08	215.98	135.35
3rd Foam (30-35 min)	25.02	287.29	191.04
4th Foam (35-40 min)	28.12	352.01	278.80
5th Foam (40-45 min)	32.12	377.67	305.36

Overall Enrichment Process

	FL [mg l^{-1}]	FM [mg l^{-1}]	FP [mg l^{-1}]
Initial Solution	3.97	39.99	33.06
Final Foam	36.33	381.87	314.03
Residual Solution	-	-	-

Annex III Enrichment of Catechins

Enrichment in Dependence of Initial Solution pH

Initial Solution [mg l^{-1}]	Initial Solution pH	EGC Relative Enrichment	C Relative Enrichment	Caff. Relative Enrichment	EC Relative Enrichment	EGCG Relative Enrichment	ECG Relative Enrichment
455	2.5	1.14	1.19	1.18	1.10	1.31	1.32
455	3.5	1.21	1.25	1.24	1.17	1.62	1.63
455	4.5	1.02	1.04	1.07	1.08	1.28	1.24

Increase in Concentration From Initial Solution to Foam Collected at Each Consecutive Foaming

Time Interval [min]	EGC [mg l^{-1}]	C [mg l^{-1}]	Caff. [mg l^{-1}]	EC [mg l^{-1}]	EGCG [mg l^{-1}]	ECG [mg l^{-1}]
Initial Solution (0 min)	13.61	8.42	7.73	44.16	25.12	14.66
1st Foam (22-50min)	16.25	11.16	8.61	61.63	31.75	23.29
2nd Foam (50-70min)	27.13	19.08	19.66	100.15	55.13	39.58
3rd Foam (70-90min)	46.72	34.05	26.89	152.47	77.41	50.58
4th Foam (90-110min)	50.61	39.03	27.80	158.95	91.11	57.51

Overall Enrichment Process

	EGC [mg l^{-1}]	C [mg l^{-1}]	Caff. [mg l^{-1}]	EC [mg l^{-1}]	EGCG [mg l^{-1}]	ECG [mg l^{-1}]
Initial Solution	54.43	33.67	30.93	176.66	100.47	58.62
Final Foam	202.44	156.12	111.22	635.78	364.42	230.03
Residual Solution	26.68	16.48	15.16	86.56	49.24	28.72

Annex IV Enrichment of Tryptanthrin

Enrichment in Dependene of Concentration in Initial Solution

Initial Solution [mg l^{-1}]	Relative Enrichment Try.
2.5	2.39
5	3.44
10	2.17
15	1.86
20	1.62

Enrichment in Dependence of Initial Solution pH

Initial Solution [mg l^{-1}]	Initial Solution pH	Try. Relative Enrichment
5	5	2.78
5	6	3.28
5	7	3.67
5	8	4.83
5	9	4.50

Enrichment in Dependence of Column Height

Initial Solution [mg l^{-1}]	Column Height [cm]	Try. Relative Enrichment
5	65	4.44
5	130	6.08

Increase in Concentration from Initial Solution to Foam Collected at Each Consecutive Foaming

Time Interval [min]	Try. [mg l^{-1}]
Initial Solution (0min)	5.65
1st Foam (14-40min)	17.63
2nd Foam (40-60min)	40.69
3rd Foam (60-80)min	45.70

Overall Enrichment Process

	Try. [mg l^{-1}]
Initial Solution	5.65
Final Foam	45.70
Residual Solution	1.58

Annex V Enrichment of Cannabinoids

Enrichment in Dependence of Concentration in Initial Solution

Initial Solution [mg l^{-1}]	CBD Relative Enrichment	CBN Relative Enrichment	THC Relative Enrichment
30	3.10	2.67	2.22
60	4.19	4.65	3.56
90	3.15	4.18	3.51
90	2.51	3.06	2.62

Enrichment in Dependence of Initial Solution pH

Initial Solution [mg l^{-1}]	Initial Solution pH	CBD Relative Enrichment	CBN Relative Enrichment	THC Relative Enrichment
60	2	3.08	3.80	2.87
60	4	3.32	4.09	3.29
60	6	3.92	4.54	3.60
60	8	4.94	5.24	4.97
60	10	6.23	6.44	6.15
60	12	3.37	3.92	3.01

Increase in Concentration from Initial Solution to Foam Collected in Each Consecutive Foaming

Time Interval [min]	CBD [mg l^{-1}]	CBN [mg l^{-1}]	THC [mg l^{-1}]
Initial Solution (0min)	28.43	17.75	16.22
1st Foam (12-40min)	86.28	61.97	49.04
2nd Foam (40-65min)	164.29	108.12	89.21
3rd Foam (60-90min)	180.32	117.67	99.80

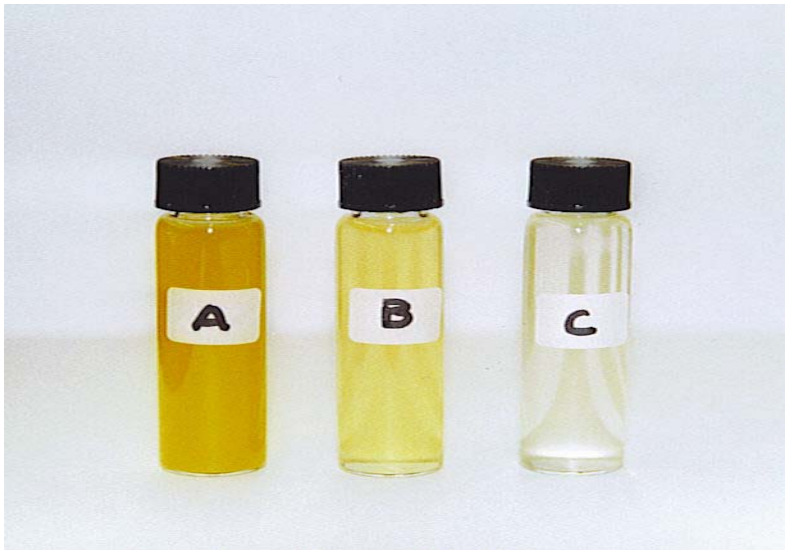
Enrichment in Dependence of Column Height

Initial Solution [mg l^{-1}]	Column Heigth [cm]	CBD Relative Enrichment	CBN Relative Enrichment	THC Relative Enrichment
60	65	3.51	4.30	3.64
60	130	6.23	6.44	6.15

Overall Enrichment Process

	CBD [mg l^{-1}]	CBN [mg l^{-1}]	THC [mg l^{-1}]
Initial Solution	28.43	17.75	16.22
Final Foam	180.32	117.67	99.80
Residual Solution	7.11	3.82	4.40

Annex VI Faradiol Esters Foam Fractionation Samples



A – Faradiol Esters Foam Fractionation - Overall Foam

B – Faradiol Esters Foam Fractionation - Initial Solution

C – Faradiol Esters Foam Fractionation - Residual Solution