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Complexation Assisted Foam Fractionation of Caffeine from Its Aqueous Solution

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Contents

List of Abbreviations

α – Alfa

β – Beta

γ – Surface tension

δ – Chemical shifts

ε – The molar absorption coefficient

µm – Micrometer

Γ_i – Surface excess

Γ_∞ – The maximum number of the sites available on the surface

°C – Degrees celsius

% – Percentage

A – The light absorption

ABS – Adsorptive bubble separation

a_i – The activity of component i

ANOVA – One-way analysis of variance

b – The path length the light travels

CO_2 – Carbon dioxide

CMC – Critical micelle concentration

C – Carbon

^{13}C – Carbon-13

c – The molar concentration of the solution

C_f – The concentration of the target component in the foamate

C_i – The concentration of the target component in the initial solution

cm – Centimeter

d – Doublets

dd – Doublet of doublets

DAD – Diode array detector

DMSO – Dimethyl sulfoxide

E_λ – The molar absorption coefficient

ER – Enrichment ratio

ESI – Electrospray ionization

FT-ICR/MS – Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

g – Gram

HPLC – High performance liquid chromatography

H – Hydrogen

H_{ax} – axial protons

H_{eq} – equatorial protons

H_3PO_4 – Phosphoric acid

^1H – Hydrogen-1

i – Component i

I – The intensity of the transmitted light

I_o – The intensity of the incident light

KH_2PO_4 – Potassium phosphate monobasic

K_2HPO_4 – Potassium phosphate dibasic

k_a – The equilibrium adsorption constant

k_d – The equilibrium desorption constant

K_L – Langmuir equilibrium adsorption constant

K_i – The equilibrium constant

L – Liter

m – multiplet

M – Molar per liter

mg – Milligram

min – Minute

ml/min – Millimeter per minute

mL – Milliliter

mM – Millimolar per liter

mm – Micrometer

MS – Mass spectrometry, Mass spectrometer

m/z – Mass to charge ratio

nm – Nanometer

NMR – Nuclear magnetic resonance

N_2 – Nitrogen gas

PB – Plateau border

pI – Isoelectric point

ppm – Parts per million

R – Recovery rate

R^2 – Coefficient of determination

$SOCl_2$ – Thionyl chloride

SPSS – Statistical package for the social sciences

TABS – Tweezing adsorptive bubble separation

t – Triplet

T – The absolute temperature

UV-Vis – Ultraviolet-visible

V – Volt

V_f – The volume of the foamate after collapse

V_i – The volume of the initial solution

v/v – Volume to volume

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1. Introduction

For couple of years, natural products have always been of great interest to chemists and pharmacists, for their unparalleled chemical and bioactive diversity and hence the great potential in the development of new drugs, functional foods and food additives. By now, a lot of extraction methods and chromatographic techniques have been developed for isolation of natural products from higher plant, marine organism and microorganism matrix (Sticher 2008, Bucar, Wube et al. 2013). However, considering that a single plant matrix may contain hundreds even thousands of other constituents, the isolation processes related could always be long and tedious (Sticher 2008), and accompanied with extensive consuming of organic solvents and considerable attention, which tends to burden the environment and also demands more investment both in equipment and energy (Backleh-Sohrt, Ekici et al. 2005). It was reported that the separation costs in the bioprocesses account for up to 90% of the whole production costs; and it would be even more with the newer separation processes (Lockwood, Bummer et al. 1997). Meanwhile, some sensitive constituents may degrade or lose their original activities during these long and harsh separation procedures. Therefore, a sustainable and gentle processing technology is of pronounced interest. Adsorptive bubble separation (ABS) offers one possibility.

ABS is started with the introduction of the inert gas into an aqueous solution containing surface-active substances, and thus bubbles are formed and move up along the riser. Then the surface-active substances attach on the bubble film and reach the top of the riser along with the bubbles, and are collected as foamate after the collapse of bubbles, while, the less surface-active substance may be collected by the foam later, and the rest would stay in the feed solution. Organic solvents are almost not necessary during the whole process, but only water and inert gas are used at ambient temperature, which makes ABS quite a promising separation technique for the bioactive substances. The glass-made device for ABS is also low in cost and easy to maintain. Besides, this separation technique is especially effective for the processing of dilute solutions (Uraizee and Narsimhan 1990) with high enrichment ratio but no co-extraction of fats or chlorophyll constituents which normally happens in solvent extraction process (Backleh-Sohrt, Ekici et al. 2005). All of these advantages make ABS a "green" and sustainable technique, which is accordance with the current growing interests from people.

ABS has already been known for almost a century. This separation technique was first described by Ostwald, and was patented in 1920 (Ostwald 1920). Afterwards, Ostwald and co-workers had further developed the basic theoretical part of the separation approach, such as collapse, spumat and other scientific background (Ostwald and Siehr 1936). At the same time, they also tried to explore this into the separation practice, such as new ABS setups evaluation (Ostwald and Mischke 1940 a) and protein separation from yeast fermentation broth (Ostwald and Mischke 1940 b). Since then, ABS has received more and more interest from researchers, not only the theoretical parts has ever been renewed, but also the application practice has been explored from waste water treatment to the other separation fields, such as metal extraction, phytonutrients and metabolites enrichment and protein enrichment (Burghoff 2012).

As natural surface-active substances that possess both polar and nonpolar groups, proteins and enzymes are tend to absorb onto the gas-liquid interface and are therefore susceptible to the ABS technique. Up to now, a lot of researches have been done in the field of ABS for the separation of proteins and enzymes. For example, separation of pepsin from rennin using ABS in 1945 (Andrews and Schutz 1945), the separation of amylase form catalase in 1966 (Charm, Morningstar et al. 1966), the separation of streptokinase from culture media (Holmstro.B 1968), the separation of bovine serum albumin from solutions containing DNA (Lalchev, Dimitrova et al. 1982) and from potato wastewater (Brown, Narsimhan et al. 1990), and the separation of lysozyme also from solutions containing DNA (Lalchev, Dimitrova et al. 1982), the separation of proteolytic enzyme from human placental extracts (Bhattacharya, Ghosal et al. 1991), the purification of alkaline protease of Rhizopus-Oryzae (Banerjee, Agnihotri et al. 1993), the separation of cellulose from the crude dilute solution (Loha, Prokop et al. 1999), the separation of bovine lactoferrin from dilute solution (Noel, Prokop et al. 2002), the separation of histidine tagged protein from tobacco extract (Crofcheck, Loiselle et al. 2003), the separation of ovalbumin as a model protein from wastewater (Maruyama, Seki et al. 2007), the purification and identification of a novel cutinase from Coprinopsis cinerea (Merz, Schembecker et al. 2009), the enrichment of α-lactalbumin and β-lactoglobulin from a whey solution (Shea, Crofcheck et al. 2009) and so on. Some researchers focused on the influencing parameters on the ABS of proteins (DeLucena, Miranda et al. 1996, Clarkson, Cui et al. 2000, Crofcheck, Maiti et al. 2004, Liu, Elmer et al. 2010), and some others on the modeling of ABS of proteins (Uraizee and Narsimhan 1990, Bhattacharjee, Kumar et al. 2001, Stevenson and Jameson 2007).

ABS was also explored in the enrichment of natural products. For example, the ABS of phenol using a cationic surfactant from aqueous solution (Grieves and Aronica 1966), the comparison of phenol separation by ABS with solvent extraction and solvent sublation (Grieves, Charewicz et al. 1974), the foam fractionation (one type of ABS) of polysaccharide mixtures (Brasch, Ngeh et al. 1983), the remove of heptachlor and hydroxychlordene using foam fractionation (Chiu and Huang 1991), the extraction and concentration of glycyrrhizic acid using foam fractionation (Ma, Xiu et al. 2002), the separation and recovery of starch and mucilage from yam tubers (Fu, Huang et al. 2005), the extraction of polyphenolics from apple juice using foam fractionation (Saleh, Stanley et al. 2006), the separation of tea saponin by two-stage foam fractionation (Yan, Wu et al. 2011), the separation of total saponins from pericarp aqueous solution of *Sapindus mukorossi* Gaerten (Li, Wu et al. 2013), the recovery of isoflavone aglycones from soy whey wastewater (Liu, Zhang et al. 2013), and also some other researches on the recovery of biosurfactants (Davis, Lynch et al. 2001, Chen, Baker et al. 2006, Chen, Baker et al. 2006, Sarachat, Pornsunthorntawee et al. 2010).

Impressively, a lot of work on the ABS of both natural products and proteins and enzymes has been accomplished in the group of Prof. Parlar at the Technical University of Munich, since 2003. For example, they eliminated the undesirable pigments flavokavine A and B from the aqueous solution of Kava Kava using foam fractionation by adjusting different parameters (Backleh, Ekici et al. 2003). The antioxidant compound carnosic acid was enriched from rosemary aqueous solution successfully with negligible degradation (Backleh, Leupold et al. 2003). In 2005, they enriched total and single whey proteins quantitatively with parameters optimized foam fractionation, using sodium dodecyl sulfate concentration as a surfactant (Ekici, Backleh-Sohrt et al. 2005). And after that, they enriched laccase C from the enzymatic bulk solution using a continuous foam fractionation method with an optimized condition with little loss of enzyme activity (Gerken, Nicolai et al. 2006), and one year later, they enriched laccase C from the culture supernatants of *P. sapidus* using foam fractionation successfully (Linke, Zorn et al. 2007).

What more significant is that the researchers in Prof. Parlar's group developed a new strategy to enhance the foaming efficiency of the enzymes which showing a weak surface activity on the surface-liquid interface. It was named Tweezing Adsorptive Bubble Separation (TABS) (Gerken, Wattenbach et al. 2005). The tweezer consists of two parts: an alkyl group with eight carbons as a tail, which tends to adsorb on the foam film; and a

chelator as a head, which bounds with the metal cation in the active center of the metalloenzyme (Fig. 1.1). As a result, not only the foamability of the protein is increased, but also the selectivity of the ABS is enhanced.

Chelator Alkyl moiety

Metalloenzyme   Tweezer   Enzyme-Tweezer Complex
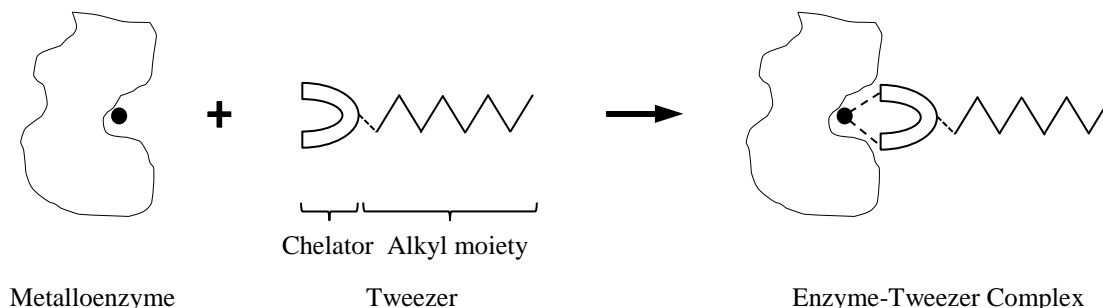
Figure 1. 1 Diagram of tweezing principle based on the research of Gerken et al. (Gerken, Wattenbach et al. 2005). (● Metal cation in the active center of the enzyme.)

Using ADA-C8 as a tweezer, synthesized from *N*-(2-Acetamido)iminodiacetic acid (ADA) and n-octyl, Gerken et al. enriched laccase C and horseradish peroxidase 13.3-fold (66.31% recovery) and 17.8-fold (85.34% recovery) in the foamate, respectively, without a significant loss of enzyme activity. Compared with ABS using trimethylammonium bromide (CTAB) as a surfactant, the enrichment for laccase was improved for more than 3-fold, and even more for horseradish peroxidase (Gerken, Wattenbach et al. 2005). Later on in 2008, bovine serum albumin was used as a tweezer to complex with bovine insulin in TABS (Nicolai, Friess et al. 2008). The results showed that unmodified bovine insulin was transferred into the foamate at pH 8 with an enrichment factor of 2.6 and a recovery of 89.7, with an immunological activity loss of nearly 30%. After the derivatiziation by *trans*-2-dodecenal, α,β-unsaturated bovine insulin-$(C12)_n$ was enriched at pH 9 with an enrichment factor of 3.3, with almost no loss of immunological activity. The significant difference in the loss of the enzyme activity in the foamate before and after derivatiziation ascribed to the binding between the alkylated insulin-C12 derivates and bovine serum albumin. Here, these experiments showed us the possibility to effectively isolate insulin from aqueous solution by TABS, at least in principle. Based on the research done before, Haller et al. (Haller, Ekici et al. 2010) used ADA-C8 again as a tweezer in the TABS of matrix metalloproteinase (MMP-9) and carboxypeptidase A (CPA) from dilute aqueous solutions. Finally, MMP-9 was enriched with an enrichment ratio of 12.0 and a recovery of 87.3%, and CPA with an enrichment ratio of 18.8 and a recovery of 95.3%. Both of them endured no significant loss in enzymatic activity.

Objectives

All of the above-mentioned ABS experiments can be divided into two groups:

a) The target constituents are naturally surface-active and are therefore able to adsorb onto the gas-liquid interface: the normal foam fractionation without additional surfactants;

b) The target constituents show weak or even no surface activity, but they are able to bind with the foam producing substances, for example, electrostatically attracting to the oppositely charged foaming–substances, or to form a complex with these foaming substances: foam fractionation with additional surfactants, such as TABS.

A lot of bioactive natural products which are normally purified using traditional extraction and chromatography methods, are not surface active, and therefore are not able to be enriched directly into the foam by ABS. However, some of them are able to complex with the other substances, so named "catcher", and may be possibly enriched by ABS, assisted by the interactions between the targets and their catchers.

Here in the present research, caffeine was foamed with different catchers from their binary aqueous solution. All the parameters, namely pH, flow rate, temperature, initial concentration, the amount of the catchers and surfactant and so on, were all varied in the experiments, in order to obtain an optimal separation condition. All the results we got were analyzed using SPSS statistical analysis software, to compare the data. The parameter values with significant better enrichment ratios verified by statistical analysis were adopted in the experiments for the comparison of the catchers. Once the best catcher was judged, it was chosen as the catcher for the enrichment of caffeine from coffee aqueous solution. And again, all the parameters were varied for an optimal separation result.

2. Theoretical background

2.1 The Classification of ABS

ABS is defined as any methods in which separation is accomplished by the preferential adsorption of the components to be separated in the highly dilute aqueous solution, at the gas-liquid interface, produced by continuously inletting of gas bubbles flowing through the column. According to Lemlich (Lemlich 1968), all these methods can be classified into two main groups, based on whether or not a foam is required for the separation: foam separation and non-foaming adsorptive bubble separation. And there are more detailed subdivisions under these two main groups (Fig. 2.1):
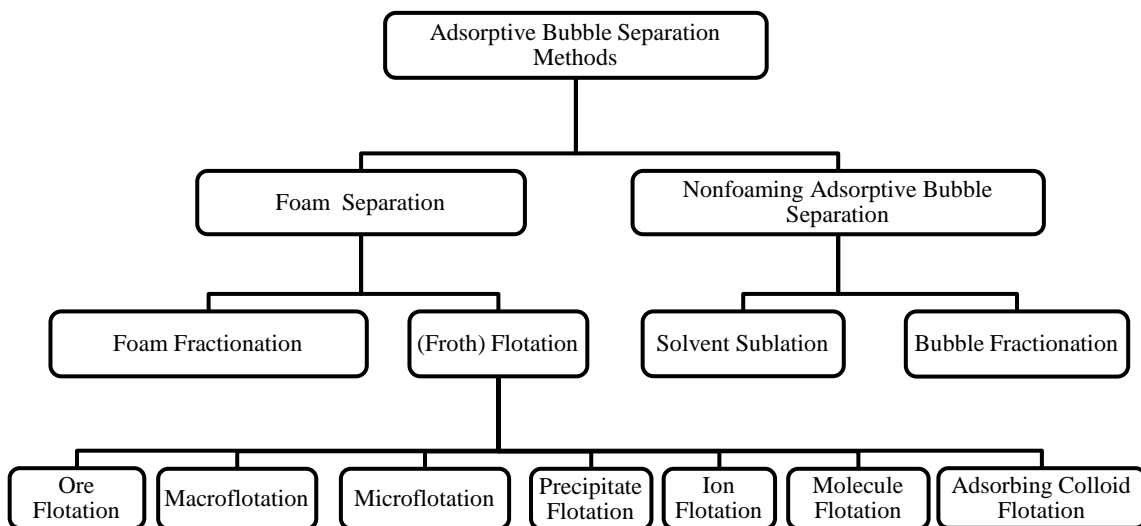
Figure 2. 1 Schematic representation of Adsorptive Bubble Separation Methods classification (Lemlich 1968)

The foam separation category is further divided into froth flotation and foam fractionation.

Froth flotation has many subdivisions: ore flotation is the special case used to describe the selective separation of ore particles in mineral; macroflotation is the removal of macro-particles; microflotation is the removal of micro-particles, especially microorganisms or colloids; precipitate flotation is the removal of an ionic species by forming of a precipitate; ion flotation involves the removal of surface-inactive ion by adding of a surfactant to yield an insoluble product; molecular flotation is the removal of surface-inactive molecules by the

adding of a surfactant to yield an insoluble product; adsorbing colloid flotation is the removal of dissolved materials which adsorb on the colloidal particles firstly.

Foam fractionation is the separation of the normal surface-active molecules, except the materials being separated specifically using the above-mentioned techniques. Compared with the other methods, foam separation is not so specific, but was studied most intensively for its importance and universality. And the researchers sometimes take foam fractionation simply as ABS. The difference between foam fractionation and foam flotation lies on that there is no solid phase in the process of foam fractionation, while in the foam flotation the formation of solid or precipitation is the basis for separation (Pinfold 1970).

Separations can still be accomplished even in the absence of foam. It is achieved by prolonging the liquid pool to form a liquid column, along which the bubbles are rising with the materials adsorbing at their surface. Then the materials deposit at the top of the column, where the bubbles pass out. This method is named as bubble fractionation. Sometimes, an immiscible liquid is placed on the top of the liquid pool to trap the materials released by the exiting bubbles. This method is solvent sublation, which increases the efficiency of bubble separation greatly (Lemlich 1968).

In the following sections of the theoretical background, we will focus on foam fractionation mainly, not just because the method being applied in this research will be foaming based, but also because most part of the fundamentals of foam fractionation discussed below is applicable to foam separation in general.

2.2   The Process of Foam Fractionation

The whole process of foam fractionation composed of three primary mechanisms basically: surface-active components preferentially adsorb at the gas-liquid interface; these adsorbed components are carried upwards by the bubbles as they go up along the column; the bubbles collapse, and a concentrated solution of the target components is collected. The surface-active substances can be separated by foam fractionation directly, because they are able to adsorb at the interface, while the surface-inactive components can also be separated by foam fractionation through complexation or the other interactions, for example oppositely charged electrostatically attraction, with the surface-active substances. Obviously, all of these possibilities are based primarily on the tendency of these components or the complexes or combinations to adsorb at the gas-liquid interface preferentially. Therefore, the acquisition

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of the principle of the adsorption process would be of great help to understand the whole process of foam fractionation, which is essential for obtaining better results.

2.2.1   Adsorption

Positive adsorption at the gas-liquid interface happens when the interaction among the solvent water molecules is stronger than that between the water solvent molecules and the solute surfactant molecules, and as a result, it would be more favorable for the surfactant molecules to stay at the surface but not in the bulk solution (Somasundaran 1972). This interference grows even stronger when the size of the nonpolar part of the surfactant is bigger. Therefore, a bigger nonpolar part in the surfactant would enhance the adsorption of the surfactant at the gas-liquid interface, and in turn, an increase of the efficiency of the foam fractionation would be expectable. Oppositely, the existence of the polar groups or double and triple bonds in the surfactant molecules would decrease the incompatibility with solvent water molecules and hence its adsorption ability at the gas-liquid interface decreased as well (Davies and Rideal 1963). The physicochemical properties of the molecules are very important for the adsorption ability at the gas-liquid interface, however, solution properties such as pH, ionic strength, concentration, and temperature will also influence the distribution of surfactant between the interface and the bulk solution (Somasundaran 1972). All the factors, which may influence the adsorption of the molecules at the gas-liquid interface and hence the efficiency of the foam fractionation, will be discussed in the later sections.

In foam fractionation, interface is generated by the injection of bubbles continuously into the bulk solution. When a fresh surface is newly created, the concentration of the monomer at the surface is lower than the equilibrium value, and hence a flux of monomer from the bulk to the interface would be resulted. This flux will cause the surface tension to decay to a value of equilibrium, where the interfacial concentration has reached its equilibrium as well. In general, there are two main models for monomer transport and adsorption, in which a subsurface that is a few molecular diameters beneath the interface is hypothesized.

Model 1: Diffusion controlled

Diffusion controlled model assumes that the monomers diffuse from the bulk solution to the subsurface, and then directly adsorb at the interface. In this model, the diffusion of the surfactants to the subsurface is the rate-controlling step, while the diffusion from subsurface to the interface is very fast.

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Model 2: Mixed kinetic-diffusion

Mixed kinetic-diffusion model also assumes that the monomers diffuse from bulk solution to the subsurface, and then diffuse to the surface. However, in this model, the rate-controlling process is the transfer of these monomers to the interface from subsurface. An adsorption barrier may present to prevent the monomers from adsorbing once the monomers have diffused to the subsurface. This barrier can ascribe to the increased surface pressure, or decreased number of vacant sites for adsorption. Also, a correct orientation of the monomer may be required due to the steric restraints of the molecules at the interface, which would cause the back diffusion of the molecules to the bulk solution, thus the timescale for equilibrium would be prolonged.

The adsorption of the surfactants at the interface is a dynamic process, and the rate of the adsorption or the change of surface excess concentration Γ_i, can be inferred from the tension decay by applying an appropriate isotherm.

The adsorption degree of the component i at the gas-liquid interface can be expressed by surface excess, Γ_i, with a unit gm·mole/cm^2. Simply, it can be taken as the concentration of component i at the gas-liquid interface. The changes of surface tension γ and surface excess Γ_i are related by Gibbs adsorption equation (Gibbs 1928):

$$d\gamma = -RT \sum \Gamma_i \, d \ln a_i \tag{1}$$

where, γ is the surface tension of the solution under consideration, R is the gas constant, T is the absolute temperature, and a$_i$ is the activity of component i. Actually, the application of Eq. (1) is severely limited in practice because of the difficulties in the measuring of the tiny changes of the surface tension, the uncertainties in identifying the components and estimating their activity. One of the most important applications is in the case of single nonionic surfactant dissolved in pure water at concentrations below the critical micelle concentration (CMC). In this situation, the Gibbs equation can be simplified to:

$$\Gamma_i = -\frac{1}{RT} \frac{d\gamma}{d \ln C_i} \tag{2}$$

where C_i is the concentration of surfactant i in the liquid.

The Gibbs adsorption isotherm, variation of Γ_i with C_i, therefore can be obtained by measuring series of surface tension γ and surface surfactant concentration. At sufficiently low concentrations, the linear isotherm, $\Gamma_i = K_i C_i$, applies, where K_i is the equilibrium constant. At sufficiently high concentrations, Γ_i may approach a constant maximum value, which corresponds to the saturation state of the surface with a monolayer adsorption. This should be the normal situation of Γ_i of the surfactant in foam, and foam techniques such as foam fractionation operate best at low concentrations before the saturation is reached. At or above the CMC, the micelles aggregates start to form and the adsorption may be interfered (Maas 1974), and as a result, the increasing rate in the activity and adsorption of the surfactant at the gas-liquid interface due to the increasing in the total concentration of the surfactant is much more slower than that below CMC (Elworthy and Mysels 1966).

A brief review of the common equilibrium adsorption isotherms was given by Eastoe and Dalton (Eastoe and Dalton 2000). Isotherms such as Henry isotherm, Langmuir isotherm, Frumkin isotherm and Freundlich isotherm, are used to relate the surfactant concentration in the bulk solution with the adsorbed amount at the interface. Here, as the most commonly used non-linear isotherm, Langmuir isotherm is introduced in details below.

Langmuir isotherm is based on a lattice-type model with the following assumptions: every adsorption site on the lattice is equivalent; the adsorption at the empty site on the lattice is not disturbed by the occupancy of the neighbor sites; and no interactions or intermolecular forces between the monomers in the lattice exist.

The adsorption rate, or the time dependent change of surface excess due to adsorption, is proportional to not only the surfactant concentration in the solution, but also the number of the vacant sites available on the surface:

$$\frac{d\Gamma}{dt} = k_a c\, \Gamma_\infty \left(1 - \frac{\Gamma}{\Gamma_\infty}\right) \tag{3}$$

where Γ_∞ is the maximum number of the adsorption sites available on the surface, k_a is the equilibrium adsorption constant, and c is the bulk surfactant concentration.

The desorption rate, or the time dependent change of surface excess due to desorption, is proportional to the number of the adsorbed components:

$$\frac{d\Gamma}{dt} = k_d \Gamma \tag{4}$$

where k_d is the equilibrium desorption constant.

At equilibrium, the adsorption rate is equal to desorption rate, and the Langmuir equilibrium adsorption constant $K_L = k_a/k_d$ is introduced:

$$\Gamma = \Gamma_\infty \left(\frac{K_L c}{1 + K_L c} \right) \tag{5}$$

Deviations from the Langmuir isotherm may ascribe to the failure of the assumption of equivalent and independent sites in the adsorption process. Intermolecular forces such as van der Waals, electrostatic effects or hydrogen bonding, exist inevitably between the molecules at the interface. The enthalpy of adsorption often becomes less negative as Γ increases, which suggests that the most energetically favorable sites are occupied first (Atkins 1994).

2.2.2   Foam Properties

Foams are highly concentrated dispersion of gas (dispersed phase) in a liquid (continuous phase) containing surface-active molecules. The surface of the bubbles are coated with surfactants which stabilize the films against rupture by providing a disjoining pressure, such as electrostatic and steric in nature, that keeps opposing faces from merging. Outside the film region, it is the surface tension that being the dominant force to determine the foam geometry. The surface tension minimizes the surface area of the bubbles and results in mean curvature (Koehler, Sascha Hilgenfeldt et al. 2000).

Foams are both a useful and problematic phase (Xie, Neethling et al. 2004). In term of a desired phase, the foam bubble size determines to a great extent the effectiveness of the separation, which is essential for foam fractionation.

2.2.2.1   The generation of foam

In foam fractionation, air or nitrogen is inlet through a frit to generate bubbles. Then the bubbles arise through the bulk solution in the column, during which, the surfactants adsorb onto the film of the bubbles. When the gas bubbles depart from the bulk solution surface, they form bubble cells with a honeycomb-like structure. These cells stack above the bulk solution surface to form a foam phase, with small amount of entraining liquid loosely

trapped in the spaces between bubbles. Foam is critical to the foam fractionation process, because they offer the necessary large gas-liquid interface for the adsorption of surfactants. Dynamic foam is a complex system with large surface area, which makes foam incline to collapse spontaneously.

2.2.2.2 The type of foam

The stability of the foam depends crucially on the surface activity of the surfactants, therefore, the bubbles coated with less surface-active surfactants form unstable foam, while, the bubbles coated with more surface-active surfactants form a stable foam, metastable foam.

The unstable foam is comprised of the bubbles with a spherical appearance, which contains relatively high amount of liquid and is only slightly distorted by neighbors. These bubbles are constantly bursting as the liquid drains from the bubble films, thus their lifespan is quite short.

The metastable foam dose not burst immediately but persists long enough for drainage to proceed, so that the bubbles contact with each other and the films between bubbles are squeezed to planar form or slightly planar lamellae with almost uniform thickness. A typical metastable bubble is considered to have 12 pentagonal faces, while in reality a typical bubble deviates from this ideal and is in fact an average of shapes.

2.2.2.3 Foam stability

The foam produced in foam fractionation usually has a liquid proportion less than 5%, which is known as dry foam (Koehler, Hilgenfeldt et al. 2000, Xie, Neethling et al. 2004). With such an amount of liquid in the foam, the bubbles are present in the form of polyhedral shapes due to the squeeze against each other (Brush and Davis 2005). Contacting with each other, the thin film known as foam lamellae is formed between two faces of neighboring polyhedral (Vitasari, Grassia et al. 2013). Three thin films meet together to form a channel or capillary, which was named as Plateau border (PB) after the Belgian physicist Plateau, and four PBs meet at a vertex (or node). These PBs form the edges of the polyhedral (Weaire and Hutzler 1999) and their actual orientation and distribution as a whole is random. The structure of the foam is shown below (Fig. 2.2).
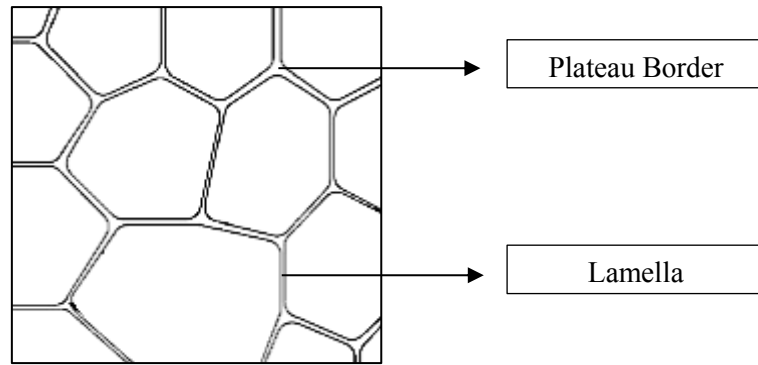
Figure 2. 2 The schematic presentation of foam structure based on Burghoff (Burghoff 2012)

Three processes, namely, foam drainage, film rupture and bubble coarsening, mainly govern the development and stability of the foam.

Foam drainage is the flow of liquid going through the interstitial spaces between bubbles, and is driven by capillary and gravity and resisted by viscous damping at the same time (Li, Li et al. 2013, Sett, Sinha-Ray et al. 2013). Foam drainage volume can be divided into three sources: lamellae, PB channels, and nodes. Accordingly, the drainage in foam takes place mainly via PBs. During drainage, unabsorbed components or surfactants with relatively weak surface-activity will flow backwards firstly, to the bulk solution, and some of them will further adsorb onto the bubbles at the lower position in column if there are free adsorption sites. Therefore, the concentration of the components in the bulk solution is modified continuously. Foam drainage process is complicated, because many parameters are involved, such as bubble size and shape, initial concentration of the components in the bulk solution, type of surfactants, unabsorbed surfactants in the interstitial liquid, the properties of fluid, addictives in the bulk solution. The drainage process in foam with larger bubble size is more rapid than that with small bubble size, since a larger cross-sectional area of the PBs is formed in large bubble foam.

The drainage process results in the thinning of the film by suction. Rupture happens when the film gets too thin and weak, which leads to the direct coalescence of neighboring bubbles and finally the collapse of some of the foam. Eventually, this would results in a decrease of the quantity of the bubbles but increase of the mean bubble volume. Film rupture is even advanced by the presence of components which are weak in surface-active, since the properties of these components determine the prevalence of the attractive van der Waal forces over the repulsive electric double-layer forces. The total gas-liquid interface would be

decreased when two bubbles coalesce into a single larger bubble, and hence greater amount of surface-active molecule will be forced to enter the interstitial liquid because of the loss of the surface area.

Gas diffusion from smaller bubbles to the larger ones and also the surface tension of the bubbles make the pressure in smaller bubbles greater than that in the larger ones, which results in the growth of some bubbles while shrink or disappear of some others. In average, the bubble size grows with time. This process is known as foam coarsening.

Foam coalescence and coarsening process both result in the uneven bubble size distribution in the foam. The bubble size would be larger where these two processes occur more frequently. A direct visual measurement of bubble diameter indicates that the average size of the bubble grows as foam is pushed up in the column.

Many parameters, such as pH of the bulk solution, concentration of the solute and surfactant, gas flow rate, height of the foam tower, additives, influence the adsorption process of the surfactants in foam fractionation techniques, and hence the efficiency of the foam fractionation would also be influenced.

2.2.3   Parameters in Foam Fractionation

pH of the bulk solution

Generally speaking, the pH value will determine the sign and the magnitude of the charge of the molecules in solution. Therefore, the adsorption behavior of these components can be positively influenced by the pH value. For some molecules, a remarkable degree of separation can be achieved in foam fractionation by adjusting the pH value of the bulk solution. An organic compound normally has several different functional groups that some types of molecules possess. Therefore, there would be a pH value, under which the net charge of the molecule is zero, and the water solubility of this molecule would be at its minimum. This pH is known as the isoelectric point (pI). Especially for proteins, the surface activity, stability and packaging at the gas-liquid interface are maximal at its pI (Charm, Morningstar et al. 1966, Noel, Prokop et al. 2002). The pI is different for different molecules when they possess different functional groups. A lot of separations with high efficiency were achieved by adjusting the pH value to pI of the target molecules (Ahmad 1975, Lockwood,

Bummer et al. 1997, Ahmad, Ahmed et al. 2009, Linke, Nimtz et al. 2009, Merz, Schembecker et al. 2009).

Concentration of the solute and surfactant

In foam fractionation, the separation is largely dependent on the concentration of the components to be separated in the bulk solution. A large number of workers have found that the lowest surfactant concentration with the desirable foaming properties in the bulk solution is the most favorable condition for separation (Somasundaran 1972). As early as 1969, Robertson (Robertson 1970) demonstrated that a low concentration of rare-earth elements is a desired property for the foam fractionation. The other researchers (Ahmad 1975, Uraizee and Narsimhan 1996) also showed that low concentration of the materials in the bulk solution is the key for an effective separation of protein. Karger and DeVivo (Karger and Devivo 1968) postulated that the premium concentration of the molecules in bulk solution for the conventional foam fractionation is between 10^{-3} to 10^{-7} M. Once the concentration is higher than CMC, a negative effect on the enrichment would be produced since micelles are formed.

Gas flow rate

Generally, low gas flow rate is beneficial for enrichment, even though the rate of separation will be lower at lower flow rates. It was found that high enrichment and low foam density was obtained at low flow rates (Schnepf and Gaden 1959, Robertson 1970). And of course, sufficient gas flow must be supplied to maintain the foam height, which is essential for good separation. The optimum flow rate is determined not only by the surfactant concentration but also by the foam stability (Somasundaran 1972).

Temperature

The main influence of temperature on foam fractionation is that the stability of the foam coated by surface-active components is different at different temperatures. The effect of temperature is complicated, because it influences many properties of foam, such as adsorption, surface elasticity and viscosity.

Height of the foam tower

Certain foam height is needed to obtain a good separation during foam fractionation. An appropriate increase in foam height brings about a significant change in the mass transfer process, due to the increase in the interfacial transfer area and the increase of drainage. The foam height is particularly essential to obtain a good enrichment and separation, when the foam is not so steady.

Addition of organic solvents

In a normal foam fractionation, water should be the solvent. It would be problematic if the components or surfactants are not well soluble in water. Addition of very small amount of organic solvent in the solvent system would be helpful to enhance the efficiency of foam fractionation.

Ionic strength

The increase of ionic strength in the bulk solution may affect the adsorption of the components at the interface. However, these effects on different foam separation techniques are apparently not always the same (Somasundaran 1972). In foam fractionation, the increase of ionic strength may enhance the adsorption of components at the gas-liquid interface, as long as the concentration of the components is maintained under its CMC and also there is no disadvantageous effect on the other properties of foam.

2.2.4 Devices for foam fractionation

There are typically three distinct zones in a foam fractionation column according to the development of foam:

Liquid pool:
This is the zone where bulk solution filled up. Gas is inlet into the liquid pool to produce bubbles, and then the components in the bulk solution absorb onto the bubbles, when they travel up along the column.

The foam phase:
This zone is the part above the liquid pool. In this zone, the geometric shape of bubbles is changed due to the foam drainage, film rupture and bubble coarsening, and the liquid volumetric fraction is significantly less than that of the bubbles in the liquid pool. The foam goes up slowly, during which, further adsorption happens.

The collection zone:

In this zone, bubbles are all polyhedral shaped and 'dry'. This foam is collected and disengaged from gas by chemical or mechanical methods.

Therefore, these three zones in the foam fractionation column also imply the basic structure of the device for foam fractionation (Fig. 2.3 a.).

As shown in Fig. 2.3 a typical foam fractionation device consist of basically a column for the bulk solution storage and the rising of foam, a gas delivery system to introduce the gas into the bulk solution, and also a foam collector, connecting with a foam breaker. The column is normally made of glass and the height and the diameter of the column chiefly depend on the foam generating methods and the desired purpose.

2.2.5 The modes for foam fractionation

There are primarily two modes of foam fractionation: simple mode and higher mode. Simple mode consists of batch mode and continuous mode, while higher mode consists of enriching mode, stripping mode, and combined mode (Lemlich 1972). Representations of various modes of foam fractionation developed by Lemlich (Lemlich 1972) are presented in Fig. 2.3 and Fig. 2.4.
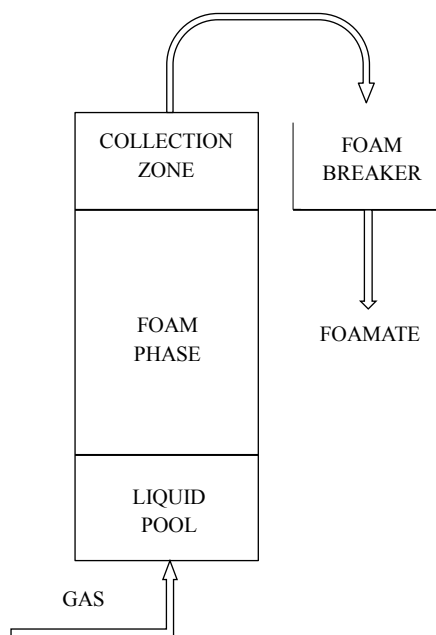
(a)
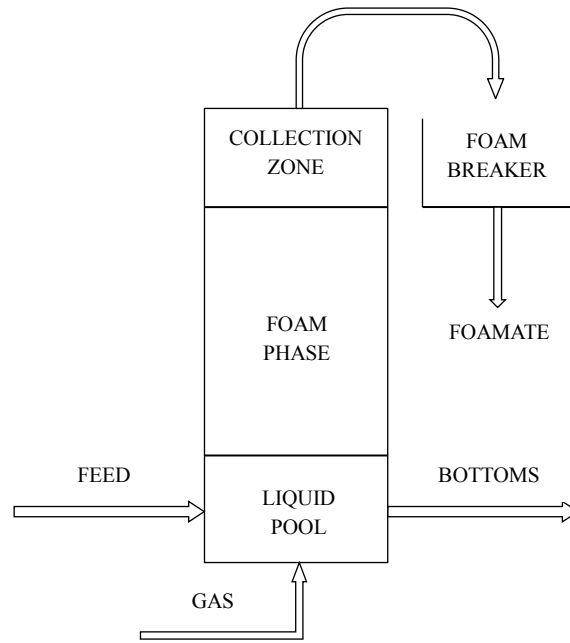
(b)

Figure 2. 3 Foam fractionation in single mode: (a) batch mode, (b) continuous mode

(a)

TOTAL
FOAM FLOW

COLLECTION
ZONE

FOAM
BREAKER

FEED

FOAM
PHASE

COLLECTOR MAKEUP (?)

LIQUID
POOL

BOTTOMS

GAS

(b)

TOTAL
FOAM FLOW

COLLECTION
ZONE

FOAM
BREAKER

FEED

FOAM
PHASE

REFLUX

NET

LIQUID
POOL

W

GAS

(c)
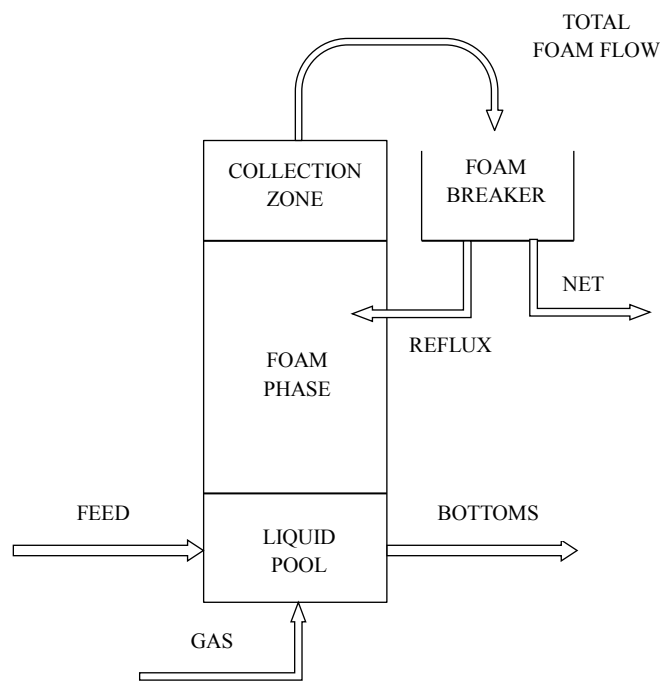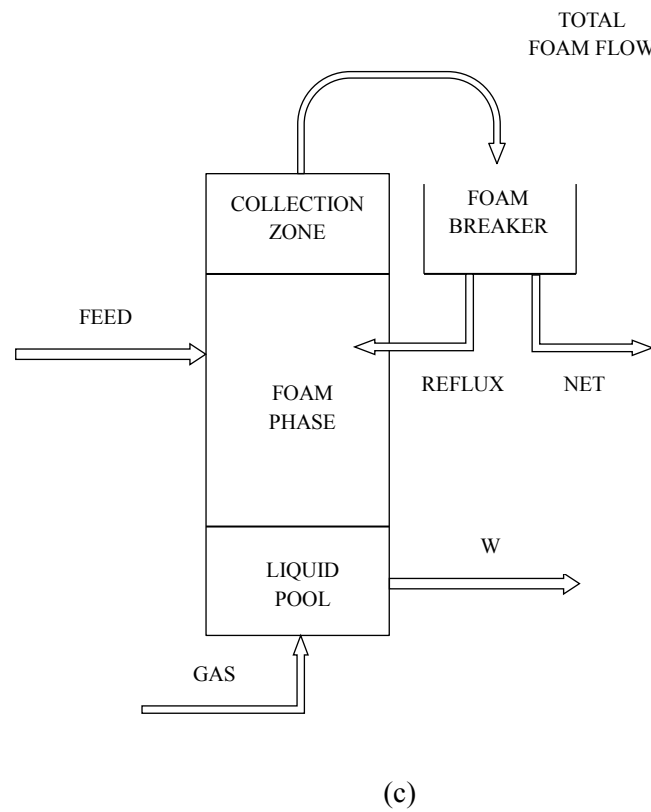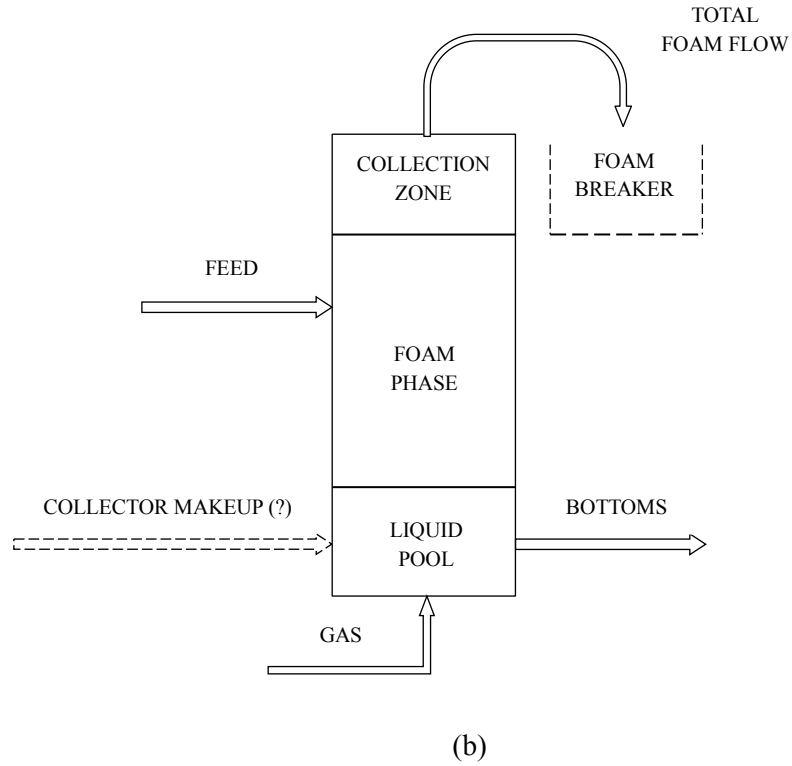
Figure 2. 4 Foam fractionation in higher mode: (a) enriching mode, (b) stripping mode, (c) combined mode

Figure 2.3(a) shows the batch mode foam fractionation in simple mode. And Fig. 2.3(b) shows the continuous mode foam fractionation of simple mode. In the batch mode, foam fractionation is achieved by inletting the N_2 gas continuously at certain flow rate, to drive the foam moving upward, and then the foam is collected. In the continuous mode, the bulk solution is renewed from time to time by exhausting the residual liquid and meanwhile, feeding the fresh liquid into the liquid pool.

Figure 2.4(a), 2.4(b) and 2.4(c) shows the enriching mode, stripping mode and combined mode of higher mode of foam fractionation respectively. In the enriching mode, part of the foamate flow back to the column at a top position counter currently, thus a reflux is formed. And this is how the enriching effect is obtained. This enriching effect has been well verified experimentally by researchers (Lemlich and Lavi 1961). Stripping mode foam fractionation is accomplished by injecting the feed solution into the foam at certain height above the liquid pool in the column. As the feed solution running down in an opposite direction with foam, the resulting counter flow and the mass transfer result in a lower solute concentration in the bottom stream. The combined mode is the combination of enriching mode and stripping mode, which makes the foam fractionation even more efficient.

2.3   Caffeine in coffee

Caffeine (1,3,7-trimethylxanthine), a member of purine alkaloids, was first discovered in tea and coffee in the 1820s (Ashihara and Crozier 2001) though has been consumed for thousands of years. Beside coffee and tea, caffeine was also found in common soft drinks, such as cola, as well as the products containing cocoa and chocolate, and also plenty of medications and dietary supplements (Barone and Roberts 1996, Andrews, Schweitzer et al. 2007). The word 'caffeine' is generated from the word coffee in German, *kaffee*, and in French *café*. According to the historians, caffeine was first consumed by the Chinese Emperor Shen Nong as early as 2737 BC, when he boiled water with the leaves from a bush, making the first port of tea with a pleasant aroma (Arab and Blumberg 2008). Many years later around 9th century, caffeine was consumed again in Ethiopia by an Arabian shepherd named Kaldi, when he saw that his goats had increased energy after the eating of the coffee berries (Heckman, Weil et al. 2010). In the late of 1800's, soft drink with caffeine was introduced by Dr. Pepper, and then followed by Coca-Cola and Pepsi Cola (Heckman, Weil et al. 2010). These caffeinated soft drinks as well as energy drinks become very prevalent. Nearly 80% of the world's population consumes a caffeinated products everyday (Ogawa

and Ueki 2007). Caffeine has become the most widely consumed stimulant drug in the world nowadays.

Caffeine concentration varies among different kinds of beverages, in which coffee has generally the highest amount of caffeine compared with others. Coffee is one of the most popular beverages in the world, and 75% of the caffeine consumed is in the form of coffee, it is also the world's most important traded commodity after oil (Fujioka and Shibamoto 2008).

The attractiveness and recognition of these beverages are mainly due to the special effects of caffeine: keeping people staying awake and improving mental alertness after fatigue (Smit and Rogers 2002). However, some other researchers found that caffeine can also be a potential contributor to reduce the risk of metabolic syndrome, including type 2 diabetes mellitus and obesity (Westerterp-Plantenga, Diepvens et al. 2006). However, a growing number of researchers believe that the consumption of caffeine may have some adverse effects on human health, such as palpitations, gastrointestinal disturbances, anxiety, tremor, increased blood pressure and insomnia (Chou and Benowitz 1994, Nurminen, Niittynen et al. 1999). Therefore, an increased demand for decaffeinated beverages has arisen.

2.3.1 Coffee decaffeination

Decaffeinated coffee was defined as "coffee from which caffeine has been eliminated", according to the International Standards Organization. The detailed requirements for decaffeinated coffee are normally stated in a specification in almost every country. In most European countries, the maximum caffeine content is set at 0.1% for roasted decaffeinated coffee, and 0.3% for instant decaffeinated coffee, respectively, on dry basis. It can be calculated therefore that a cup of brewed coffee with 10 g of roasted coffee beans, will contain no more than 10 mg of caffeine, while in instant coffee with 2 g of the product will be 6 mg. This figure is also generally accepted elsewhere in the world, no matter by legislation or otherwise. While in the USA, no specific legislation exists, but the manufacturers generally claim that more than 97% of the caffeine in coffee beans has been eliminated, both for roasted and instant decaffeinated coffee.

Decaffeination is the name of the process through which caffeine is removed, which dates back to the year of 1905 in Germany. In commercial practice, this process is normally applied to green coffee, and then the coffee is roasted and grinded, or prepared as the instant coffee. Theoretically, this decaffeinated coffee should be identical with normal coffee, except

the amount of caffeine. However, there will be some differences such as flavor and taste, due to the co-elimination of other components in coffee.

There are three processes for the decaffeination of coffee, which are normally used.

Direct solvent decaffeination

The first commercial process for decaffeination, with a product name Kaffee Hag and still available nowadays, was developed and patented in Bremen, Germany, in the year of 1905. In this process, benzene was used as the solvent to extract caffeine from previously steamed green beans. However, chlorinate hydrocarbons were used to replace benzene when they are available with a reasonable price, because of the flammability and toxicity of benzene. Trichloroethylene was particularly favored later on, and then was replaced by methylene chloride. A number of other organic solvents have been proposed, but only ethyl acetate and vegetable oils are believed to be used in commercial production of decaffeinated coffee (Clarke 2003). What very interesting in the decaffeination process is that, moisture of green coffee beans, first by steaming and then soaking in water, is necessary for an extraction with high efficiency. A direct use of dry organic solvent extracted relatively little caffeine, or very slowly, in spite of the fact that the solubility of caffeine in methylene chloride is moderate. People ascribed these phenomena to that the swelling of coffee beans assists the diffusion of caffeine, especially from the caffeine-chlorogenic acid complex.

In the direct solvent decaffeination method, a large amount of solvent is needed. For example, 4 kilos of methylene chloride is required for the decaffeination of one kilo of coffee. And also, the operation time can be as long as 10 hours. All of these increase the costs of decaffeination, and may cause various potential health and environmental problems. These concerns have promoted the exploration of the new alternative methods or solvents for decaffeination.

Indirect solvent decaffeination

A new approach for the decaffeination of coffee was patented in 1941, in which water was used to eliminate caffeine from green coffee beans. In this method, water is saturated with non-caffeine solutes, which exist in green coffee beans, in order to prevent their co-extraction by water. After the extraction by water, caffeine enriched water solution is contacted counter currently with an organic solvent at around 80 ℃. As a result, the caffeine content in this water is decreased to below than 0.05% from about 0.5%, and hence, after

stripping of the residual organic solvent, this water is then recycled to the extraction system for further extraction of caffeine in the green coffee beans.

This approach is somewhat more complicated but less time consuming (around 8 hours), and is more efficient but with less energy, compared with the direct solvent extraction method. However, this method may result in a slightly higher loss of non-caffeine substances, such as aroma precursors which may affect the aroma and/or taste of coffee after roasting.

Activated carbon decaffeination

A company named Coffex in Amsterdam proposed the decaffeination of coffee using activated carbon, which is pre-coated with other coffee extracted substances or some substitute components with similar molecular structure or size, especially with carbohydrates. Therefore, the charcoal will adsorb as much caffeine as possible, other than the other substances. Afterwards, the charcoal can be reactivated for reuse, and the use of organic solvents is avoided.

Supercritical carbon dioxide decaffeination

Recently, supercritical fluids have been used as the solvent for the decaffeination of coffee beans in large-scale commercial processes. The selectivity of this supercritical fluid is very important for the decaffeination process, since the extraction of the other components besides caffeine is not expected. As a nonpolar solvent, carbon dioxide is proved to be quite selective for caffeine. Normally, CO_2 flow rate, temperature and pressure are adjusted to optimize the extraction efficiency. However, soaking or steaming the green coffee beans with water prior to decaffeination is very essential for the increase of extraction rate. The low yield rate of extraction without water treatment can be ascribe to the formation of the caffeine and chlorogenic acids complex in coffee beans, which would prevent the caffeine from solvating into the CO_2 fluid (Peker, Srinivasan et al. 1992). Meanwhile, in order to enhance the extraction rate of supercritical CO_2 fluid, water or ethanol is also added into the extraction flow, as a co-solvent, due to the high polarity of caffeine.

Using supercritical CO_2 as a solvent for decaffeination, polluting of organic solvent is prevented, and the expensive post-treatment cost of the extract for solvent reduction is eliminated, without significant loss of coffee flavor, which is released only during roasting.

However, there are several significant disadvantages of supercritical fluid extraction as well. First, the phase equilibrium of the solvent/solute system in this technology is complex, which makes the design of extraction conditions difficult; second, the most popular solvent, carbon dioxide, is non-polar and therefore, it is only useful in the extraction of non-polar solutes. The use of co-solvents can enhance the extraction rate of the polar compounds, but they may make the downstream process more complicated. Third, the use of high pressure increases the costs for the equipment and their maintenance, and the operation costs can also be high. Therefore, supercritical fluid extraction will only be used when significant advantages exist.

2.3.2 Caffeine complex

It has been known for a long time that caffeine complexes with polyphenols and aromatic hydroxyl acids molecules in aqueous solution (Cai, Gaffney et al. 1990, D'Amelio, Fontanive et al. 2009), such as methyl gallate, 3-nitrobenzoic acid (Martin, Lilley et al. 1986 a), 5-chlorosalicylic acid (Shefter 1968), gallic acid and quercitin (Jobstl, Fairclough et al. 2005), pyrogallol (Gould 1968), theaflavin (Charlton, Davis et al. 2000), catechins (Hayashi, Ujihara et al. 2004), and cyclodextrins (Spencer, Cai et al. 1988). The caffeine-chlorogenic acid complex was firstly isolated from green coffee beans almost one century ago (Gorter 1907). Sondheimer et al (Sondheimer, Covitz et al. 1961) studied the solution behavior of the complex, by means of solvent partition method and a spectrophotometric procedure. They proposed that the complex is formed by caffeine and chlorogenic acid in the ratio of 1:1 in aqueous solution, in which the benzene ring, the conjugated double bond, and the phenolic groups contribute to the stability, while the H-bonding contributed by hydroxyl groups is not involved. However, some other researchers illustrated that beside the π stacking interaction, hydrogen bonding is also responsible for the stabilization of the complex (Horman and Viani 1972, Martin, Lilley et al. 1986 a, Martin, Lilley et al. 1986 b). Horman and Viani (Horman and Viani 1972) evaluated the association constant for the formation of the complex, and also, based on the data from nuclear magnetic resonance (NMR), they proposed that the caffeine-chlorogenic acid complex may be described as a 1:1 hydrophobically-bound π-molecule, in which the plane of caffeine is parallel to the plane of the caffeoyl aromatic ring, and the five and six membered rings of caffeine are equally involved in the complex formation. They believed that the complex is a time-average of many other conformations involving relative twisting, sliding and rocking of the two components, rather than absolutely fixed. Martin et al. (Martin, Lilley et al. 1986 b)

elucidated the crystal structure of the caffeine-potassium chlorogenate complex. In their research, a striking resemblance between the crystal structure and the complex model proposed by Horman and Viani was found, suggesting that the complexes discussed by Horman and Viani are the precursors in crystal formation and growth. Using high-resolution ^{1}H-NMR, D'Amelio et al. (D'Amelio, Fontanive et al. 2009) investigated the caffeine complexation by chlorogenic acid in aqueous solution as well as caffeine-chlorogenic acid complex in freshly prepared coffee brews. Confirmed some previous findings of the complex, their NMR reinvestigation also proved the existence of this complex in the real beverage. A possible conformation of caffeine-chlorogenic acid complex is shown in Figure 2.5.
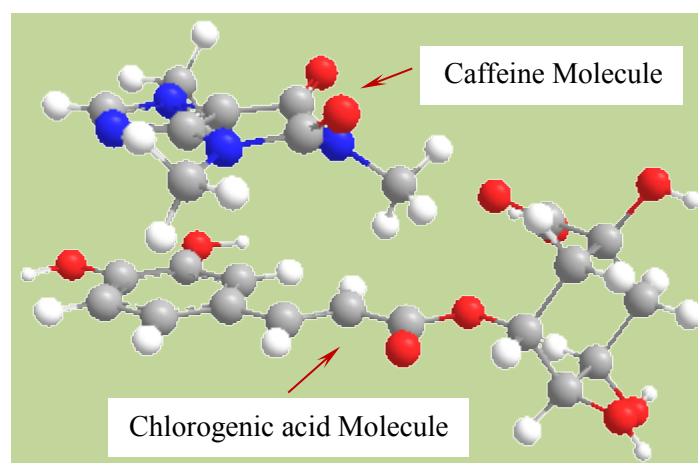
Figure 2. 5 A possible conformation of caffeine-chlorogenic acid complex (Horman and Viani 1972, Martin, Lilley et al. 1986 a, Martin, Lilley et al. 1986 b)

Here, we are trying to enrich/eliminate caffeine from its aqueous solution using foam fractionation technique. Firstly, chlorogenic acid was used as a catcher in the foam fractionation of caffeine, since it can form a complex with caffeine. Then, some other compounds similar to chlorogenic acid were synthesized, and were used as catchers later on in the foam fractionation of caffeine. The foamability and foam stability of the catchers were all tested before foaming, and saponin was added when the system could not produce enough foam. The complexation between caffeine and the catchers was also proved. In the foaming experiments of caffeine with each catcher, all parameters that may influence the foaming efficiency were optimized. The catcher with a better performance was used in the enrichment/elimination of caffeine from green coffee sample.

3. Materials and Methods

3.1 Materials

3.1.1 Green coffee sample

One thousand grams of green coffee beans (Wellenkamp, Sjöström & Co. GmbH, Bremen) were purchased from the local market in Freising, Germany. The package was closely sealed and stored in a cool, dry and dark place, before and after sampling.

3.1.2 Chemicals and solvents

- 1,4-Dioxane: anhydrous, 99.8%, Sigma-Aldrich (Steinheim, Germany)
- 1-Octanol: Gas chromatography grade, Riedel-de Haën (Seelzer, Germany)
- Acetic acid: HPLC grade, Merck (Darmstadt, Germany)
- Acetonitrile: HPLC grade, VWR (Fontenay-sous-Bois, France)
- Aceton: for analysis, Neolab (Heidelberg, Germany)
- Caffeine: anhydrous, HPLC grade, Fluka (Buchs, Germany)
- Chloroform: for analysis, ROTH (Karlsruhe, Germany)
- Chlorogenic acid: HPLC grade, Sigma-Aldrich (Steinheim, Germany)
- N,N'-Dicyclohexylcarbodiimide: 99%, Sigma-Aldrich (Steinheim, Germany)
- Dimethyl sulfoxide (DMSO): for analysis, Merck (Darmstadt, Germany)
- Ethanol: for analysis, Merck (Darmstadt, Germany)
- Ethyl Acetate: for analysis, Fluka (Steinheim, Germany)
- Formic acid: HPLC grade, Merck (Darmstadt, Germany)
- n-Hexane: for analysis, Merck (Darmstadt, Germany)
- Methanol: HPLC grade, Sigma-Aldrich (Steinheim, Germany)
- Nitrogen Gas (N_2): pure, Linde (München, Germany)
- Octylamine: 99%, Sigma-Aldrich (Steinheim, Germany)
- Phosphoric acid (H_3PO_4): 85 wt.% solution in water, Aldrich (Steinheim, Germany)
- Potassium phosphate dibasic (K_2HPO_4): ≥98%, Sigma-Aldrich (Steinheim, Germany)
- Potassium acetate: ≥99%, Sigma-Aldrich (Steinheim, Germany)

- Potassium phosphate Monobasic (KH_2PO_4): HPLC grade, ≥99.5%, Sigma-Aldrich (Steinheim, Germany)

- Saponin: pure, Riedel-de Haën (Seelzer, Germany)

- Thionyl Chloride ($SOCl_2$): ≥99.0%, Sigma-Aldrich (Steinheim, Germany)

- Tetrahydrofuran: anhydrous, 99.9%, Sigma-Aldrich (Steinheim, Germany)

- Water (Distilled): in house distillation system at CTA

- Water (Bi-distilled/de-ionized): "Milli-Q_{185} Plus", Millipore (Darmstadt, Germany)

3.1.3 Equipments and Software

- Foam fractionation system

The foam fractionation apparatus was assembled with series of glass wares in our lab. Figure 3.1 in the section of 3.2.5 depicted the operation unit.

- High Performance Liquid Chromatograph

UltiMate 3000 HPLC system, Thermo Fisher Scientific (Sunnyvale, United States):

Solvent rack: SR-3000
Pump: LPG-3400SD
Autosampler: WPS-3000 SL (Analytical)
Column compartment: TCC-3000SD
Detector: DAD-3000 (Photometer)
Software: Chromeleon 6.80 for Windows

- UV- spectrophotometer

UV-1800 240V IVDD, Shimadzu (Tokyo, Japan)
Software: UV Probe 2.34 for Windows

- Electronic balance

AX224, Sartorius (Göttingen, Germany);
AX2202, Sartorius (Göttingen, Germany)

- Ultrasonic bath

RK 510, Bandelin electronic (Berlin, Germany)

- pH-Meter

  Education line EL20, Mettler Toledo (Schwerzenbach, Swiss)

- Pipette

  Eppendorf Research plus 0.5-5 ml, Eppendorf (Hamburg, Germany)

  Eppendorf Reference 100-1000 µl, Eppendorf (Hamburg, Germany)

  Eppendorf Reference 10-100 µl, Eppendorf (Hamburg, Germany)

  Eppendorf Reference 2-20 µl, Eppendorf (Hamburg, Germany)

- Rotary evaporator

  Rotavapor-R, Büchi (Swiss)

- Magnetic stirrer

  MR Hei-Standard, Heidolph Instruments (Schwabach, Germany)

- Nuclear magnetic resonance

  Bruker DMX 500 spectrometer, Bruker (Billerica, MA, USA)

- Mass spectrometer (MS)

  HCT Ultra electrospray ionization (ESI) - Ion Trap mass spectrometer, Bruker Daltonics (Bremen, Germany)

  Bruker Fourier Transform Ion Cyclotron Resonance Mass Spectrometry, Bruker Daltonics (Bremen, Germany)

- Statistical analysis

  IBM Statistical Package for the Social Sciences (SPSS), IBM (Chicago, USA)

3.2 Methods

3.2.1 Preparation of caffeine-chlorogenic acid complex

The caffeine-chlorogenic acid complex was prepared according to the method from Sondheimer et al. (Sondheimer, Szymansk.Cd et al. 1961). Seventy-five mg of chlorogenic acid and 50 mg of potassium acetate were dissolved in ethanol (95%), and followed by 150

mg caffeine. After storage in the fridge at 0 °C for 48 hours, the mixture of the solution was filtered, which yielded the crystal of the product.

3.2.2 Synthesis of catchers

The synthesis of n-octyl caffeate:

n-Octyl caffeate was synthesized according to the method reported (Nagaoka, Banskota et al. 2002), with a small modification. 1.02 g (5.6 mmol) caffeic acid was dissolved in 25 ml dioxane, followed by 0.6 ml (8.2 mmol) $SOCl_2$ under N_2. The mixture was stirred at 100 °C for 3 hours. Then 1.33 ml (8.4 mmol) 1-octanol was added dropwise to the mixture, and stirred for another 6 hours. After the remove of the solvent under reduced pressure, the residue was subjected to a silica gel column chromatograph, using n-hexane–aceton (3:1) as a mobile phase, to give the crude caffeic acid ester: n-octyl caffeate. To get a pure form of n-octyl caffeate, recrystallization using n-hexane-diethyl ether as a solvent was also conducted.

The synthesis of n-octyl caffeamide:

n-Octyl caffeamide was synthesized according to the literature (Sugiura, Naito et al. 1989). 900 mg (5 mmol) caffeic acid was dissolved in 25 ml tetrahydrofuran. Then, 1030 mg (5 mmol) dicyclohexylcarbodiimide and 645 mg (5 mmol) octylmine were added to the solution successively, and the mixture was stirred for 7 hours at 50 °C. The solvent in the mixture was removed under reduced pressure, and the residue was subjected to the silica gel column chromatograph, using n-hexane–ethyl acetate (1:1) as a mobile phase, to give the desired caffeic acid octyl amide: n-octyl caffeamide.

3.2.3 NMR and MS analysis

To confirm the structure of the compounds obtained, their ^1H, ^{13}C NMR and MS spectra were all recorded.

Dissolved in D_2O or MeOD, the NMR spectra of the compounds were recorded on a Bruker DMX 500 spectrometer (proton frequency: 500.13 MHz) at 298K or 303K, using CD_3OD as an external reference (δ_H 3,30 ppm, δ_C 49.0 ppm).

The MS spectra were acquired on an HCT Ultra electrospray ionization (ESI) - Ion Trap mass spectrometer in both positive and negative mode using the following parameters:

source temperature, 300 ℃; gas flow, 5 L/min; capillary, 4000 V; skimmer, 40 V; capillary exit, 98.5 V; scan range, 50 – 500 *m/z*. Samples were dissolved in ACN (LC-MS grade) to obtain a solution of 100 ppm, and then infused directly for record.

Ultrahigh-resolution MS spectra were all acquired in negative mode, on a Bruker Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR/MS), equipped with a 12-Tesla superconducting magnet and an Apollo II electrospray source.

3.2.4   Evaluation of foamability and foam stability

Two ml of catchers' aqueous solution in a 5 ml volumetric flask was hand shaked for 1 minute. The height of the foam was recorded as the index of foamability. Afterwards, the height of the foam recorded at two minute was taken as the index of foam stability of the catcher. The concentration of the catcher in water was 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} M. The solvent for the catcher was mainly water; however, a small amount of organic solvent was also used when the catcher is insoluble in pure water, such as ethanol.

3.2.5   Evaluation of the complexation of caffeine and its catchers

The Beer-Lamber law (Equation 6) describes the absorption of the light travelling through a solution.

$$A = -log\frac{I}{I_o} \tag{6}$$

Where, A is the absorption, *I* is the intensity of the transmitted light, and I_o is the intensity of the incident light.

Actually, the absorption of the light is determined by the properties of the solution through which the light travels. So, the absorption can also be expressed as:

$$A = \varepsilon \cdot b \cdot c \tag{7}$$

Where, ε is the molar absorption coefficient, which is also expressed as E_λ, in units of $M^{-1} \cdot cm^{-1}$, *b* is the path length the light travels in units of cm, and *c* is the molar concentration of the solution in the unit of M.

The molar absorption coefficient is one of the unique properties of the substance and hence is constant at each fixed wavelength. When complexation or aggregation happens, the molar

absorption coefficient of this substance would be changed. Therefore, UV-Vis spectroscopy is one of the most suitable methods for quantitative study of the aggregation or complexation of the substances (Antonov, Gergov et al. 1999) because of its simplicity and high sensitivity, and several researches on caffeine aggregation or complexation with other ligands using UV-Vis spectroscopy based colorimetric titration method have already been published (Kapuscinski and Kimmel 1993, Zdunek, Piosik et al. 2000, Woziwodzka, Gwizdek-Wisniewska et al. 2011). Here, we used the same method to analyze the complexation between caffeine and its catchers.

Caffeine stock solution was prepared by dissolving a weighted amount in the 0.1 M phosphate buffer with a pH of 7, to obtain a final concentration of 50 mM. Chlorogenic acid and the other catchers were also dissolved in the same buffer, to obtain a solution of 0.25 mM.

The mixtures for the light absorption measurements were prepared by adding an increasing amount of caffeine stock solution into the catcher solution (shown in Table 1), and then buffer solution was added to obtain a final volume of 4 ml. For a different catcher, the number of the mixed solution adopted was varied in a small range. The solutions prepared were well mixed and stood still for 30 min before the light absorption measurements.

Table 1 The volumetric composition of the solutions of catcher titrated with caffeine

Number	Catcher (0.25 mM) (ml)	Caffeine (50 mM) (ml)	Phosphate buffer (0.1 M, pH 7.0) (ml)
1	1	0.02	2.98
2	1	0.05	2.95
3	1	0.1	2.9
4	1	0.2	2.8
5	1	0.4	2.6
6	1	0.6	2.4
7	1	0.8	2.2
8	1	1.0	2.0
9	1	1.2	1.8
10	1	1.5	1.5
11	1	2.0	1.0
12	1	2.5	0.5

Light absorption spectra were measured using Shimadzu UV-1800 UV- spectrophotometer. The 1 ml aliquot mixed solution was placed in a quartz cuvette, with a light path of 1 cm. Then the spectra were recorded at 1 nm intervals in a wavelength range of 250 to 500 nm, using buffer as a black control. The data was stored in a digital form and was converted to the form of molar absorption coefficient.

3.2.6 Foam fractionation apparatus and operation

The apparatus for foam fractionation used in this research is in batch mode, which is mainly composed of six parts: N_2 tank with switch and valve; N_2 inlet copper tube with an inner diameter of 0.3 cm; flowmeter; bubble frit consisting of a glass tube and a fused porous glass end (porosity grade 3: 16-40 μm); foam riser or column with an inner diameter of 18 cm; and foam collector. In this apparatus, the foam collector is settled upon the foam riser, while the frit is fixed at its bottom. The N_2 flow is inlet into the column through the frit by the tube connected with the N_2 tank, controlled by a switch and valve and monitored by a flowmeter. A graphical depiction of the apparatus is illustrated in Fig. 3.1.
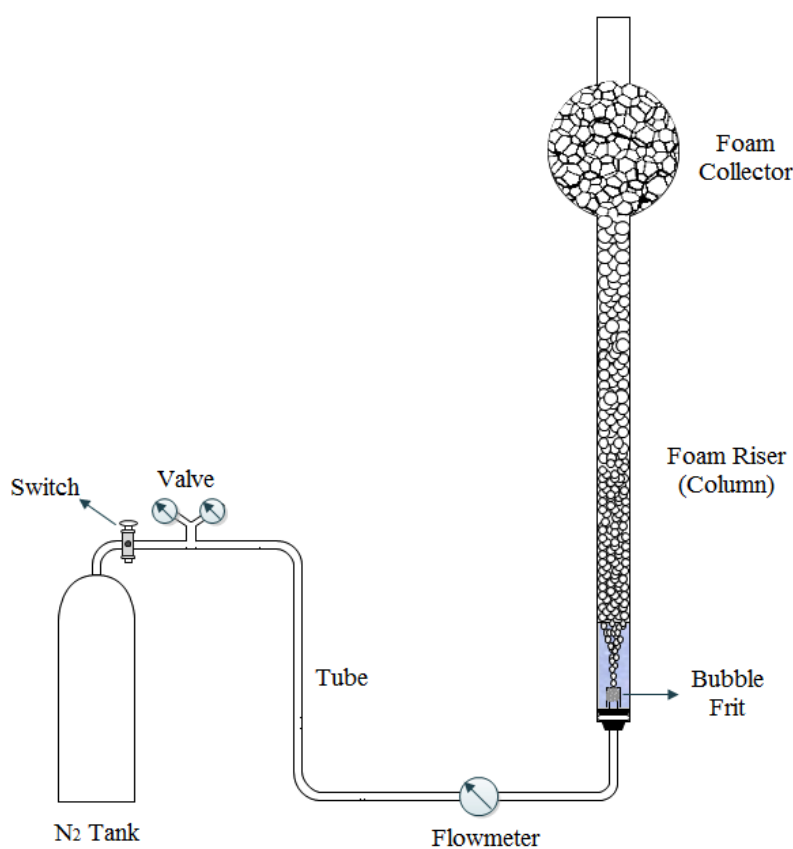
Figure 3. 1 Diagram of batch mode foam fractionation apparatus

In the operation process of foam fractionation, N_2 is inlet continuously into the bulk solution holded at the bottom of the riser, to produce enough bubbles. When the bubbles go up along the riser, they become dryer and dryer, and finally accumulate in the foam collector as foamate. In this research, a small amount of ethanol was used to liquefy the foamate.

Firstly, the initial solution contains caffeine alone, caffeine and the catcher (chlorogenic acid, n-octyl caffeate, n-octyl caffeamide, caffeic acid) was foamed. Caffeine was dissolved in distilled water and then stored in the fridge as a stock solution. The stock solution for each catcher was also obtained by dissolving the catcher into distilled water or organic solvents, or the mixture of distilled water and organic solvents, depending on the solubility of the catcher in water. Before foam fractionation, certain amount of stock solutions of both caffeine and catcher were mixed well and then diluted with distilled water to prepare the initial solution. The stock solutions of both caffeine and catcher were newly prepared every three days, while the initial solution for foam fractionation was prepared before foaming. In each binary system, a small amount of saponin would be added if the solution itself was not able to produce adequate foam. Before the formal foam fractionation experiments, some preliminary tests were conducted firstly, such as the adjustment of the flow rate for the production of the stable foam. After that, all the parameters, such as pH value, flow rate, amount of saponin, the height of column, temperature and so on, were all varied in each series of foam fractionation experiments for each binary system of caffeine and catchers, to investigate their influence on the foam fractionation. The results were statistically analyzed using SPSS software, to compare the means of separation efficiency obtained from varied values of each parameter. After the comparison, the parameters with better results for each binary system were combined in one foaming experiment, in order to get the best foaming efficiency. Finally, the catcher with a best foaming result was used for the enrichment of caffeine from coffee sample, using foam fractionation.

3.2.7 HPLC analysis and preparation of the calibration curves

The HPLC programs for caffeine, each binary system of caffeine and catchers, and also the green coffee sample were optimized, and then the calibration curves for all the standards were prepared.

HPLC analysis of caffeine alone:

Column: Thermo ODS Hypersil, 150×4.6 mm, 5 μm

Eluent A: Water

Eluent B: Methanol

Temperature: 20 ℃

Detection wavelength: 280 nm

HPLC program is shown below:

Time (min)	Flow rate (ml/min)	A (%)	B (%)
0	1.0	75	25
10	1.0	75	25

HPLC analysis of caffeine and chlorogenic acid:

Column: Thermo ODS Hypersil, 150×4.6 mm, 5 μm

Eluent A: 0.1% (v/v) Formic acid in water

Eluent B: 0.1% (v/v) Formic acid in Water-Acetonitrile (40:50, v/v)

Temperature: 20 ℃

Detection wavelength: 280 nm and 320 nm

HPLC program is shown below:

Time (min)	Flow rate (ml/min)	A (%)	B (%)
0	1.0	88	12
10	1.0	83	17
11	1.0	88	12

HPLC analysis of caffeine and n-octyl caffeate:

Column: Thermo ODS Hypersil, 150×4.6 mm, 5 μm

Eluent A: Acetonitrile

Eluent B: Water

Temperature: 20 ℃

Detection wavelength: 280 nm and 320 nm

HPLC program is shown below:

Time (min)	Flow rate (ml/min)	A (%)	B (%)

0	1.5	20	80
3	1.5	20	80
5	1.5	70	30
10	1.5	70	30
12	1.5	20	80

HPLC analysis of caffeine and n-octyl caffeamide:

Column: Hamilton HxSil C 18, 150×4.6 mm, 5 μm

Eluent A: Water

Eluent B: Acetonitrile

Temperature: 20 ℃

Detection wavelength: 280 nm and 320 nm

HPLC program is shown below:

Time (min)	Flow rate (ml/min)	A (%)	B (%)
0	0.5	60	40
15	0.5	60	40

HPLC analysis of caffeine and caffeic acid:

Column: Hamilton HxSil C 18, 150×4.6 mm, 5 μm

Eluent A: Methanol

Eluent B: 0.3% (v/v) H_3PO_4 (85 wt. % in water)

Temperature: 20 ℃

Detection wavelength: 280 nm and 320 nm

HPLC program is shown below:

Time (min)	Flow rate (ml/min)	A (%)	B (%)
0	1.0	20	80
6	1.0	20	80

HPLC analysis of green coffee sample:

Column: Hamilton HxSil C 18, 150×4.6 mm, 5 μm

Eluent A: Methanol

Eluent B: 0.3% H_3PO_4 (85 wt. % in water)

Temperature: 20 ℃

Detection wavelength: 280 nm and 320 nm

HPLC program is shown below:

Time (min)	Flow rate (ml/min)	A (%)	B (%)
0	0.5	5	95
20	0.5	35	65
25	0.5	40	60
30	0.5	40	60
33	0.5	5	95
35	0.5	5	95

Table 2 The calibration equations for the quantification of each substance in different foaming systems

Foaming system	Substances	Calibration equation	Coefficient of determination (R^2, %)
Caffeine alone	Caffeine	$y = 0.0398x - 0.0107$	99.9990
Caffeine-chlorogenic acid complex	Caffeine Chlorogenic acid	$y = 0.0395x - 0.0211$ $y = 0.0475x - 0.0181$	99.9978 99.9999
Caffeine & n-octyl caffeate	Caffeine n-Octyl caffeate	$y = 0.0272x - 0.0228$ $y = 0.0180x - 0.0414$	99.9991 99.9910
Caffeine & n-octyl caffeamide	Caffeine n-Octyl caffeamide	$y = 0.0740x + 0.0053$ $y = 0.0001x^2 + 0.0508x - 0.0293$	100.0000 100.0000
Caffeine & caffeic acid	Caffeine Caffeic acid	$y = 0.0265x + 0.0005$ $y = 0.0378x + 0.0042$	99.9990 99.9999
Coffee	Caffeine Chlorogenic acid	$y = 0.0001x^2 + 0.0726x + 0.0714$ $y = 0.2433x + 0.2844$	99.9990 100.0000

Note: x, the amount of the standards analyzed (ng); y, the area of the peak (mAU*min).

In order to quantify the amount of caffeine and catcher in the corresponding samples, the calibration curves were prepared base on the above-obtained HPLC programs, using the UltiMate 3000 HPLC system. Only one solution with a proper concentration of each standard was prepared, since different data points (at least 4) for the calibration curve of each standard was easily obtained by the injection of different volumes of standard solution, using Chromeleon 6.80 software (Table 2). The regression of the data points was also achieved by the software automatically.

Once the calibration curve was prepared, the quantification of the corresponding substances in samples was accomplished automatically. Examples of the chromatograms of HPLC analysis are shown in Figure 3.2.
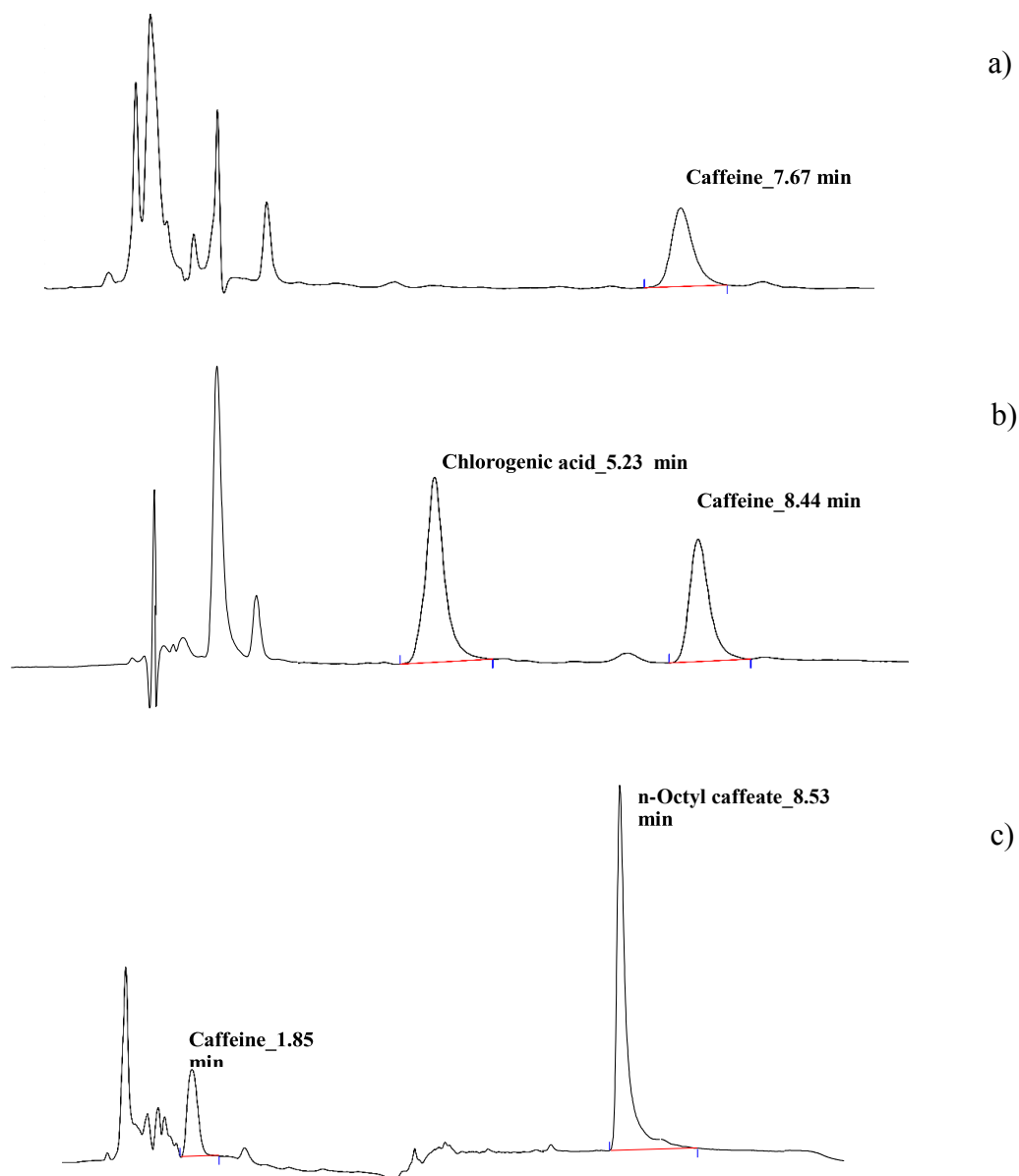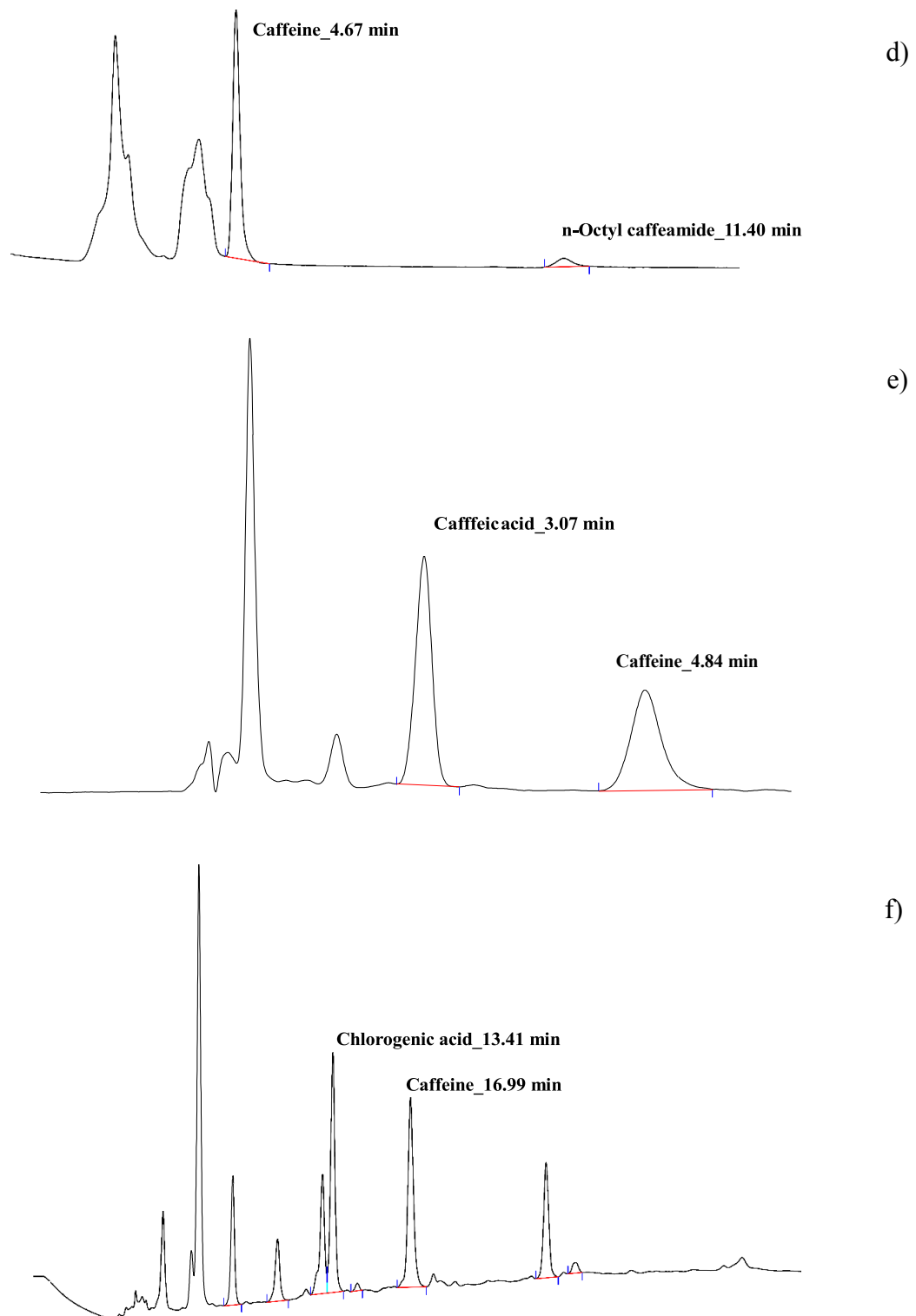
Figure 3. 2 HPLC analysis of caffeine in each aqueous solution using the corresponding programme: a) caffeine alone; b) caffeine-chlorogenic acid complex; c) caffeine and n-octyl caffeate; d) caffeine and n-octyl caffeamide; e) caffeine and caffeic acid; f) green coffee

3.2.8 Evaluation of the foam fractionation efficiency

Generally, the efficiency of foam fractionation is evaluated by the comparison of the concentration or the total amount of the target components in the initial solution before foaming with that in the foamate after foaming. For these purpose, enrichment ratio (ER) and recovery rate (R) were used for the comparison of the concentration and total amount, respectively.

The equations are shown as below:

$$ER = \frac{C_f}{C_i} \qquad (8)$$

$$R\ (\%) = \frac{C_f \cdot V_f}{C_i \cdot V_i} \times 100 \qquad (9)$$

Where:

C_f is the concentration of the target component in the foamate

C_i is the concentration of the target component in the initial solution

V_f is the volume of the foamate after collapse

V_i is the volume of the initial solution

For *ER*, a value ≤ 1 means the concentration of the component in the foamate is lower than that in the initial solution; therefore, no enrichment is achieved. An *ER* value ≥ 1 means the concentration in the foamate is higher than that in the initial solution, and the component is enriched. A high value of *ER* indicates a high foaming efficiency.

R can be explained as the percentage of the target component transferred into the foam phase. This value depends not only on the concentration of the component in the foamate, but also on the volume of the foamate. A value of 1 means a complete transfer of the target component into the foamate, though this value can never be reached in practice.

3.2.9 Statistical analysis

The results of the experiments were analyzed statistically with SPSS 13.0 software. One-way analysis of variance (ANOVA) was used to compare the means, for there was only one variance in each series of experiments. The Duncan's Multiple Range post hoc test was selected above the other post hoc tests due to its descriptive presentation of significant

differences between group means. Significance level was established at $\alpha = 0.05$. All the experiments were done at least in duplicate.

3.2.10 Preparation of the green coffee initial solution

Green coffee beans were grilled into fine powder before foam fractionation. Then a portion of the powder was dispensed into a certain volume of distilled water to form a coffee mixture. The mixture was filtered and the supernatant was then used as the initial solution for the foam fractionation. A small amount of saponin was added to make sure that enough bubbles could be produced during the foaming process. This initial solution was always prepared newly before foaming.

4.   Results and Discussion

4.1   Foam fractionation of caffeine alone

4.1.1   Evaluation of the foamability and foam stability of caffeine

Firstly, caffeine aqueous solution with a concentration from 10^{-7} M to 10^{-3} M was tested for its foamability. However, the solution at different concentrations was not able to produce any foam, or collapsed very quickly if any. Then, the caffeine solution with a concentration of 10^{-5} M at different pH values (from 3 to 10) was tested for its foamability. Similar results were obtained.

4.1.2   Foam fractionation efficiency

For foam fractionation, the generation of adequate foam is certainly a necessity. The foam can be either produced by the solution system of the subject investigated itself or with the assistance of additional foam arising substances when the solution system is failed to generate enough foam. As indicated in section 4.1.1, caffeine aqueous solution was not able to produce any stable foam ample for the foam fractionation, even with different concentrations or at different pH values. Therefore, saponin, a natural-sourced surface active substance, was added to the aqueous solution of caffeine to enhance both of the foamability and foam stability.

A different amount of saponin ranging from 5 mg/100ml to 100 mg/100ml was tested for the foam properties. Finally, a saponin concentration ranging from 12.5 mg/100ml to 50 mg/100ml in the aqueous solution of caffeine was chosen for further foaming test, since within this range of saponin concentration the foam with optimal size and water content was generated, which is beneficial for the obtaining of good separation efficiency. Saponin with an amount below 12.5 mg/100ml in the initial solution was not able to produce adequate foam, while an amount above 50 mg/100ml in the solution produced too much foam, which is small and wet.

The flow rate is also a very important parameter for obtaining of good separation efficiency in the foam fractionation. In the primary test, it was found that too high a flow rate produced a large amount of bubbles with a small size and rich in bulk solution. Also, the time for the

foam to reach to the collector was shortened; the separation efficiency was hence decreased because of the low collapse rate. A low flow rate ensured the timespan for the drainage process, in which separation of different substances and enrichment happened. Here in this research, a flow rate below than 50 ml/min was adopted.

After the primary test, the parameters including pH, amount of saponin, caffeine concentration, flow rate, temperature, height of column and NaCl amount, which may have an influence on the separation, were all investigated.

4.1.2.1 Influence of initial pH value

Caffeine aqueous solution with an initial pH value from 3 to 10 was investigated for their influence on the foam fractionation efficiency (shown in Fig. 4.1). The other parameters were kept constant: caffeine initial concentration, 1×10^{-5} M; saponin, 25 mg/100ml; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃.

The results obtained were all statistically analyzed using SPSS statistic software to compare the means.
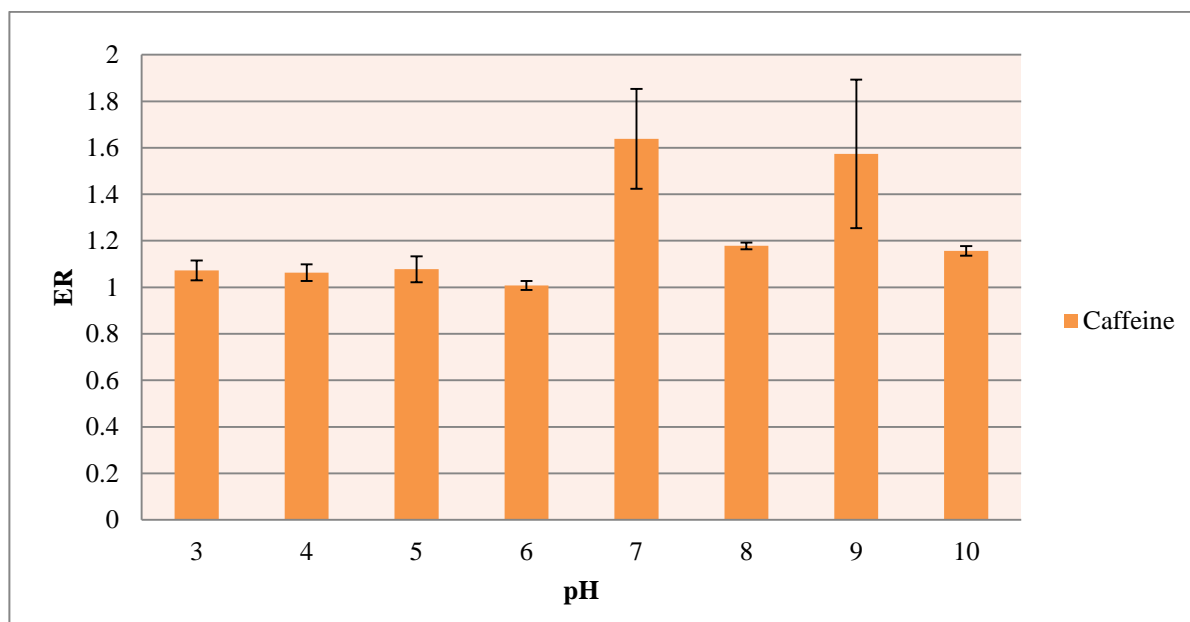
Figure 4. 1 pH-dependent enrichment ratio (ER) of caffeine in the foam fractionation process of caffeine

As shown in Fig. 4.1, all the values of enrichment ratio obtained at different pH values are around the value of 1, which means that almost no enrichment was achieved during these

foam fractionation experiments. The comparison of the values by statistical analysis shows that higher values of enrichment ratio were all obtained under alkaline condition: 1.64 at pH 7 and 1.57 at pH 9. Moreover, the values obtained at a pH value above 7 are not different statistically.

Generally, the pH value of the solution will determine the solubility of the molecules in the aqueous solution, by influencing the charge properties of the functional groups in the molecules. As mentioned before, the solubility of the molecules would be at its minimum at the pH value of isoelectric point (pI), since the net charge of the molecule reaches at zero. It has been proved by a lot of researchers that high foam fractionation efficiency of the molecules, especially proteins, achieved at this pH point. In the molecule of caffeine, the N atom at position 9 that is not bound to $-CH_3$ group has a planar geometry, of which the lone electron pair is not delocalized into the ring system. As a result of this special N atom, caffeine can act as an electron donor, or a Lewis base, and is more inclined to dissolve into a solution of acidic. At a pH value of alkaline in the present experiment, the solubility of caffeine in water decreased or even reached its minimum; therefore, a better result for foam fractionation was obtained. However, the solubility of caffeine in water may not fluctuate significantly along with the variation of the pH value, which makes the enrichment ratio of caffeine changed only slightly at different pH values.

4.1.2.2   Influence of saponin amount

Since the concentration of surfactant may affect the foam fractionation efficiency, foaming experiments with saponin amount in a range of 12.5 mg/100ml to 50 mg/100ml were conducted. The other parameters were kept constant: caffeine initial concentration, 1×10^{-5} M; pH, 7.0; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown below (Fig. 4.2).

In the range of the surfactant concentration adopted in these series of experiments, stable foam with a proper size was generated. Here the results show us apparently that the enrichment ratio is higher at a low surfactant concentration. However, the statistical analysis indicated that the separation efficiency is not influenced significantly by the variation of the surfactant concentration.

Normally, the stability of the foam depends largely on the surface activity of the surfactant, or on the number or the concentration of the giving surfactant. The bubbles with more

surface-active surfactants or higher concentration of the giving surfactant will form more stable foam. However, extra stable foam inclines to prevent the occurrence of the drainage process by reducing the collapse and coarsening of the bubbles. Therefore, low enrichment ratio of foam fractionation can be caused by excessive foam stability. In these foaming experiments, too stable of the foam as a reason of low separation efficiency was excluded, since collapse was clearly observed. Then, we concluded that the caffeine molecules could not be attached on the surface of the bubbles provided by the surfactant molecules, but flowed back along with the drainage to the bulk solution during the rising of the foam in the column, in the condition here provided.
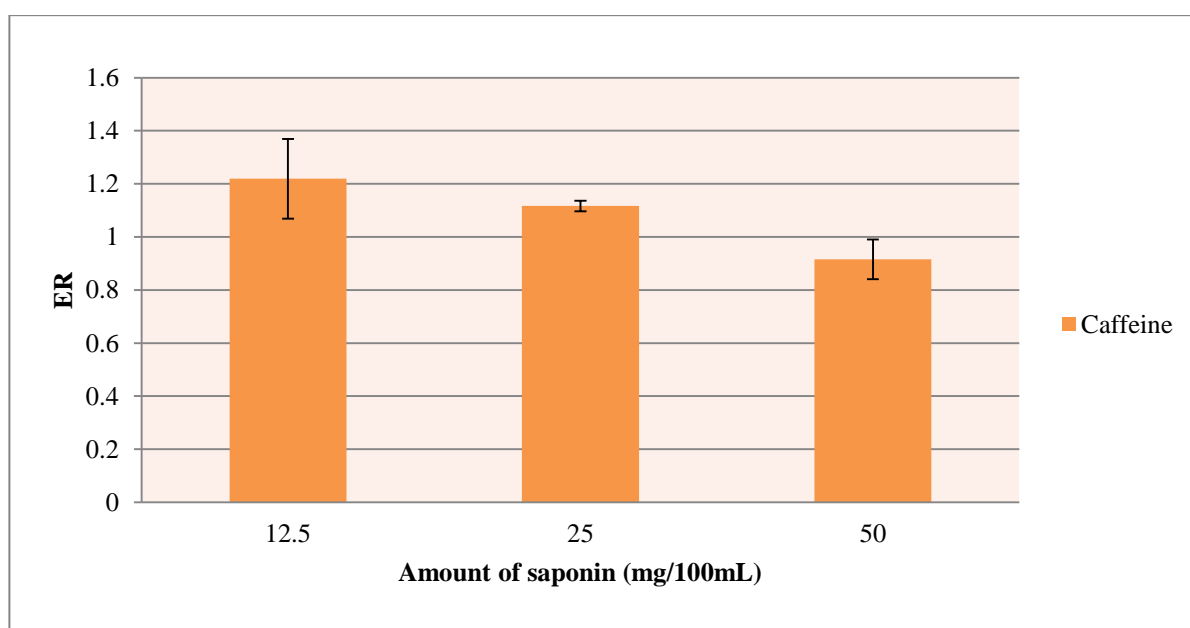
Figure 4. 2 Saponin amount-dependent enrichment ratio (ER) of caffeine in the foam fractionation process of caffeine

However, the experiments of foam fractionation of caffeine in aqueous solution should be continued, since it cannot be certain that the variation of the other parameters will not influence the separation behavior.

4.1.2.3   Influence of caffeine initial concentration

To investigate the influence of initial caffeine concentration on the foam fractionation efficiency, caffeine aqueous solution with a concentration of 1.0×10^{-4} M to 1.0×10^{-6} M was foamed, with the other parameters keeping constant: saponin amount, 25 mg/100ml; pH, 7.0;

flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 °C. The results are shown below in Fig. 4.3.

Figure 4. 3 Concentration-dependent enrichment ratio (ER) of caffeine in the foam fractionation process of caffeine

The enrichment ratio of the foaming experiments with an initial caffeine concentration of 1.0×10^{-6} M is shown to be most effective, proved by statistical analysis of the results.

In the process of foam fractionation, the separation efficiency is largely dependent on the initial concentration of the substances to be separated. Base on the former researches (Somasundaran 1972) (Robertson 1970) (Ahmad 1975) (Uraizee and Narsimhan 1996) (Karger and Devivo 1968), it is believed that a low concentration of the target substance, normally between 1.0×10^{-3} M to 1.0×10^{-7} M, is favorable for foam fractionation. Here our results seem to be constant with this 'principle', but it also suggests that the foam fractionation of caffeine was ineffective, since the concentration in the foam was almost the same with that in the bulk solution.

4.1.2.4   Influence of flow rate

Flow rate is also a very important parameter in foam fractionation technique. Two different values of flow rate were adopted in the following experiments to investigate its influence on the enrichment ratio of caffeine. The other parameters were kept constant: saponin amount, 25 mg/100ml; pH, 7.0; caffeine initial concentration, 1×10^{-5} M; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 °C.
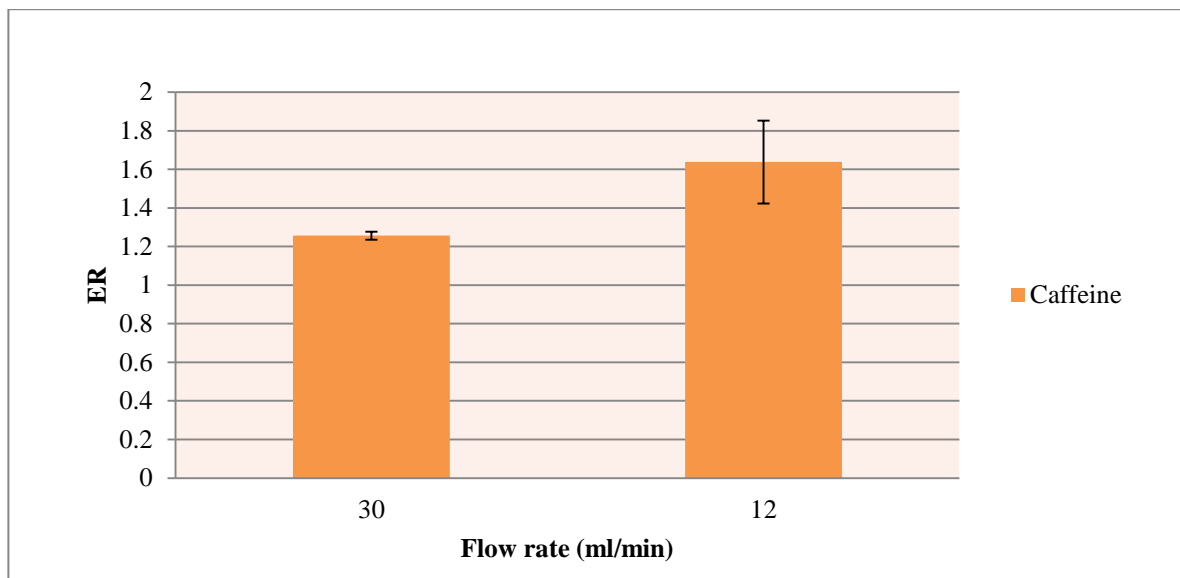
Figure 4. 4 Flow rate-dependent enrichment ratio (ER) of caffeine in the foam fractionation process of caffeine

The results shown in Fig. 4.4 indicated that although both of the values of enrichment ratio for caffeine are not ideal, the enrichment ratio at a flow rate of 12 ml/min is higher than that at a flow rate of 30 ml/min, which is also proved by the statistical analysis.

In the foam fractionation experiment, a low flow rate provides the foam more timespan to accomplish the processes of collapse and drainage, in which the molecules with weaker surface activity flow back to the bulk solution while the ones with a stronger surface activity incline to retain on the interface and finally accumulate in the foamate. Based on the knowledge we have learnt in the above sections, we know that caffeine molecule is not surface active, but easily flow back along with the drainage. Here, this view is confirmed once again by the present results, because of the slight enrichment of caffeine obtained. However, the importance of the flow rate as a parameter should be emphasized.

4.1.2.5 Influence of height of the column

The influence of the height of the column on foam fractionation was also investigated by varying the height from 9 cm to 38 cm in the following experiments. The other parameters were kept constant: caffeine initial concentration, 1×10^{-5} M; saponin, 25 mg/100ml; flow rate, 12 ml/min; pH, 7.0; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Fig. 4.5.
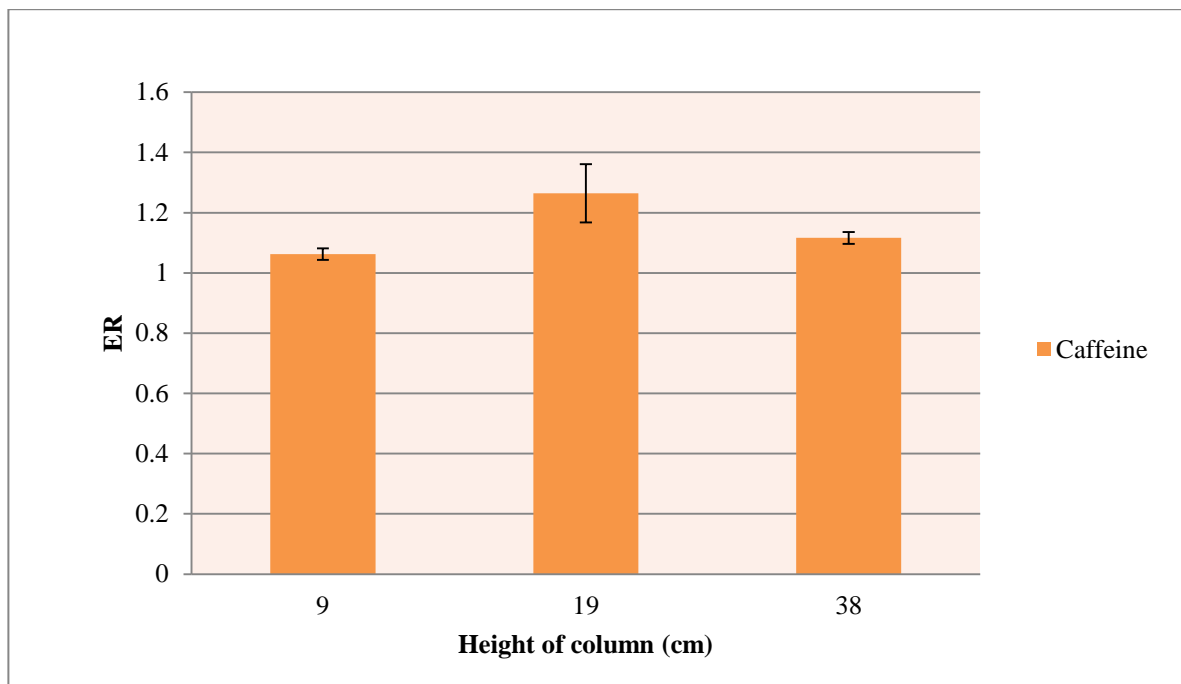
Figure 4. 5 Column height-dependent enrichment ratio (ER) of caffeine in the foam fractionation process of caffeine

A higher column for the foam fractionation provides longer time for the foam to rise up along the column, and thus the time for drainage is prolonged. As a result, less solvent is held in the foam and the substances retained in the foam would be more concentrated.

Here in our foaming separation experiments, all the values of enrichment ratio obtained with a different column height are close to the value of 1, which means almost no enrichment was obtained. And also, the statistical analysis proved that all the results do not have a difference in statistic. Therefore, the variation of the height of the column does not have an influence on the foam efficiency of caffeine under the condition in the present experiment.

4.1.2.6   Influence of temperature

Temperature can also influence the efficiency of foam experiment, since the stability of the foam coated by surfactant varies at different temperature. Here, foam experiments with a varying bulk solution temperature from 1-2 ℃ to 40 ℃ were conducted. The other parameters were kept constant: caffeine initial concentration, 1×10^{-5} M; saponin, 25 mg/100ml; flow rate, 12 ml/min; pH, 7.0; height of the column, 38 cm; initial solution volume, 40 ml.
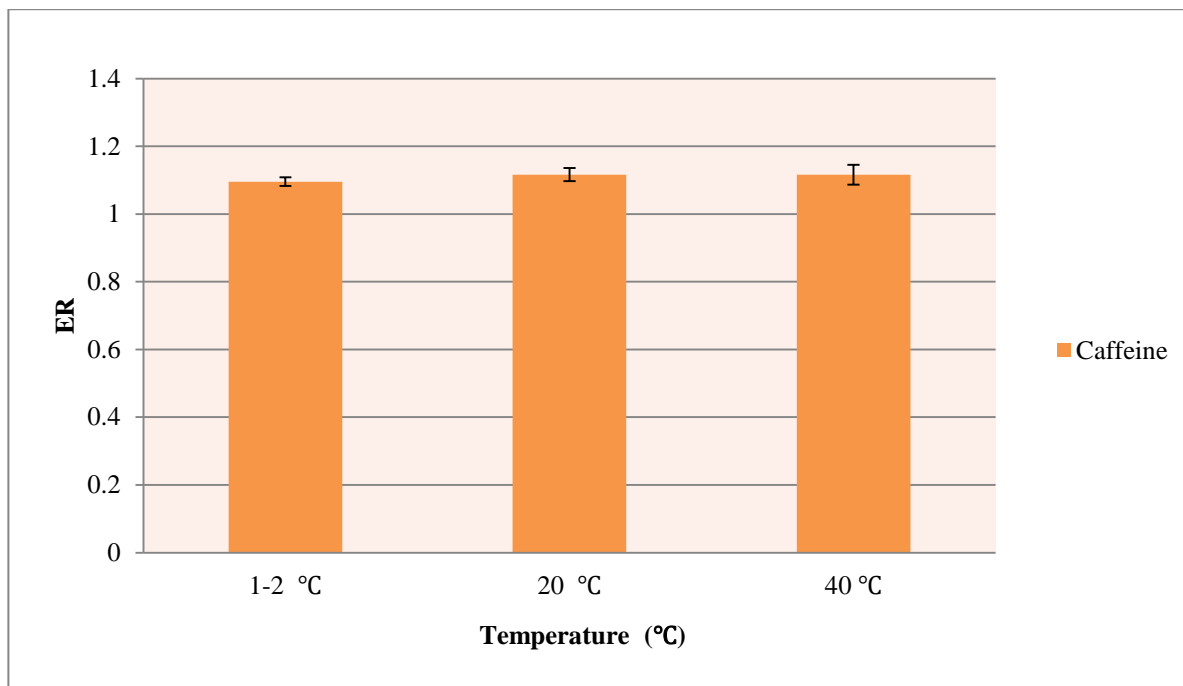
Figure 4. 6 Temperature-dependent enrichment ratio (ER) of caffeine in the foam fractionation process of caffeine

However, as can be seen in Fig. 4.6, a value around 1 was obtained for enrichment ratio in all the experiments conducted, which means no caffeine was enriched in the foam phase. Besides, the temperature did not influence the enrichment ratio of caffeine under the condition provided here, indicated by the results of the statistical analysis.

4.1.2.7   Influence of NaCl concentration

Foaming experiments with a NaCl concentration ranging from 0 to 2 g/100ml were conducted to investigate the influence of the amount of NaCl on the foaming efficiency. The other parameters were kept constant: caffeine initial concentration, 1×10^{-5} M; saponin, 25 mg/100ml; flow rate, 12 ml/min; pH, 7.0; height of the column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃.

As indicated in Fig. 4.7, the enrichment ratio of caffeine obtained at a NaCl concentration of 2 mg/100 ml is shown to be the highest. However, the results from statistical analysis proved that these values have no difference in statistic. Therefore, the enrichment ratio of caffeine did not change along with the variation of the NaCl concentration added into the foaming system.
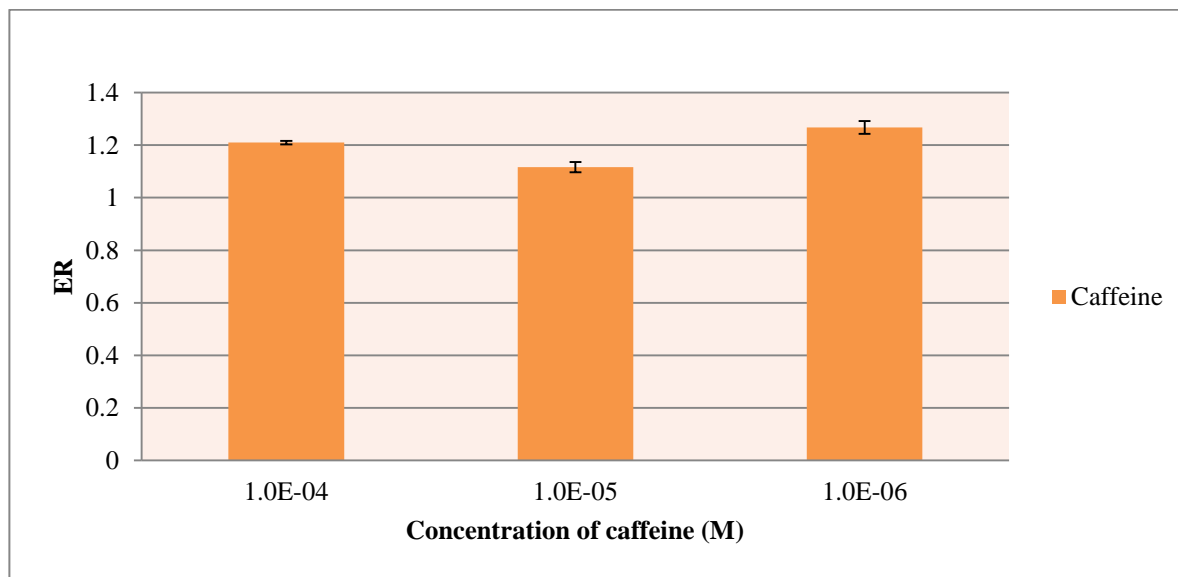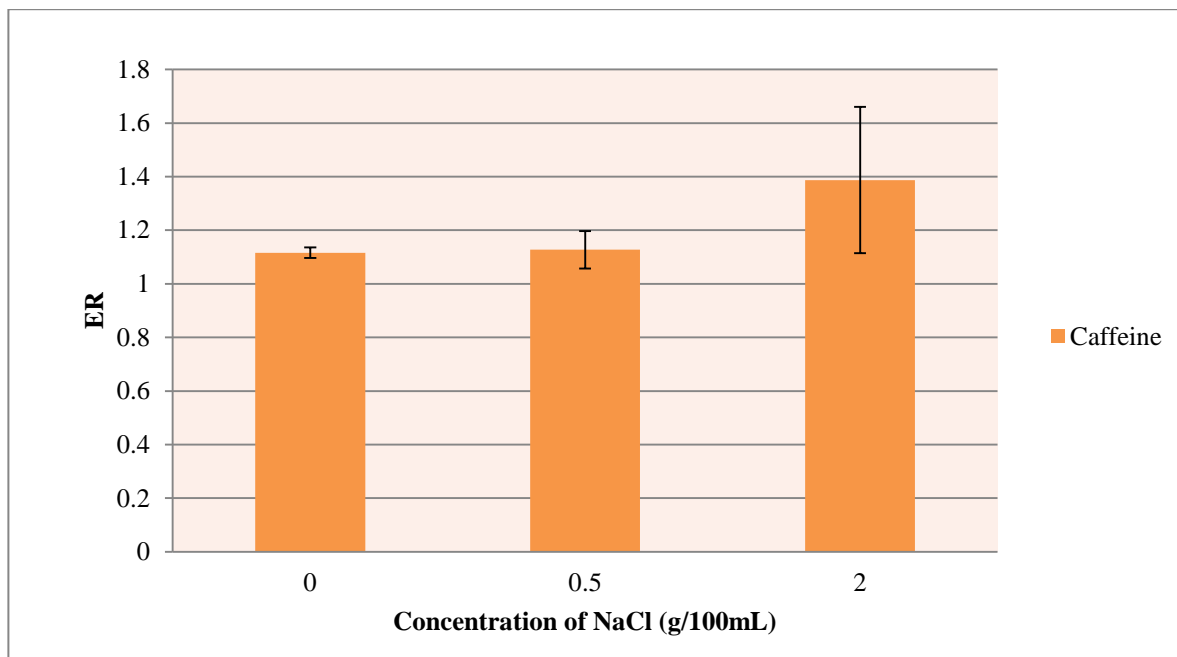
Figure 4. 7 NaCl concentration-dependent enrichment ratio (ER) of caffeine in the foam fractionation process of caffeine

4.1.2.8   Overall evaluation of the foam fractionation of caffeine

In all of the parameters investigated above, only three of them (pH value, caffeine initial concentration and flow rate) were shown to influence the foaming efficiency of caffeine slightly. Subsequent experiments adopting the values of these three parameters with a better performance were conducted to optimize the foam fractionation. However, the enrichment was not improved significantly ($ER = 3.01$), even with a longer column and a lower flow rate. Therefore, we come to the conclusion that caffeine in aqueous solution alone cannot be enriched effectively by foam fractionation under the present condition in this research.

Concerning the structure information of caffeine, we may find some reason for the inefficiency of foam fractionation for it.

Firstly, caffeine is a polar substance which contains two heterocyclic rings, a six membered pyrimidine ring and a five membered imidazole ring; secondly, caffeine possesses three hydrogen-bond acceptors: the imidazole nitrogen (position 9) and the two oxygen atoms (position 2 and 6); and last, there is no non-polar chains exist in the molecule of caffeine. All the three facts make caffeine fairly soluble in water and are more prone to stay in the water phase, but not at the bubble gas-liquid interface.

Further investigations were conducted afterwards using the complexation based foam fractionation, aiming to enhance the enrichment efficiency of caffeine from the aqueous solution.

4.2 Foam fractionation of caffeine-chlorogenic acid complex

4.2.1 Preparation of caffeine-chlorogenic acid complex

Caffeine-chlorogenic acid complex was obtained as a light yellow needle crystal. Twenty-four signals were found from the ^{13}C NMR spectrum (125 MHz, D_2O), corresponding to 24 carbon atoms in the complex ($12\times (-CH_3 + -CH) + 2\times (-CH_2) + 10\times (-C) = 24$ (C)). The chemical shifts of carbon atoms and hydrogen atoms from the complex were also compared with that from the monomer caffeine (Sitkowski, Stefaniak et al. 1995) (Kan, Borer et al. 1980) and chlorogenic acid molecules (Nakatani, Kayano et al. 2000) (Chan, Lim et al. 2009) (Morishita, Iwahashi et al. 1984) (Shi, Zhao et al. 2008), and chemical shift differences were noticed (See Fig. 4.8 and 4.9; Table 3 and 4). For example, the chemical shift of hydrogen atoms from $-CH_3$ at position 1 in caffeine shifts from 3.37 to 3.19 in the complex, the ones at position 3 shifts from 3.55 to 3.36, and the ones at position 7 shifts from 4.01 to 3.82; however, the chemical shift of the hydrogen at position 8 shifts to an opposite direction: from 7.58 in the monomer to 7.76 in the complex (See the values in bold and italic in Table 3 and also the simulated NMR spectrum in Figure 4.10, the other differences are not described here in details). These shifts (both in values and directions) of the chemical shift of the hydrogen in caffeine moiety indicate that their chemical environment is changed because of the formation of the complex. The complex was further confirmed by comparison of the ^{1}H-NMR spectrum with the data reported, since our results are completely consistent with the ones reported (D'Amelio, Fontanive et al. 2009).
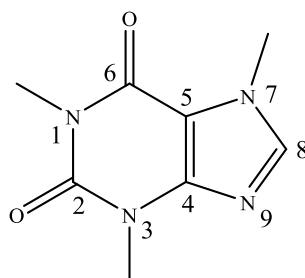
Figure 4. 8 The chemical structure of caffeine

Table 3 ^{1}H and ^{13}C NMR (D_2O) chemical shifts observed in the complex and the monomer molecules of caffeine (Sitkowski, Stefaniak et al. 1995)

Number of carbon in caffeine (Figure 4.8)	[13]C (δ/ppm)		[1]H (δ/ppm)	
	Complex	Monomer	Complex	Monomer
1-CH$_3$	29.259	27.5	*3.192*	*3.37*
2	153.424	151.3		
3-CH$_3$	31.2	29.3	*3.357*	*3.55*
4	149.232	148.3		
5	108.864	107.1		
6	157.026	154.9		
7-CH$_3$	34.751	33.2	*3.817*	*4.01*
8	144.534	141.2	*7.762*	*7.58*

Table 4 ^{1}H and ^{13}C NMR (D_2O) chemical shifts observed in the complex and the monomer molecules of chlorogenic acid (Nakatani, Kayano et al. 2000)

Number of carbon in chlorogenic acid (Figure 4.9)	[13]C (δ/ppm)		[1]H (δ/ppm)	
	Complex	Monomer	Complex	Monomer
1	78.316	75.4		
2	39.964	36.7	2.049, 2.247 (m, H$_{eq}$ and H$_{ax}$)	2.20 (dd, J=4, 15 Hz, H$_{ax}$); 2.13 (m, H$_{eq}$)
3	72.686	73.0	5.363 (ddd, J=11.5, 6.5, 5 Hz)	5.34 (ddd, J=3, 3, 4 Hz)
4	74.404	74.8	3.915 (dd, J=3.5, 10 Hz)	3.63 (dd, J=3, 9 Hz)
5	72.251	68.3	4.295 (dd, J=3.5, 6.5 Hz)	4.14 (ddd, J=3, 9, 9 Hz)
6	38.787	41.5	2.085, 2.192 (m, H$_{ax}$ and H$_{eq}$)	1.95 (dd, J=9, 14 Hz, H$_{ax}$); 2.13 (m, H$_{eq}$)
1'	127.727	127.9		
2'	115.405	115.1	6.837 (d, J=2 Hz)	7.04 (d, J=2 Hz)
3'	145.534	146.79		
4'	148.340	149.4		
5'	116.944	116.4	6.715 (d, J=8 Hz)	6.76 (d, J=8 Hz)
6'	123.381	122.9	6.803 (dd, J=2, 8 Hz)	6.93 (dd, J=2, 8 Hz)
7'	146.923	146.80	7.364 (d, J=16 Hz)	7.58 (d, J=16 Hz)
8'	115.610	115.8	6.187 (d, J=16 Hz)	6.30 (d, J=16 Hz)
9'	170.292	169.0		
-COOH	182.279			

Note: H$_{ax}$, axial protons; H$_{eq}$, equatorial protons.
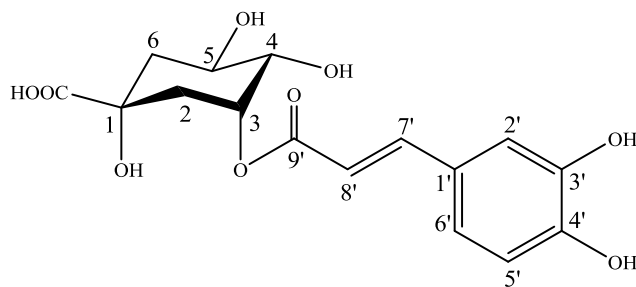
Figure 4. 9 The chemical structure of chlorogenic acid
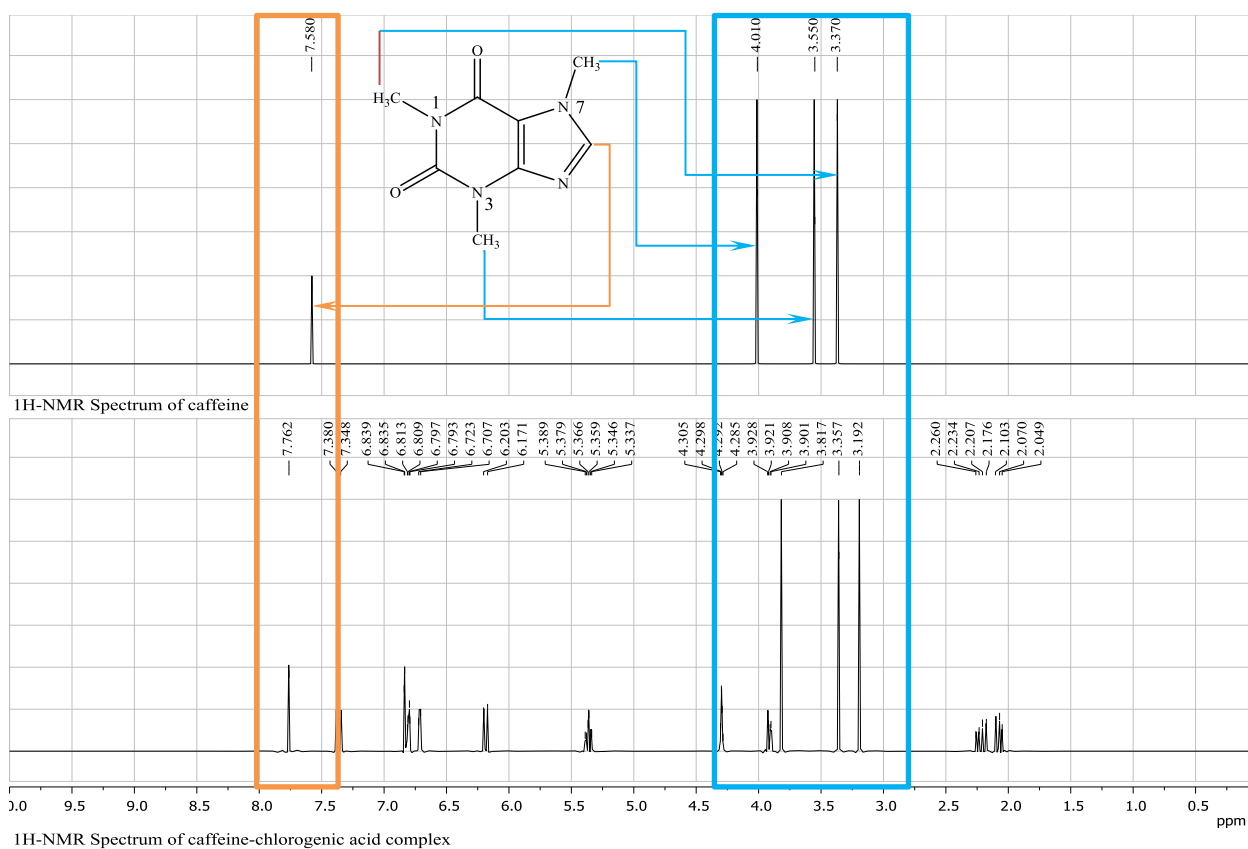
Figure 4. 10 Visualized comparison of the ^{1}H NMR chemical shifts in caffeine moiety from both monomer caffeine and the complex (These spectra are not the real ones but simulated from the real data, using MestReNova 9.0.1 for Windows, MestreLab research)

The HPLC analysis indicated that this complex is not stable in the separation condition adopted here but decomplexed to the monomers; however, it proved that the ratio of caffeine and chlorogenic acid is exactly 1 to 1 in molar in this complex (see Figure 4.11).
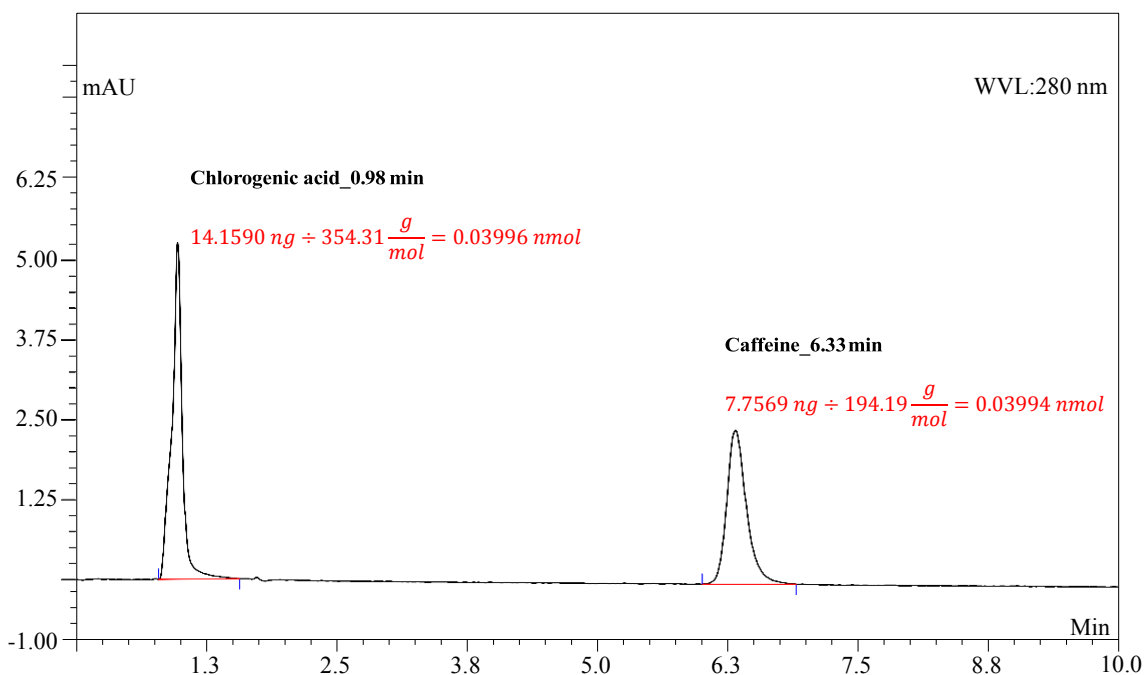
Figure 4. 11 HPLC analysis of caffeine-chlorogenic acid complex (the HPLC program is shown in the section of 3.2.7, but a different column was used here: Hamilton HxSil C 18, 150×4.6 mm, 5 μm)

4.2.2 Evaluation of the foamability and foam stability of caffeine-chlorogenic acid complex

Only negligible foam was produced by the aqueous solution of caffeine-chlorogenic acid complex at different concentrations, and the foam collapsed also very quickly because of the poor stability. The foamability of this aqueous solution did not showed any fluctuation along with the variation of the pH value. Therefore in order to make the solution of this complex foamable, a small amount of saponin was added before each foaming experiment.

4.2.3 Evaluation of the complexation of caffeine and chlorogenic acid in aqueous solution

The absorption spectra of chlorogenic acid titrated with caffeine are presented in Fig. 4.12, in the form of molar absorption coefficient (E_λ). Wavelength ranging from 320 nm to 350 nm was chosen as the band to reflect the absorption changes of chlorogenic acid only, as caffeine has negligible absorption over 320 nm. The bathochromic shifts (or red shifts) are clearly visible in these spectra, suggesting that aromatic chromophore interactions happened

between caffeine and chlorogenic acid molecules, and a new absorbing component (complex of caffeine and chlorogenic acid) appeared in the mixture. The presence of an isosbestic point at around 336 nm in the spectra indicates that two components of chlorogenic acid were predominantly present in the mixture of the titration solution: monomer of chlorogenic acid and the caffeine-chlorogenic acid complex.
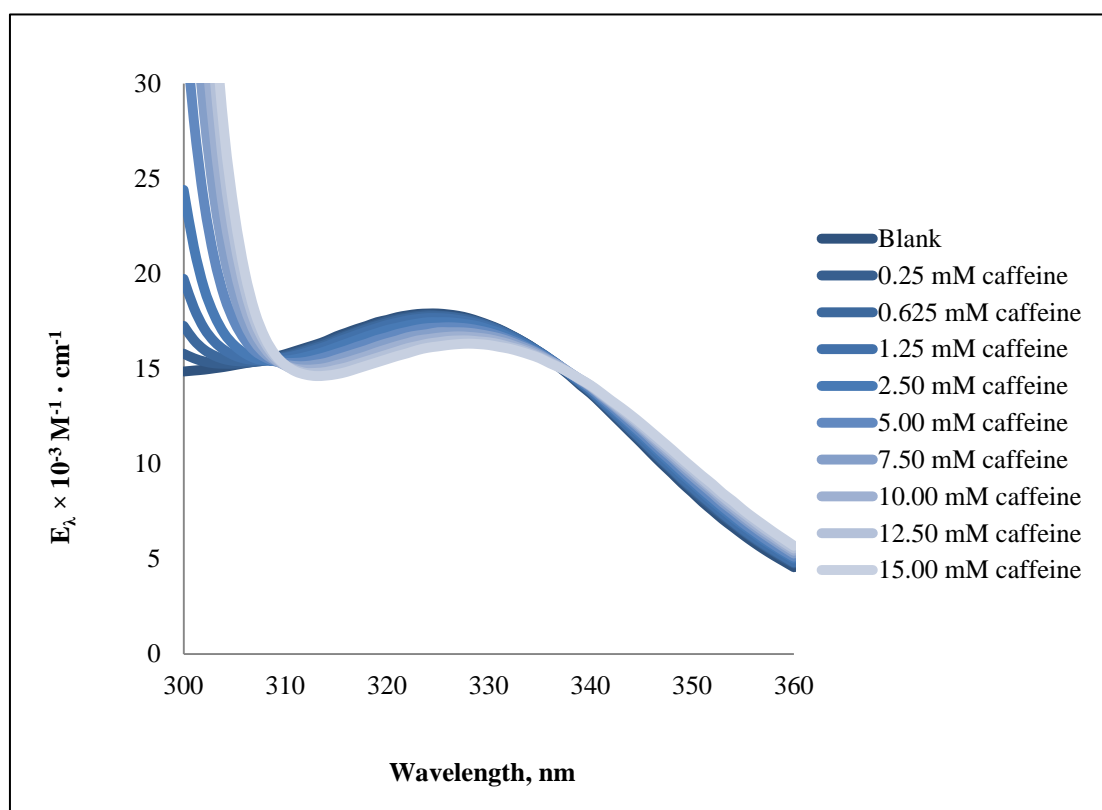
Figure 4. 12 Spectrophotometric titration of chlorogenic acid (initial concentration 0.042 mM) with caffeine (concentration ranging from 0.25 to 15 mM)

4.2.4   Foam fractionation efficiency

4.2.4.1   Influence of initial pH value

The influence of pH value on the efficiency of foam fractionation of caffeine-chlorogenic acid complex was investigated by varying the pH value from 3 to 7 in series of foaming experiments. At a pH value above 7, chlorogenic acid would be degraded, and even nonreversible when the pH value is above 10.5 (Friedman and Jurgens 2000). Therefore, the pH value above 7 was not adopted here. The other parameters were kept constant: the complex initial concentration, 1×10^{-5} M; saponin, 25 mg/100ml; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 °C. The results are

shown in Figure 4.13, and also, all the results were compared using SPSS statistical software (Table. 5).

As indicated from the results, the foaming efficiency of caffeine and chlorogenic acid both reached their maximum at pH 3, with an enrichment ratio of 3.95 and 3.68 respectively. And the significance of these values was proved by statistical analysis. At pH 4, the foaming efficiency of both caffeine and chlorogenic acid decreased to 1.5. After that, the enrichment ratios of the both increased gradually to the second high peak about 3 at pH 7.

What very interesting showing in Figure 4.13 is that the value of enrichment ratio for both caffeine and chlorogenic acid are very close to each other at each pH point, which means that they are sharing a similar fluctuation trend along with the varying values of pH. Therefore, we may come to the conclusion that the caffeine-chlorogenic acid complex can be enriched by foam fractionation technique here in this research. And the results here are also in accordance with the assumption that the complex formed between caffeine and chlorogenic acid is in the ratio of 1 to 1 in aqueous solution in former reports (Sondheimer, Covitz et al. 1961, Horman and Viani 1972).

Figure 4. 13 pH-dependent enrichment ratio (ER) of caffeine and chlorogenic acid in the foam fractionation process of caffeine-chlorogenic acid complex

Table 5 Statistical analysis of the enrichment ratio of caffeine (the upper one) and chlorogenic acid (the lower one), obtained from the pH-dependent foaming experiments ([a] Number of sample)

Caffeine		Subset for alpha = 0.05				
pH Value	N [a]	1	2	3	4	5
4.00	3	1.4719	1.4719			
5.00	3		1.6812	1.6812		
6.00	3			2.1650		
7.00	3				2.9340	
3.00	3					3.9480
Sig.		.058	.111	.083	1.000	1.000

Chlorogenic acid		Subset for alpha = 0.05		
pH Value	N	1	2	3
5.00	3	1.3658	1.3658	
4.00	3	1.3961	1.3961	
6.00	3		1.7377	
7.00	3			3.0964
3.00	3			3.6812
Sig.		.056	.191	.243

4.2.4.2   Influence of saponin concentration

Foam fractionation experiments with a different saponin concentration ranging from 12.5 mg/100ml to 50 mg/100ml were conducted to investigate the influence of saponin amount on the foaming efficiency of caffeine and chlorogenic acid. The other parameters were kept constant: pH value, 3; the complex initial concentration, 1×10^{-5} M; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Figure 4.14.

As indicated from Figure 4.14, a saponin concentration of 25 mg/100ml is supposed to be more beneficial for the foaming experiments of the complex, compared with the other two concentrations. However, the statistical analysis proved that there is no difference between the enrichment ratio obtained at 25 mg/100ml and 50 mg/100ml saponin amount (the results for statistical analysis were not shown here). The enrichment ratio obtained with a saponin amount of 12.5 mg/100ml was much lower than that of the others.

Generally speaking, the stability of the foam is very important for the foaming experiment. Unsteady foam will not be able to provide persistent and adequate gas-liquid interface for the absorption of the molecules in the solution, but burst constantly as the liquid drains from the bubble films. Therefore, to achieve an enrichment ratio acceptable, certain amount of surface active substance have to be added when the solution system is unable to produce adequate amount of foam. Here in the present experiment, a concentration of 12.5 mg/100ml saponin added is definitely not enough, since the foam observed was not steady and no enrichment was achieved, while 50 mg/100ml saponin seemed to be too much, since the enrichment ratio was not further increased compare with that of 25 mg/100ml saponin concentration.

Figure 4. 14 Saponin concentration-dependent enrichment ratio (ER) of caffeine and chlorogenic acid in the foam fractionation process of caffeine-chlorogenic acid complex

We also notice that the values of enrichment ratio for caffeine and chlorogenic acid obtained at each saponin concentration in all of the three sets of experiments are very close to each other, which may be regarded as an evidence for that caffeine and chlorogenic acid were enriched as a 1 to 1 (in molar) complex into the foam.

4.2.4.3   Influence of caffeine-chlorogenic acid initial concentration

An aqueous solution with caffeine-chlorogenic acid complex concentration ranging from 1×10^{-6} to 1×10^{-4} M was foamed to investigate their influence on the foaming efficiency. The

other parameters were kept constant: pH value, 3; saponin concentration, 25 mg/100ml; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Figure 4.15.

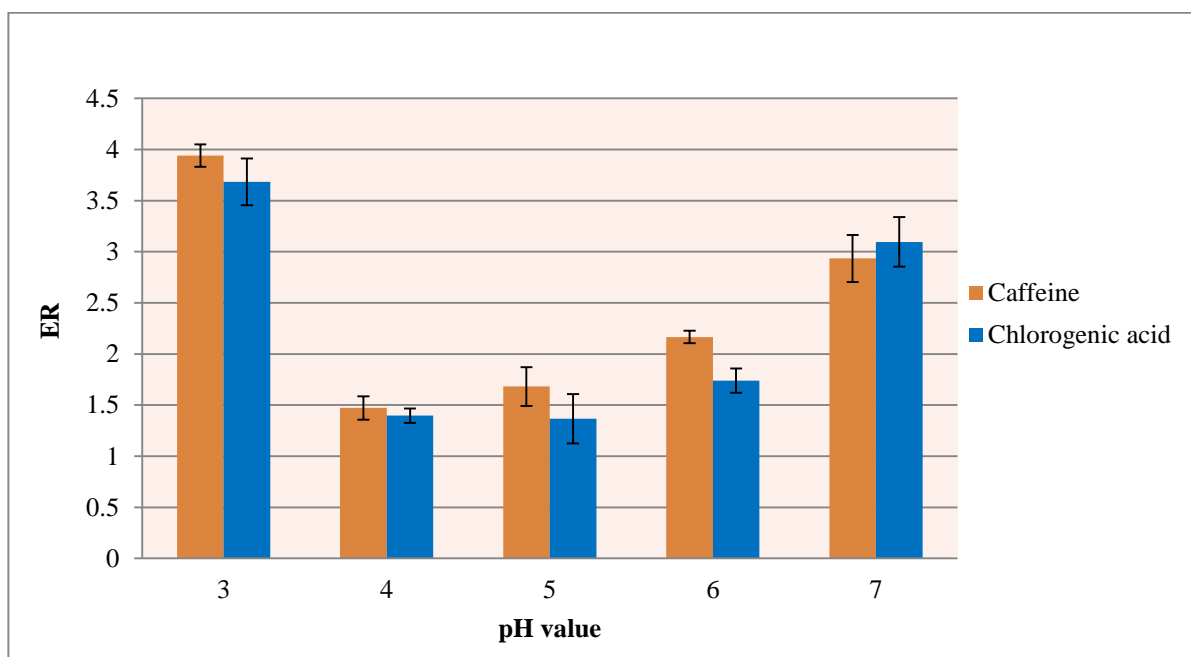**Figure 4. 15** Complex concentration-dependent enrichment ratio (ER) of caffeine and chlorogenic acid in the foam fractionation process of caffeine-chlorogenic acid complex

For caffeine, the enrichment ratio increased from 2.04 to 6.27 when its initial concentration decreased from 1×10^{-4} M to 1×10^{-6} M in the aqueous solution. For chlorogenic acid, similar results were observed: the enrichment ratio increased from 1.77 to 6.10 when the concentration decreased from 1×10^{-4} to 1×10^{-6} M. The results here prove again that a lower initial concentration of the target substances is more favorable for the foam fractionation.

4.2.4.4   Influence of gas flow rate

The gas flow rate ranging from 12 ml/min to 60 ml/min was also investigated for their influence on the foaming efficiency of caffeine-chlorogenic acid complex. The other parameters were kept constant: pH value, 3; saponin concentration, 25 mg/100ml; caffeine-chlorogenic acid complex initial concentration, 1×10^{-5} M; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Figure 4.16.

A low gas flow rate provided more time for drainage, therefore, the enrichment ratio for both caffeine and chlorogenic acid were in the trend of growth, when the flow rate decreased from 60 ml/min to 12 ml/min.

Figure 4. 16 Flow rate-dependent enrichment ratio (ER) of caffeine and chlorogenic acid in the foam fractionation process of caffeine-chlorogenic acid complex

4.2.4.5   Influence of height of the column

Foam experiments with a column height ranging from 9 cm to 38 cm were also conducted for its influence on the foaming efficiency of caffeine and chlorogenic acid. The other parameters were kept constant: pH value, 3; saponin concentration, 25 mg/100ml; caffeine-chlorogenic acid complex initial concentration, 1×10^{-5} M; flow rate, 12 ml/min; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Figure 4.17.

As expected, the enrichment ratio of both caffeine and chlorogenic acid increased gradually along with the growing column height from 9 cm to 38 cm. Therefore, it is quite possible that the foaming efficiency could be further increased with a column higher than 38 cm. Here in this experiment, the aim is to learn the importance of the column height in the foaming experiment of caffeine-chlorogenic acid complex. The experiments with a higher column were done afterwards in the final optimization test.

Figure 4. 17 Column height-dependent enrichment ratio (ER) of caffeine and chlorogenic acid in the foam fractionation process of caffeine-chlorogenic acid complex
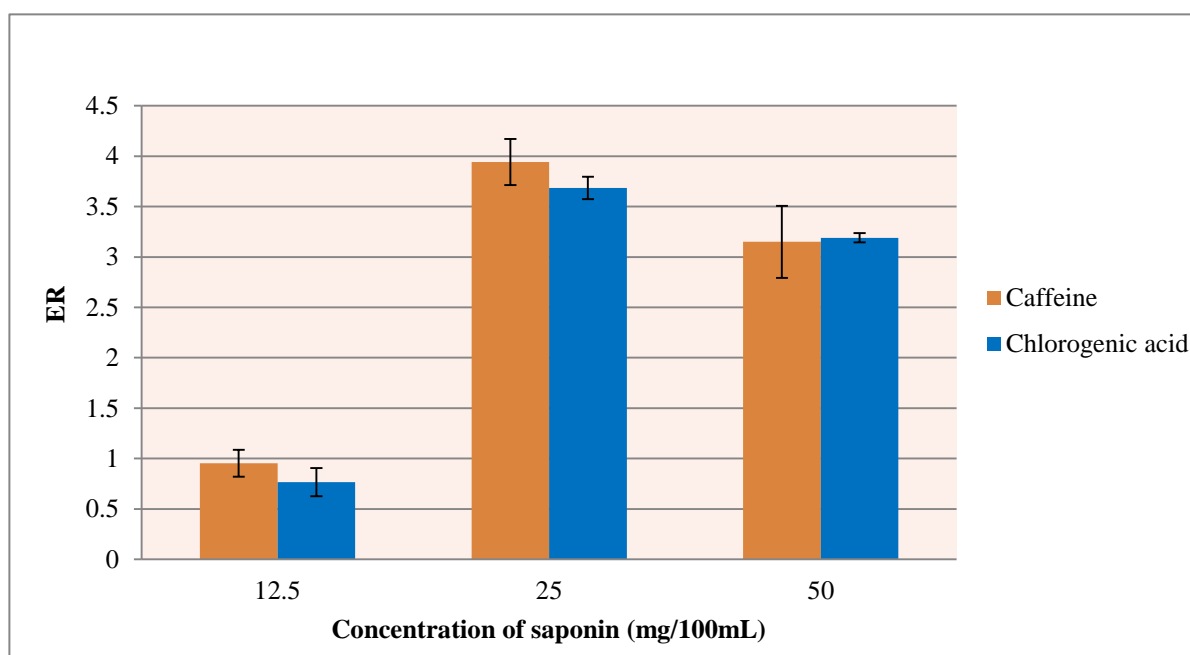
4.2.4.6 Influence of the temperature

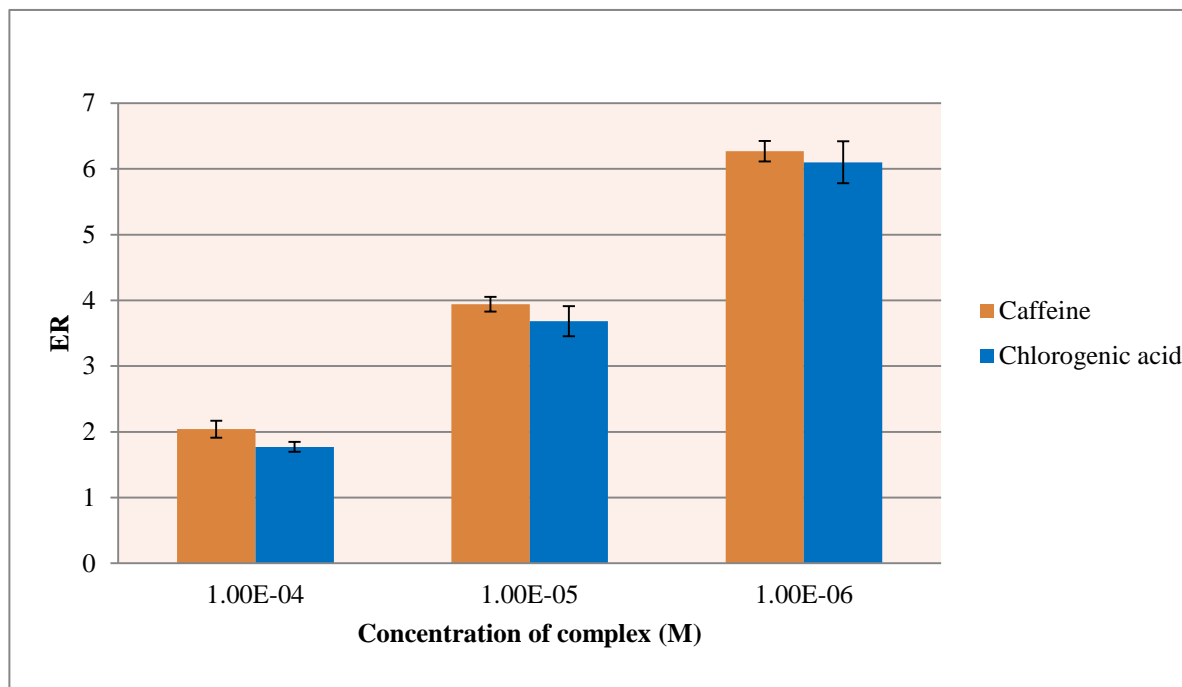**Figure 4. 18** Temperature-dependent enrichment ratio (ER) of caffeine and chlorogenic acid in the foam fractionation process of caffeine-chlorogenic acid complex

Temperature was also varied in the foam experiments here, since it may also influence the foaming efficiency of caffeine and chlorogenic acid. The other parameters were kept constant: pH value, 3; saponin concentration, 25 mg/100ml; caffeine-chlorogenic acid complex initial concentration, 1×10^{-5} M; flow rate, 12 ml/min; column height, 38 cm; initial solution volume, 40 ml. The results are shown in Figure 4.18.

As shown in Figure 4.18, a better enrichment ratio was obtained at 20 ℃ for both caffeine and chlorogenic acid, compared with that at 2 ℃ or 40 ℃. The effect of temperature on foam fractionation is complicated; since it may influence the stability of foam through many aspects, such as adsorption, surface elasticity and viscosity. At a lower operation temperature, such as at 2 ℃, the stability of the foam would be increased since the elasticity and viscosity of the solution are increased. As a result of this, the bursting and drainage would be decreased, which may depress the efficiency of foam fractionation. At a high temperature, such as 40 ℃, the stability of the foam may decrease, which is also not favorable for foam fractionation. Moreover, the complexation of caffeine and chlorogenic acid may be also influenced by temperature. As dominated by the π stacking interaction (Horman and Viani 1972, Martin, Lilley et al. 1986 b), the formation constant of the caffeine complex would be decreased at the condition of high temperature (Gattuso, Manfredi et al. 2011). Another force that also stabilizes the complex is the hydrogen bonding interaction (Horman and Viani 1972, Martin, Lilley et al. 1986 a, Martin, Lilley et al. 1986 b). High temperature increases the vibrations of the atoms, thus may weaken or even break the hydrogen bonds in the complex. Therefore, the low enrichment ratio at 40 ℃ was the result of the synergy influence of both foam stability and the stability of the complex.

4.2.4.7   Influence of NaCl concentration

As a parameter which may also influence the foaming efficiency, the initial solution with different NaCl concentration ranging from 0 g/100ml to 5 g/100ml was foamed. The other parameters were kept constant: pH value, 3; saponin concentration, 25 mg/100ml; caffeine-chlorogenic acid complex initial concentration, 1×10^{-5} M; flow rate, 12 ml/min; column height, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Figure 4.19.
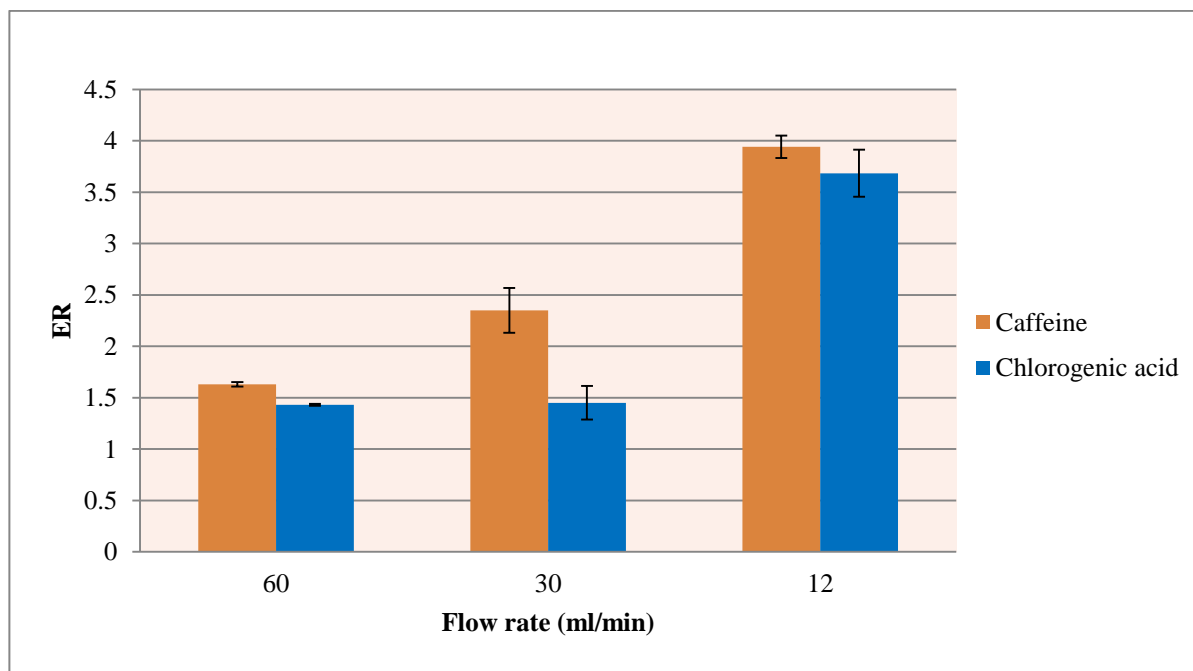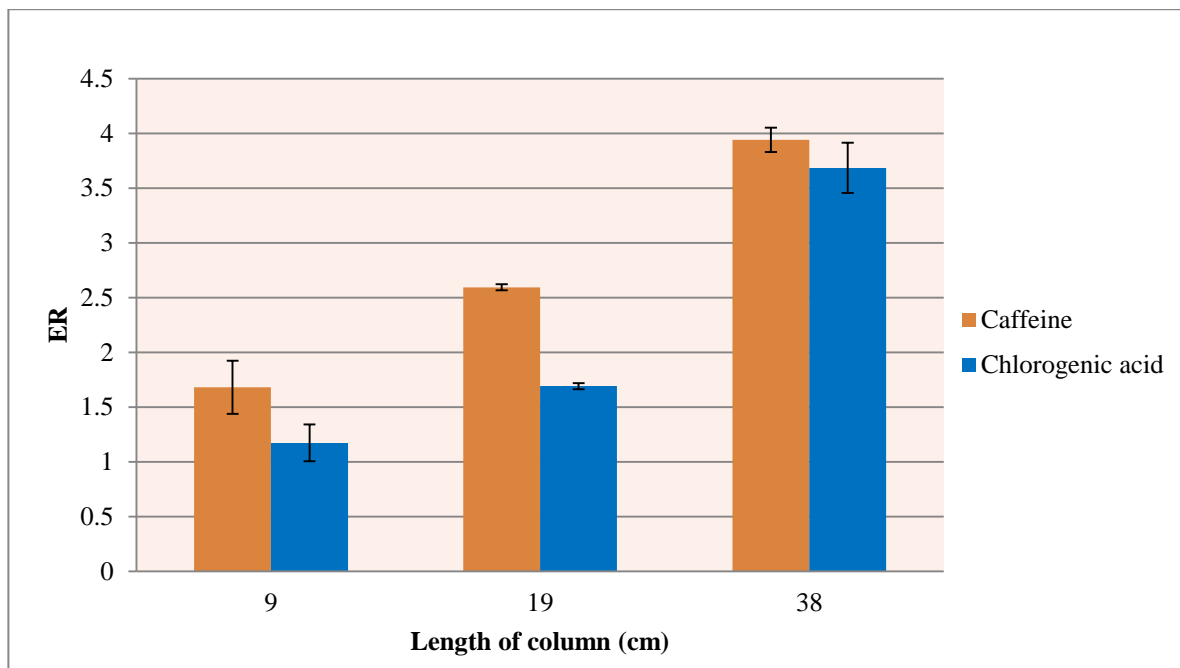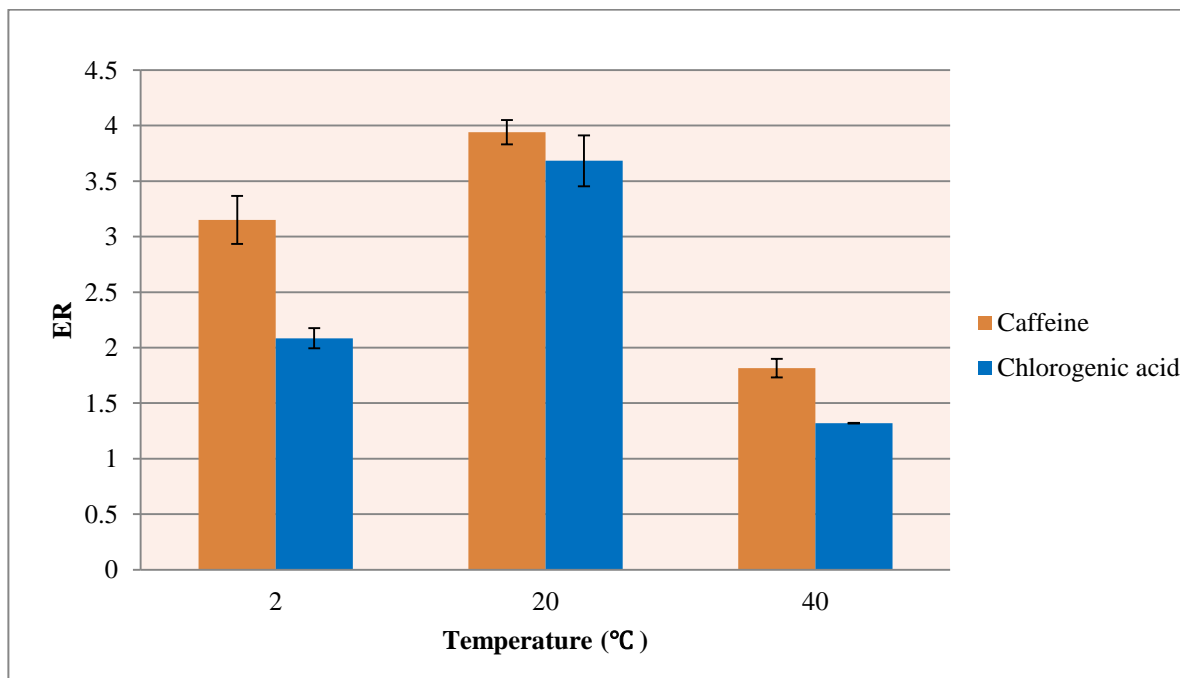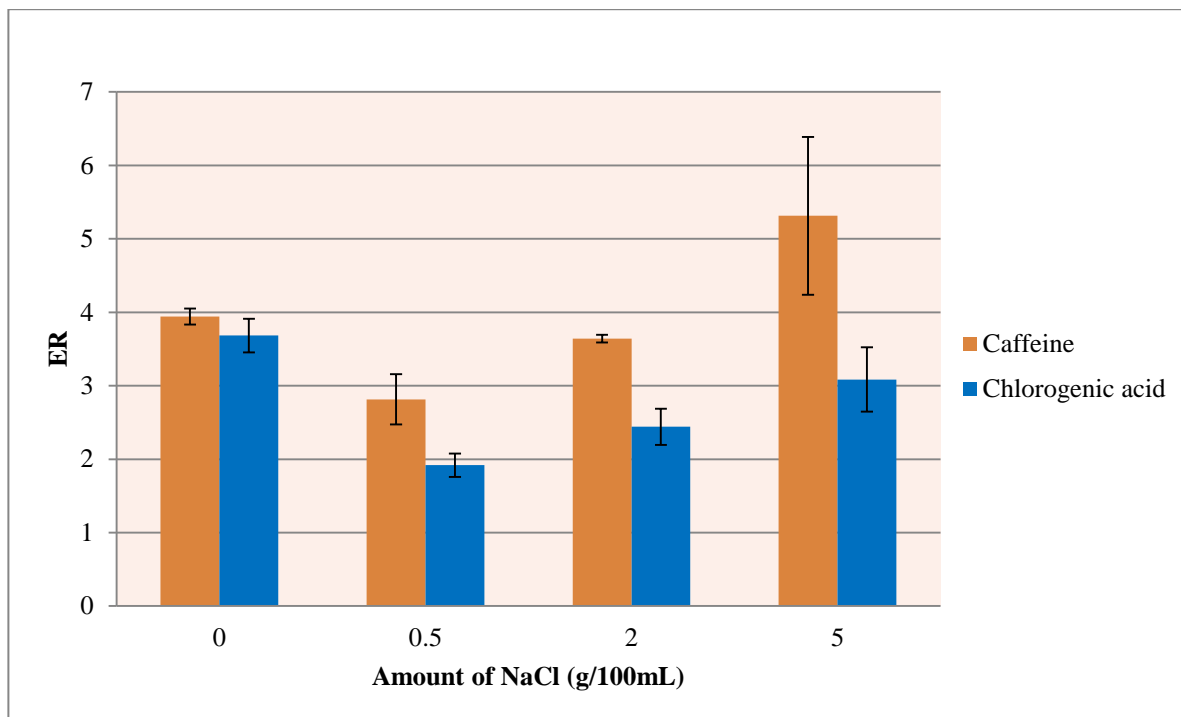
Figure 4. 19 NaCl amount-dependent enrichment ratio (ER) of caffeine and chlorogenic acid in the foam fractionation process of caffeine-chlorogenic acid complex

As shown in Figure 4.19, the enrichment ratio of both caffeine and chlorogenic acid decreased when small amount of NaCl (0.5 g/100ml) was added into the initial solution. After that, the enrichment ratio of both was increased gradually when more NaCl (2 g/100ml and 5 g/100ml) was added. Meanwhile, we noticed that the gap of the enrichment ratio between caffeine and chlorogenic acid was increased along with the growing amount of NaCl.

Beside the π stacking interaction, hydrogen bonding is also thought to be responsible for the stabilization of the complex (Horman and Viani 1972, Martin, Lilley et al. 1986 a, Martin, Lilley et al. 1986 b). High concentration of ions in the aqueous solution will interfere with the formation of the hydrogen bond, and hence decrease the amount of the complex. Meanwhile, a high concentration of ions will also enhance the adsorption of the components at the gas-liquid interface, which would increase the enrichment ratio of both caffeine and chlorogenic acid. Here the present results suggested that the foam efficiency was affected by these two effects simultaneously along with the increasing amount of NaCl: the enrichment ratio of caffeine and chlorogenic acid as complex was reduced because of the concentration of the complex was decreased resulting from the interference of the hydrogen bonding by an low ionic concentration; the enrichment ratio of caffeine and chlorogenic acid as single

substance was increased because of their enhanced absorption to the gas-liquid interface at a higher ionic concentration. Therefore, the final results showing here is the summation of these two effects: the former one dominated before the NaCl reaching a concentration of 0.5 g/100ml, while the later one dominated at the higher concentration.

4.2.4.8   Overall evaluation of the foam fractionation of caffeine-chlorogenic acid complex

As the main interaction in caffeine-chlorogenic acid complex, π stacking and hydrogen bonding are predominantly stabilizing this complex in aqueous solution. Therefore, all the factors, such as pH value, temperature and ionic strength, which may influence the stability of these interactions, would influence the stability of the complex, and finally determine the foaming efficiency of this complex or caffeine and chlorogenic acid.
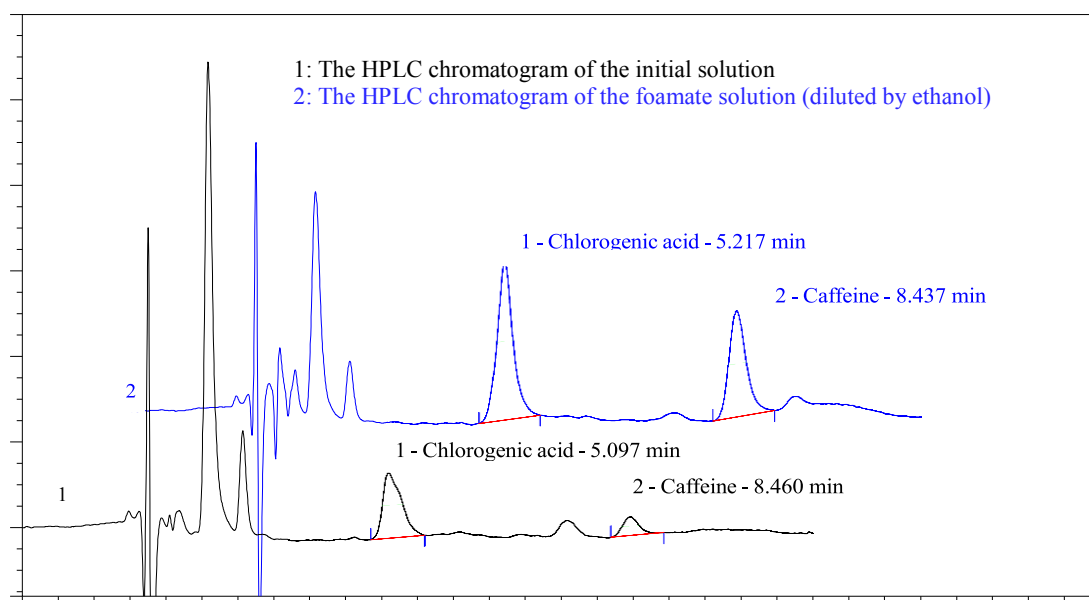
Figure 4. 20 HPLC analysis of the initial (in black) and foamate (in blue) solution of caffeine-chlorogenic acid complex.

Here in the present foaming experiments, all the parameters, namely pH value, saponin amount, caffeine-chlorogenic acid initial concentration, flow rate, column height, temperature and NaCl concentration, are all proved to have an influence on the foaming efficiency of this complex. Therefore, further experiments combining all the parameters with a better separation result were conducted. The parameters were as follows: pH value, 3; saponin amount, 25 mg/100ml; caffeine-chlorogenic acid initial concentration, 1×10^{-6} M; flow rate, 12 ml/min; column height, 60 cm, temperature, 20 °C and NaCl concentration, 5

g/100ml. After a foaming of 45 min, an enrichment ratio of 11.22 and 7.29, a recovery rate of 10.4 and 9.1 for caffeine and chlorogenic acid were obtained, respectively. As indicated in Figure 4.20, the concentration of caffeine and chlorogenic acid in the foamate solution is obviously higher than that in the initial solution.

4.3   Foam fractionation of caffeine and n-octyl caffeate

4.3.1   Synthesis of n-octyl caffeate

n-Octyl caffeate (Figure 4.21) was obtained as a light yellow needle crystal, with a yield of 41.5%. The NMR data are shown as follows: ^{1}H NMR (500 MHz, MeOD) δ: 0.895 (3H, t, J=7.0 Hz, H-8′), 1.330 (10H, m, H-3′ – H-7′), 1.678 (2H, m, H-2′), 4.144 (2H, t, J=6.5 Hz, H-1′), 6.252 (1H, d, J=15.5 Hz, H-8), 6.760 (1H, d, J=8.0 Hz, H-5), 6.932 (1H, dd, J=8.5, 2.0 Hz, H-6), 7.028 (1H, d, J=2.0 Hz, H-2), 7.520 (1H, d, J= 15.5 Hz, H-7); ^{13}C NMR (125 MHz, MeOD) δ: 14.534 (C-8′), 23.798, 27.160, 29.879, 30.464, 30.464, 33.061 (C-2′ – C-7′), 65.581 (C-1′), 114.903 (C-2), 115.027 (C-8), 116.388 (C-5), 122.992 (C-6), 127.574 (C-1), 146.813 (C-7), 146.825 (C-3), 149.602 (C-4), 169.403 (C-9). These data were also compared with those in literatures (Etzenhouser, Hansch et al. 2001, Nagaoka, Banskota et al. 2002, Jayaprakasam, Vanisree et al. 2006, Uwai, Osanai et al. 2008, Jaikang, Chaiyasut et al. 2011, Xiang, Su et al. 2011) to confirm the structure of the product. Important ESI-MS data are as follows (m/z, (fragment, %)): 291.00 ([M − H]$^-$, 100) in the ESI-MS negative mode; 315.20 ([M + Na]$^+$, 100) in the positive mode; 291.00 ([M − H]$^-$, 51), 178.80 ([M − H − C$_8$H$_{16}$]$^-$, 100), 160.80 ([M − H − OC$_8$H$_{16}$ − 2H]$^-$, 58), 134.80 ([M − H − COOC$_8$H$_{16}$]$^-$, 62) in the ESI-MS/MS negative mode. ESI-FT-ICR/MS for [M − H]$^-$ (C$_{17}$H$_{23}$O$_4$): calculated 291.15964, found 291.16018. All the original spectra are listed in Appendix.
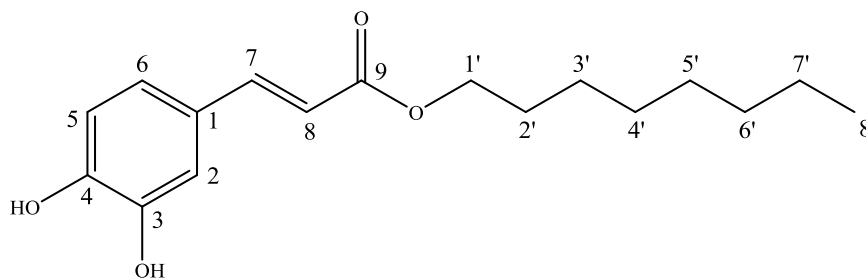
Figure 4. 21 The structure of n-octyl caffeate

4.3.2 Evaluation of the foamability and foam stability of n-octyl caffeate

8.7 mg n-octyl caffeate was dissolved in 10 ml DMSO to obtain a solution of 3×10^{-3} M, and then a diluted solution was prepared by adding distilled water into it. The foamability and foam stability of n-octyl caffeate under different pH values were determined using the method described in the former sections. The results are shown in Fig. 4.22. Similar experiments were also done using the diluted solution of n-octyl caffeate with a concentration ranging from 1×10^{-7} M to 1×10^{-3} M, at the pH value with a best foaming behavior obtained above. The results are shown below in Fig. 4.23.
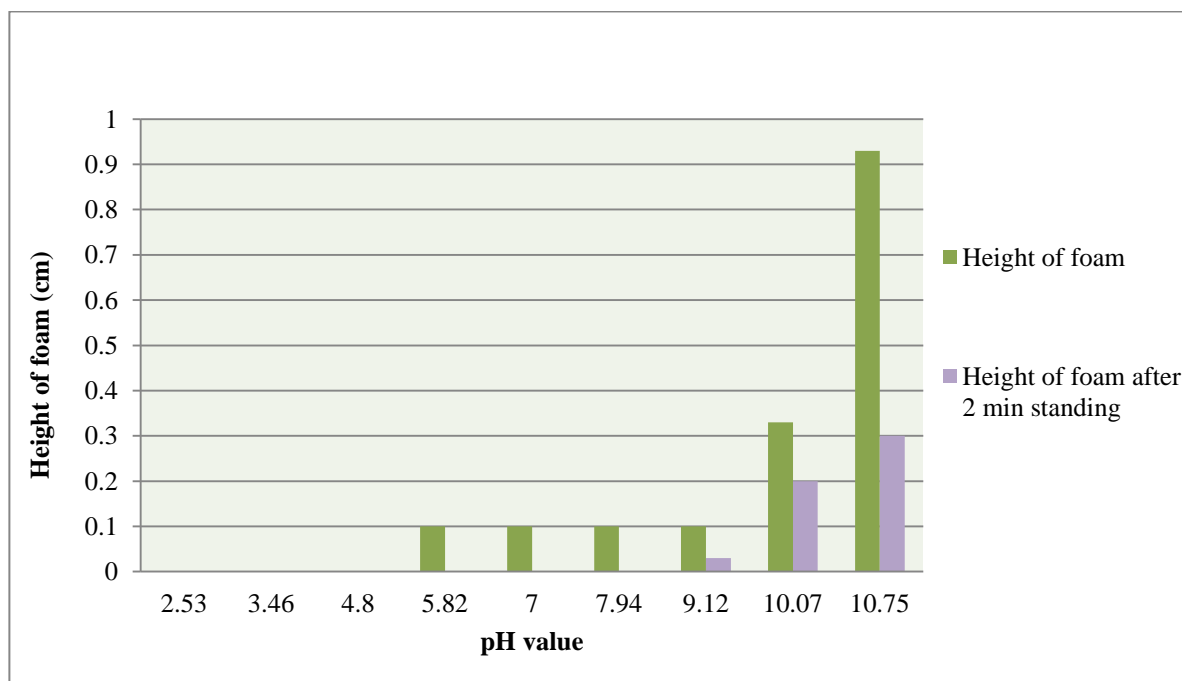
Figure 4. 22 Foam properties of n-octyl caffeate DMSO/water solution (concentration, 3×10^{-5} M), depending on the pH value

As shown in Fig. 4.22, both the foamability and foam stability of n-octyl caffeate with a concentration of 3×10^{-5} M were very weak except the ones at a pH value around 10. The foamability increased quickly from a height of 0.33 cm at pH 10.07 to 0.93 cm at pH 10.75; however the foam collapsed very soon, as indicated by the data of foam stability. The poor solubility of n-octyl caffeate at a pH value blow 7 was indicated by the tiny particles observed in the aqueous solution. Above pH 7, the color of the solution changed gradually from colorless to light yellow at pH 7.94, until light green at pH 10.75. The different

65

solubility of n-octyl caffeate at different pH values may explain the difference in the foamability and foam stability. Therefore, the poor foamability and foam stability could be a result of the poor solubility of n-octyl caffeate.
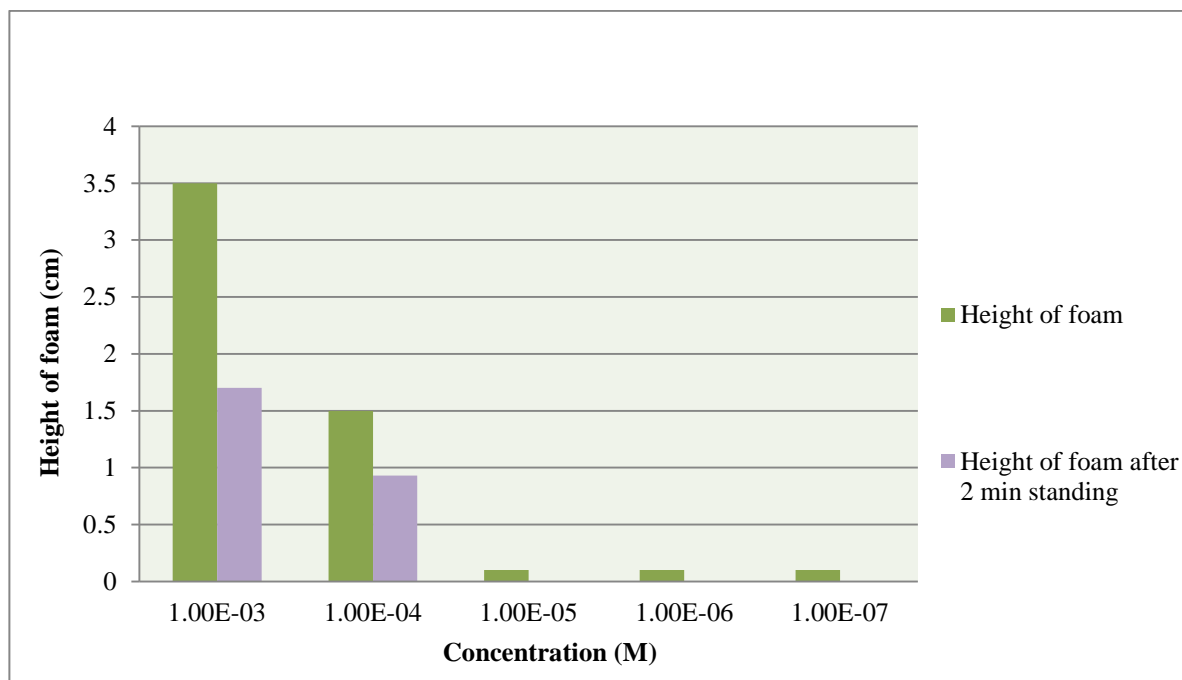
Figure 4. 23 Foam properties of n-octyl caffeate DMSO/water solution (pH 10.5), depending on the concentration

All the results shown in Fig. 4.23 were obtained at the pH value very close to 10.75, at which n-octyl caffeate was dissolved completely into the solution. The results here indicate that the foamability and foam stability of n-octyl caffeate was increased very significantly, when the concentration was higher than 1×10^{-4} M.

Even a higher concentration of n-octyl caffeate at a pH value above 7 increased the foam properties significantly; lower concentrations and pH values below 7 were adopted in the following experiment in this section, concerning its poor solubility in aqueous solution and poor stability at pH above 7.

4.3.3 Evaluation of the complexation between caffeine and n-octyl caffeate in aqueous solution

The absorption spectra of n-octyl caffeate titrated with caffeine are presented in Fig. 4.24, in the form of molar absorption coefficient (E_λ). Wavelength ranging from 320 nm to 350 nm was chosen as the band to reflect the absorption change of n-octyl caffeate only, as caffeine

has negligible absorption over 320 nm. The bathochromic shifts (or red shifts) are clearly visible in these spectra, suggesting that aromatic chromophore interactions happened between caffeine and n-octyl caffeate molecules, and a new absorbing component (complex of caffeine and n-octyl caffeate) appeared in the mixture. The presence of an isosbestic point at around 336 nm in the spectra indicates that two components of n-octyl caffeate are predominantly present in the mixture: monomer of n-octyl caffeate and the caffeine–n-octyl caffeate complex.
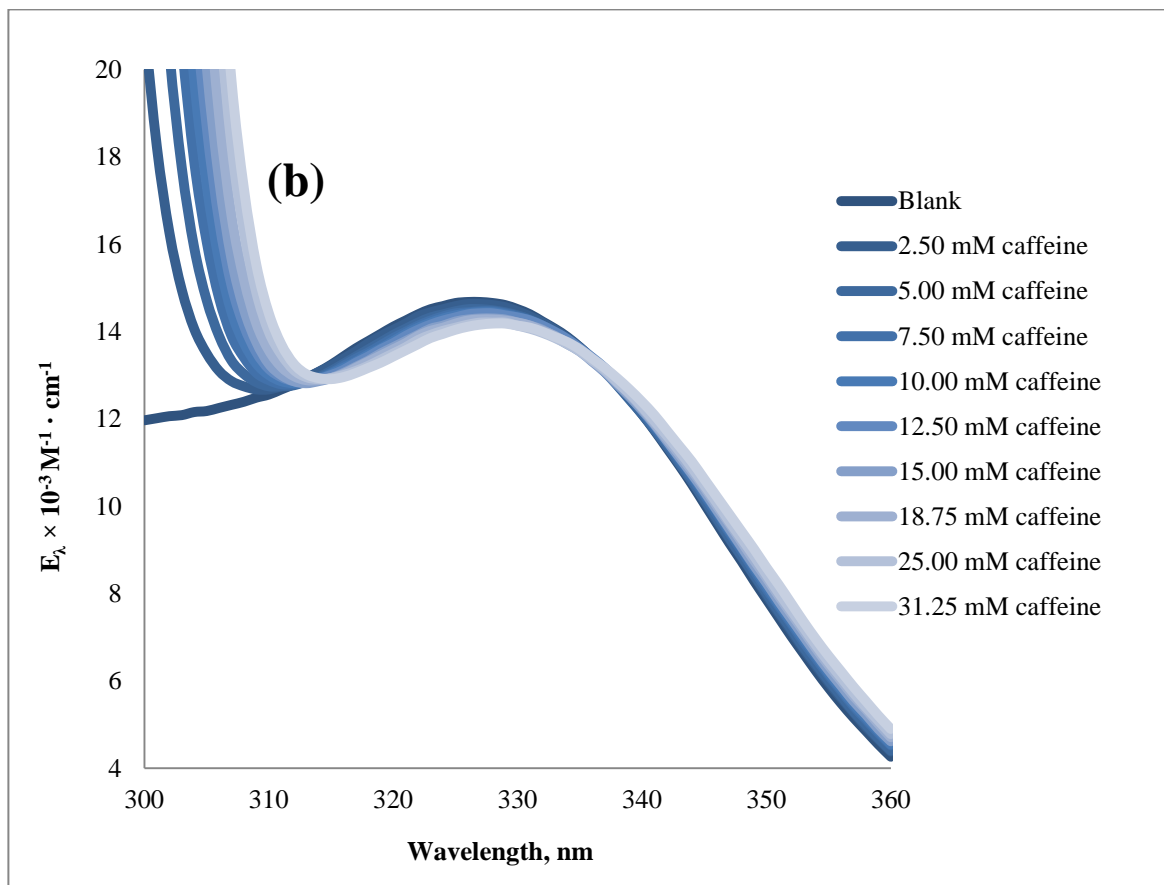
Figure 4. 24 Spectrophotometric titration of n-octyl caffeate (initial concentration 0.042 mM) with caffeine (concentration ranging from 2.5 mM to 31.25 mM)

4.3.4 Foam fractionation efficiency

In the following foaming experiments of caffeine and n-octyl caffeate binary aqueous solution, certain amount of saponin was also added before foaming, since n-octyl caffeate was not able to produce adequate foam at the conditions adopted below. Also, small amount of DMSO was added in the initial solution, in order to increase the solubility of n-octyl caffeate.

4.3.4.1 Influence of pH value

pH value as a parameter which may influence the foam efficiency was investigated by varying the pH value from 3 to 7 in the foaming experiments of caffeine and n-octyl caffeate binary aqueous solution. It was proved by former research (Friedman and Jurgens 2000) that compounds such as chlorogenic acid and caffeic acid that containing a caffeic acid based structure are not stable at pH value above 7. The HPLC analysis in our experiments also proved this (results not showing here), therefore, the pH value above 7 was not adopted here. The other parameters were kept constant: saponin concentration, 25 mg/100ml; caffeine and n-octyl caffeate initial concentration, 1×10^{-5} M; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Fig. 4.25.

As shown in Fig. 4.25, the values of enrichment ratio of caffeine are all below 1, which means that no enrichment was achieved during the foaming experiments. The statistical analysis proved that these values at a pH value above 6 are higher than that at pH value below 6, which is similar to the results obtained from the foaming experiments of caffeine alone. Compared with caffeine, n-octyl caffeate showed very high separation efficiency, especially at pH 7. The huge gap in the enrichment ratio of caffeine and n-octyl caffeate indicated that these two substances were not transferred as a complex, but being separately under the condition described here.

Figure 4. 25 pH-dependent enrichment ratio (ER) of caffeine and n-octyl caffeate in their foam fractionation process

4.3.4.2 Influence of saponin amount

The influence of saponin amount on the foaming efficiency was also investigated using a saponin concentration from 12.5 mg/100ml to 50 mg/100ml in the experiments. The other parameters were kept constant: pH original, 5.8; caffeine and n-octyl caffeate initial concentration, 1×10^{-5} M; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Fig. 4.26.

Here, still no enrichment for caffeine was achieved in the foaming experiments, and statistical analysis proved that there was no difference between the values of enrichment ratio obtained under different saponin amount. For n-octyl caffeate, the enrichment ratio was decreased significantly along with an increasing amount of saponin in the initial solution, which should ascribe to the suppression of the drainage process by the generation of the wet foam at a high surfactant concentration.

Figure 4. 26 Saponin amount-dependent enrichment ratio (ER) of caffeine and n-octyl caffeate in their foam fractionation process

4.3.4.3 Influence of caffeine and n-octyl caffeate ratio

The influence of caffeine and n-octyl caffeate ratio on the foaming efficiency was also investigated by varying the ratio to 1:1, 1:0.2 and 1:5 in the foam experiments. Here the concentration of caffeine in the aqueous solution was not changed (1×10^{-5} M), but the

amount of n-octyl caffeate was increased or decreased. The other parameters were kept constant: pH original, 5.8; saponin amount, 25 mg/100ml; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 °C. The results are shown in Fig. 4.27.

The results here indicated that caffeine was not enriched at each ratio of caffeine and n-octyl caffeate, since all the values of enrichment ratio are below 1. Compared with caffeine, the enrichment ratio for n-octyl caffeate was much higher, especially an enrichment ratio of 51.35 at the caffeine and n-octyl caffeate ratio of 1:5.

Figure 4. 27 Caffeine/n-octyl caffeate ratio-dependent enrichment ratio (ER) of caffeine and n-octyl caffeate in their foam fractionation process

4.3.4.4   Influence of flow rate

To investigate the influence of the flow rate on the foam efficiency of caffeine, experiments with different flow rate were conducted. The other parameters were kept constant: pH original, 5.8; saponin amount, 25 mg/100ml; caffeine and n-octyl caffeate initial concentration, 1×10^{-5} M; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 °C. The results are shown in Fig. 4.28.

Figure 4. 28 Flow rate-dependent enrichment ratio (ER) of caffeine and n-octyl caffeate in their foam fractionation process

The results indicate that caffeine was not enriched under the condition here in these experiments, and the statistical analysis proved that the enrichment ratio of caffeine did not change along with the varying flow rate. However, the separation efficiency of n-octyl caffeate was influenced by the flow rate dramatically: an enrichment ratio of 4.6 at the flow rate of 30 ml/min versus 41.8 at the flow rate of 12 ml/min. These results for n-octyl caffeate proved again that a low flow rate is beneficial for better separation efficiency.

4.3.4.5   Influence of column height

The height of the column was also varied in the following foaming experiments, to investigate its influence on the foam efficiency of caffeine and n-octyl caffeate. The other parameters were kept constant: pH original, 5.8; saponin amount, 25 mg/100ml; caffeine and n-octyl caffeate initial concentration, 1×10^{-5} M; flow rate, 12 ml/min; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Fig. 4.29.

The enrichment ratio obtained for caffeine was still below the value 1, and was not influenced by the fluctuation of the column height. Compared with caffeine, the enrichment ratio of n-octyl caffeate was increased significantly with an increasing height of column. The enrichment ratio of n-octyl caffeate did not increase much when the height of the column increased from 19 cm to 38 cm, which indicates that a height of 38 cm is approaching the optimal column height for the best separation efficiency of n-octyl caffeate.

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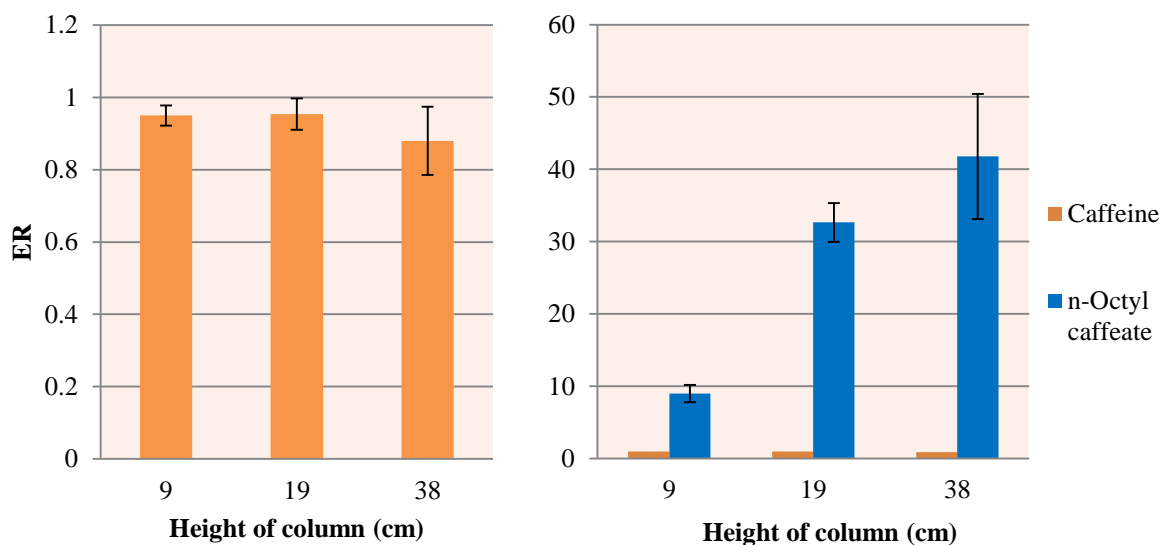
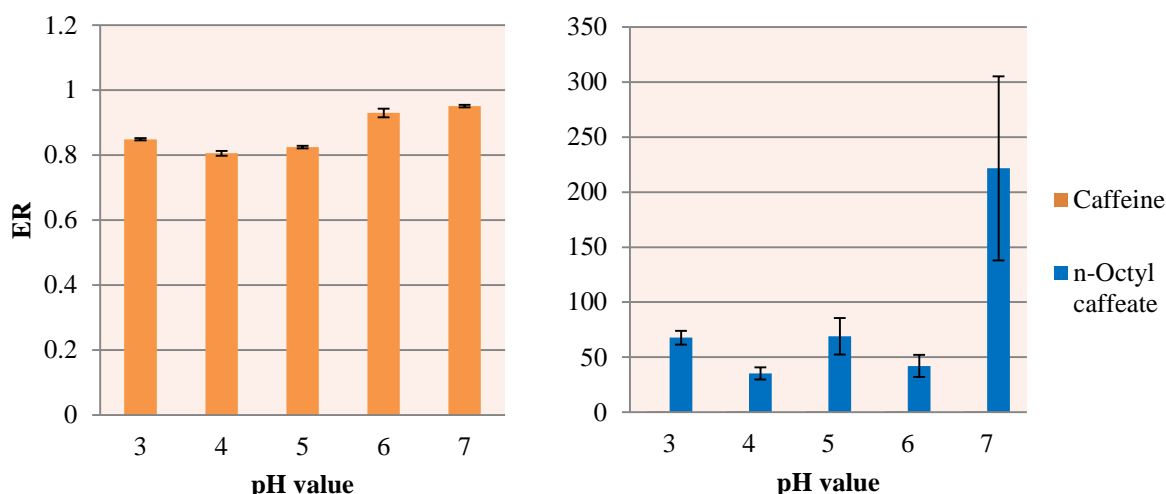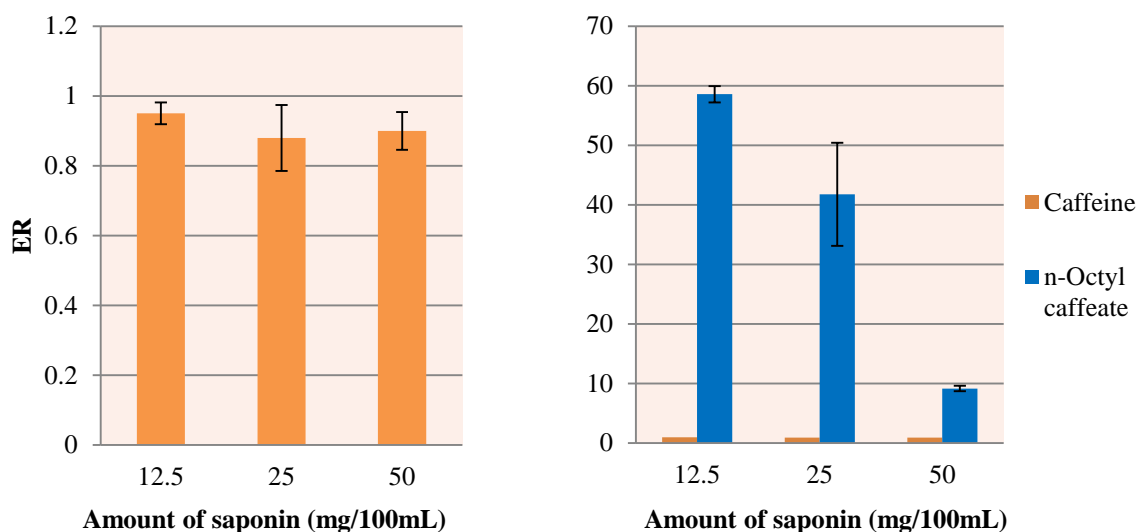**Figure 4. 29** Column height-dependent enrichment ratio (ER) of caffeine and n-octyl caffeate in their foam fractionation process

4.3.4.6 Influence of temperature

The temperature was also varied in the foaming experiments, to investigate its influence on the separation efficiency of caffeine and n-octyl caffeate. During the experiments, the other parameters were kept constant: pH original, 5.8; saponin amount, 25 mg/100ml; caffeine and n-octyl caffeate initial concentration, 1×10^{-5} M; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml. The results are shown in Fig. 4.30.

The results indicate that temperature did not influence the separation efficiency of both caffeine and n-octyl caffeate significantly, since the statistical analysis proved that the values of the enrichment ratio obtained were not different statistically. By the way, caffeine was not enriched at all, while n-octyl caffeate was enriched moderately.

4.3.4.7 Influence of NaCl amount

To investigate the influence of NaCl concentration on the foaming efficiency of caffeine and n-octyl caffeate, different amount of NaCl was added into the foaming experiments. The other parameters were kept constant: pH original, 5.8; saponin amount, 25 mg/100ml; caffeine and n-octyl caffeate initial concentration, 1×10^{-5} M; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Fig. 4.31.

72
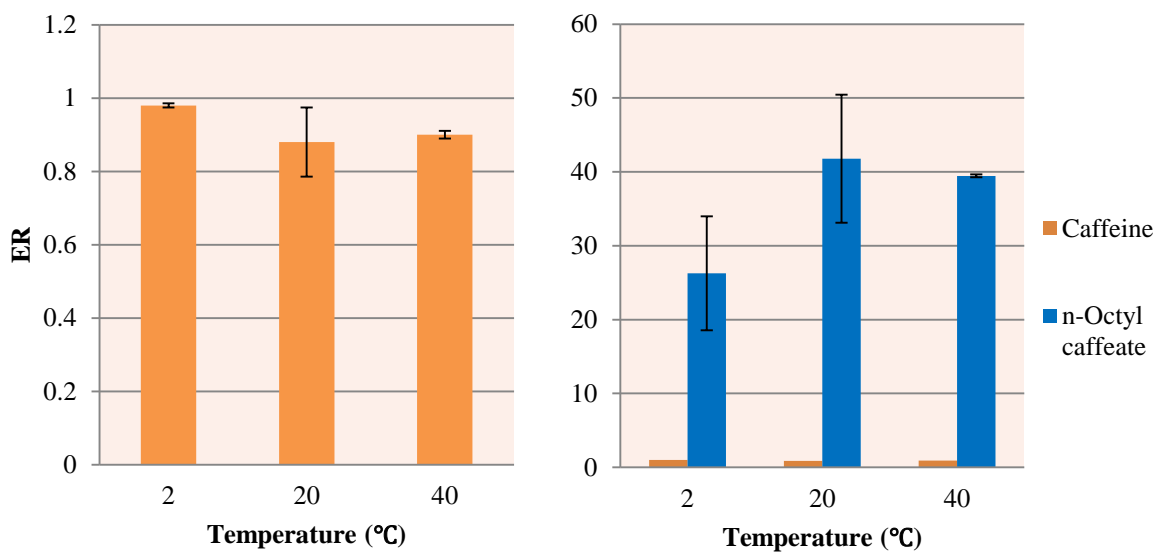
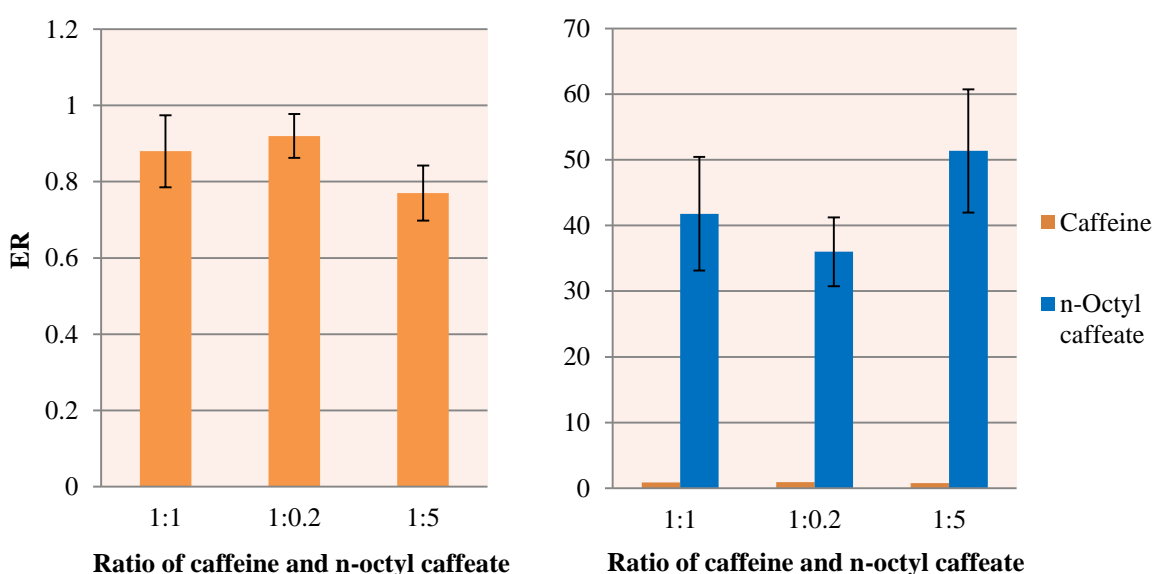**Figure 4. 30** Temperature-dependent enrichment ratio (ER) of caffeine and n-octyl caffeate in their foam fractionation process

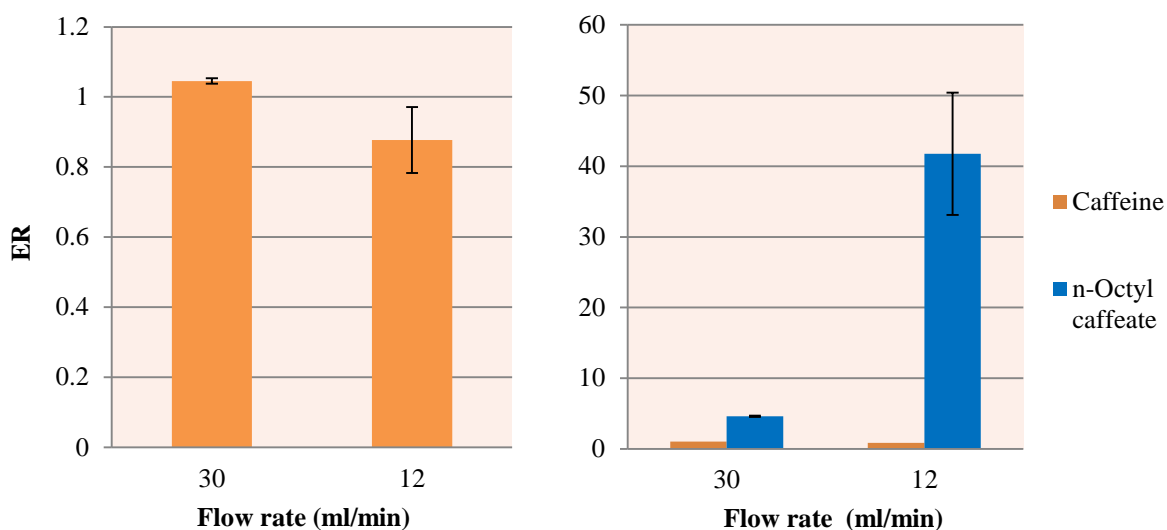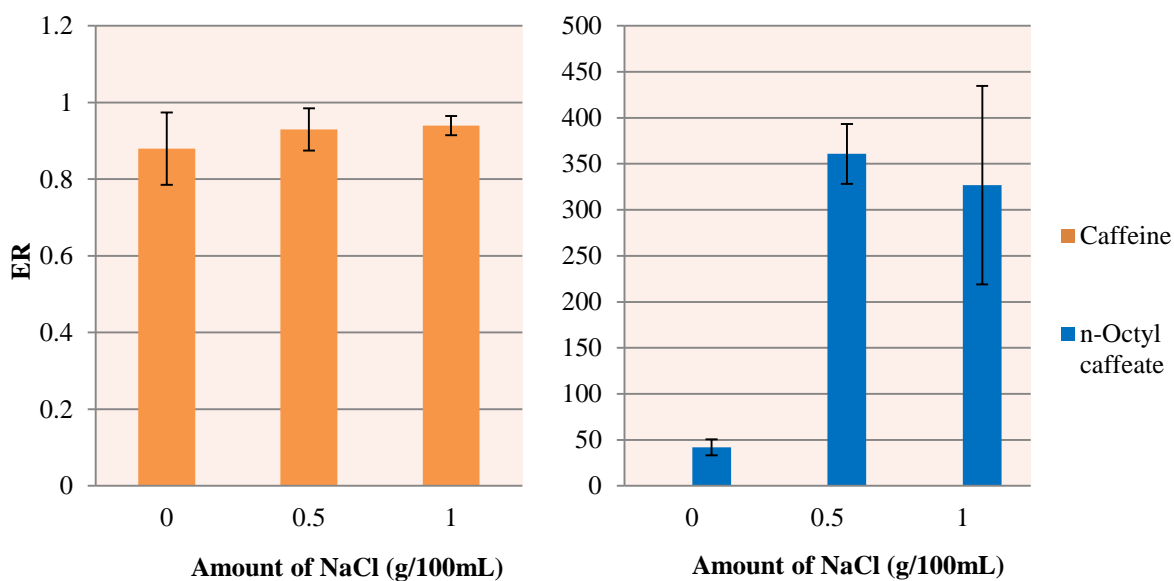**Figure 4. 31** NaCl amount-dependent enrichment ratio (ER) of caffeine and n-octyl caffeate in their foam fractionation process

As indicated in Figure 4.31, no enrichment was achieved for caffeine. In comparison, the enrichment ratio of n-octyl caffeate was greatly increased when a small amount of NaCl was added: an enrichment ratio of 360.81 at NaCl amount of 0.5 g/100ml, and 326.84 at NaCl amount of 1 g/100ml.

4.3.4.8   Overall evaluation of the foam experiments of caffeine and n-octyl caffeate binary aqueous solution

In all the experiments conducted here in the foam fractionation of caffeine and n-octyl caffeate from their binary aqueous solution, caffeine was not able to be enriched into the foam phase, and even worse results were obtained compared with the situation of caffeine foamed alone, while the enrichment of n-octyl caffeate was highly efficient. (An example of the HPLC chromatogram is shown in Figure 4.32; the change in size of the peaks from both caffeine and n-octyl caffeate in initial, residual and foamate solution indicates a huge difference of enrichment behavior of these two compounds.). Therefore, we come to the conclusion that caffeine and n-octyl caffeate cannot be enriched as a complex, and also the existence of n-octyl caffeate in the aqueous solution depressed the enrichment of caffeine instead of promoting it.

According to the results of the spectrophotometric titration of n-octyl caffeate with caffeine, the complex could surely be formed. The results obtained above may ascribe to two reasons: the amount of the complex formed is fairly low under the condition provided in each of the experiments here, since the complexation is influenced by temperature, pH value, and ionic strength and so on; the complex was dissociated in the process of foaming. Although we are not able to prove either of it, we may find some theory arguments to support our assumption here. Concerning the difference in the structure of chlorogenic acid and n-octyl caffeate, the quinic acid moiety in the former molecule is replaced by an alkane chain containing 8 carbons in the later one. This change may have two consequences: the stability of the complex is weakened, since it eliminate the hydrogen bonding contributed by quinic acid moiety in the complex, which was supposed to stabilize the complex (Horman and Viani 1972, Martin, Lilley et al. 1986 b); n-octyl caffeate was much more hydrophobic than chlorogenic acid, which makes it more favorable to be absorbed at the gas-liquid interface. Therefore, the complex of caffeine and n-octyl caffeate would be less stable and much easier to be decomposed in the foaming process, which brings high separation efficiency for n-octyl caffeate, but poor one for caffeine. Moreover, the competition of the adsorption site on the gas-liquid interface between caffeine and n-octyl caffeate results in even lower separation efficiency of caffeine compared with that of caffeine foaming alone.
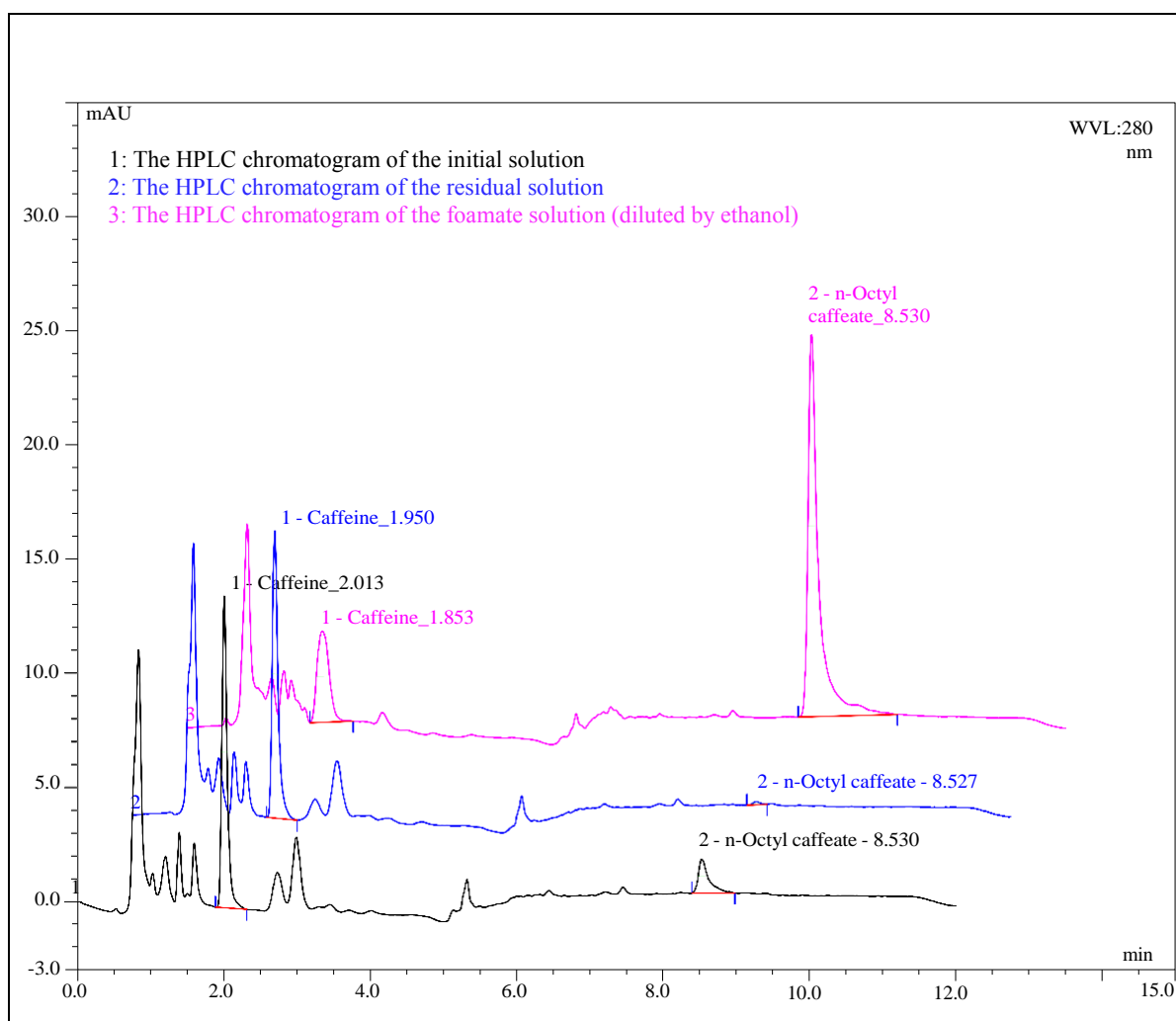
Figure 4. 32 HPLC analysis of the initial (in black), residual (in blue) and foamate (in purple) solution of caffeine and n-octyl caffeate binary system. The parameters adopted are as follows: pH original, 5.6; saponin concentration, 25 mg/100ml; caffeine and n-octyl caffeate initial concentration, 1×10^{-5} M; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃

4.4   Foam fractionation of caffeine and n-octyl caffeamide

4.4.1   Synthesis of n-octyl caffeamide

n-Octyl caffeamide (Figure 4.33) was obtained as a light yellow needle crystal, with a yield of 28.5%. The NMR data are shown as follows: ^{1}H NMR (500 MHz, MeOD) δ: 0.893 (3H, t, *J*=7.0 Hz, H-8′), 1.309 (10H, m, H-3′ – H-7′), 1.541 (2H, m, H-2′), 3.259 (2H, t, *J*=7.0 Hz, H-1′), 6.348 (1H, d, *J*=15.5 Hz, H-8), 6.745 (1H, d, *J*=8.5 Hz, H-5), 6.888 (1H, dd, *J*=8.5, 2.0 Hz, H-6), 6.989 (1H, d, *J*=2.0 Hz, H-2), 7.362 (1H, d, *J*= 15.5 Hz, H-7); ^{13}C NMR (125

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MHz, MeOD) δ: 14.519 (C-8′), 23.799, 28.127, 30.484, 30.501, 30.527, 33.073 (C-2′ – C-7′), 40.553 (C-1′), 114.827 (C-2), 116.339 (C-5), 118.189 (C-8), 122.107 (C-6), 128.159 (C-1), 142.113 (C-7), 146.730 (C-3), 148.773 (C-4), 169.215 (C-9). These data were also compared with those in literatures (Sattar, Glasl et al. 1990, Adam 1995, Nomura, Kashiwada et al. 2003, Yingyongnarongkul, Apiratikul et al. 2006, Roleira, Siquet et al. 2010, Xiang, Su et al. 2011, Trabelsi, Oueslati et al. 2014) to confirm the structure of the product. Important ESI-MS data are as follows (m/z, (fragment, %)): 290.00 ([M − H]$^-$, 100) in the ESI-MS negative mode; 314.20 ([M + Na]$^+$, 100), 330.20 ([M + K]$^+$, 100) in the positive mode; 290.00 ([M − H]$^-$, 61), 135.00 ([M − H − CONC$_8$H$_{16}$]$^-$, 100) in the ESI-MS/MS negative mode. ESI-FT-ICR/MS for [M − H]$^-$ (C$_{17}$H$_{24}$O$_3$N): calculated 290.17562, found 290.17617. All the original spectra are listed in Appendix.
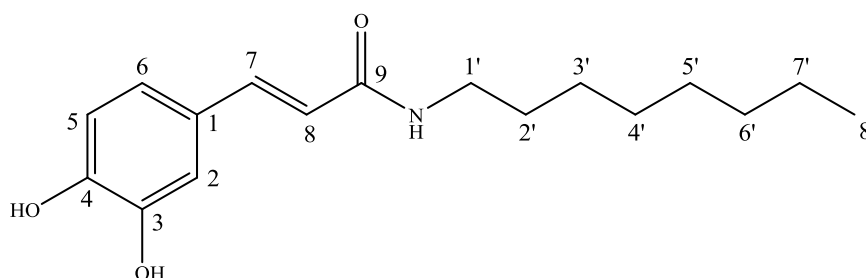
Figure 4. 33 The structure of n-octyl caffeamide

4.4.2 Evaluation of the foamability and foam stability of n-octyl caffeamide
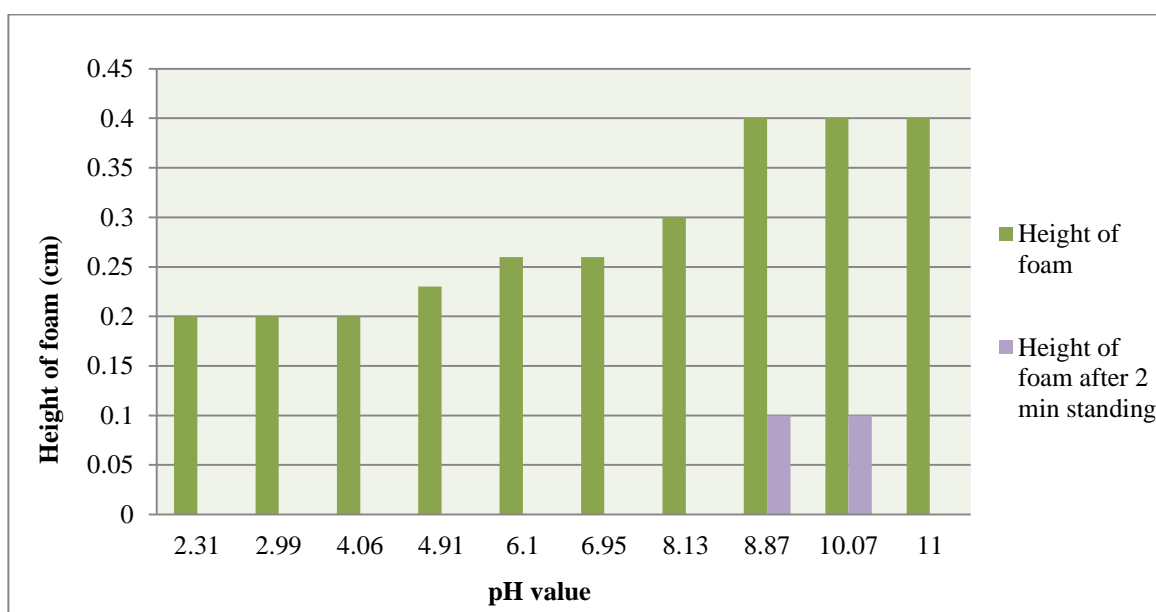
Figure 4. 34 Foam properties of n-octyl caffeamide DMSO/water solution (concentration, 3×10^{-5} M), depending on the pH value

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Because of the poor solubility of n-octyl caffeamide in water, certain amount of organic solvent, such as DMSO or ethanol was used to enhance its solubility. 8.7 mg of n-octyl caffeamide was firstly dissolved in 10 ml of DMSO to obtain a solution of 3×10^{-3} M. Then the solution was diluted with distilled water for the determination of foamability and foam stability of n-octyl caffeamide under different pH values. Based on the results obtained above, foam properties of n-octyl caffeamide with a concentration range from 3×10^{-7} M to 1×10^{-3} M were also determined. The results are shown below (Fig. 4.34 and 4.35).

As shown in Fig. 4.34, the foamability of n-octyl caffeamide increased gradually along with the increasing pH value. At around pH 9, the foamability reached the maximum. However, the foam collapsed so quickly that almost no foam left at the end of 2 min.

The foam properties of n-octyl caffeamide at different concentrations were determined at pH 10. The biggest foam height index for both foamability and foam stability were observed at high concentration of n-octyl caffeate (1×10^{-3} M); they decreased dramatically when the concentration decreased.

Compared with n-octyl caffeate, the solubility of n-octyl caffeamide was increased; however, its foamability and foam stability were all decreased.
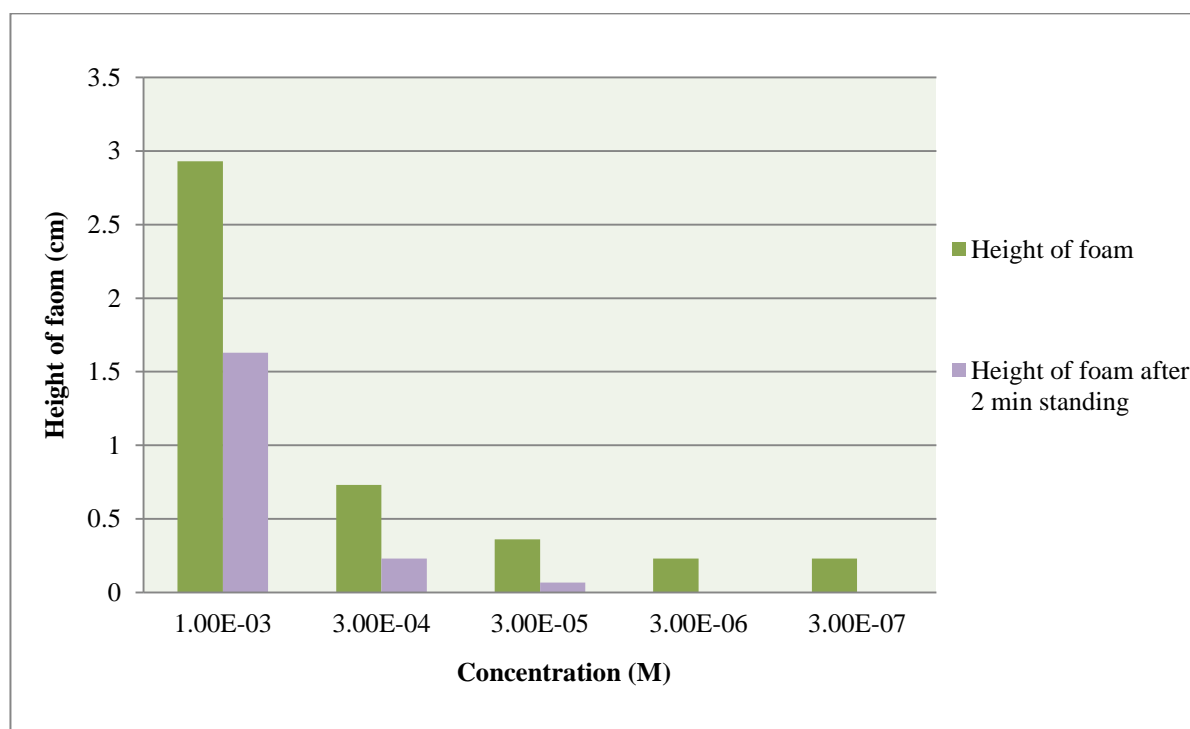
Figure 4. 35 Foam properties of n-octyl caffeamide DMSO/water solution (pH 10.0), depending on the concentration

4.4.3 Evaluation of the complexation between caffeine and n-octyl caffeamide in aqueous solution

The absorption spectra of n-octyl caffeamide titrated with caffeine are presented in Fig. 4.36, in the form of molar absorption coefficient (E_λ). Wavelength ranging from 320 nm to 350 nm was chosen as the band to reflect the absorption changes of n-octyl caffeamide only, as caffeine has negligible absorption over 320 nm. The bathochromic shifts (or red shifts) are clearly visible in these spectra, suggesting that aromatic chromophore interactions happened between caffeine and n-octyl caffeamide molecules, and a new absorbing component (complex of caffeine and n-octyl caffeamide) appeared in the mixture. The presence of an isosbestic point at around 334 nm in the spectra indicates that two components of n-octyl caffeamide are predominantly present in the mixture: monomer of n-octyl caffeamide and the caffeine–n-octyl caffeamide complex.
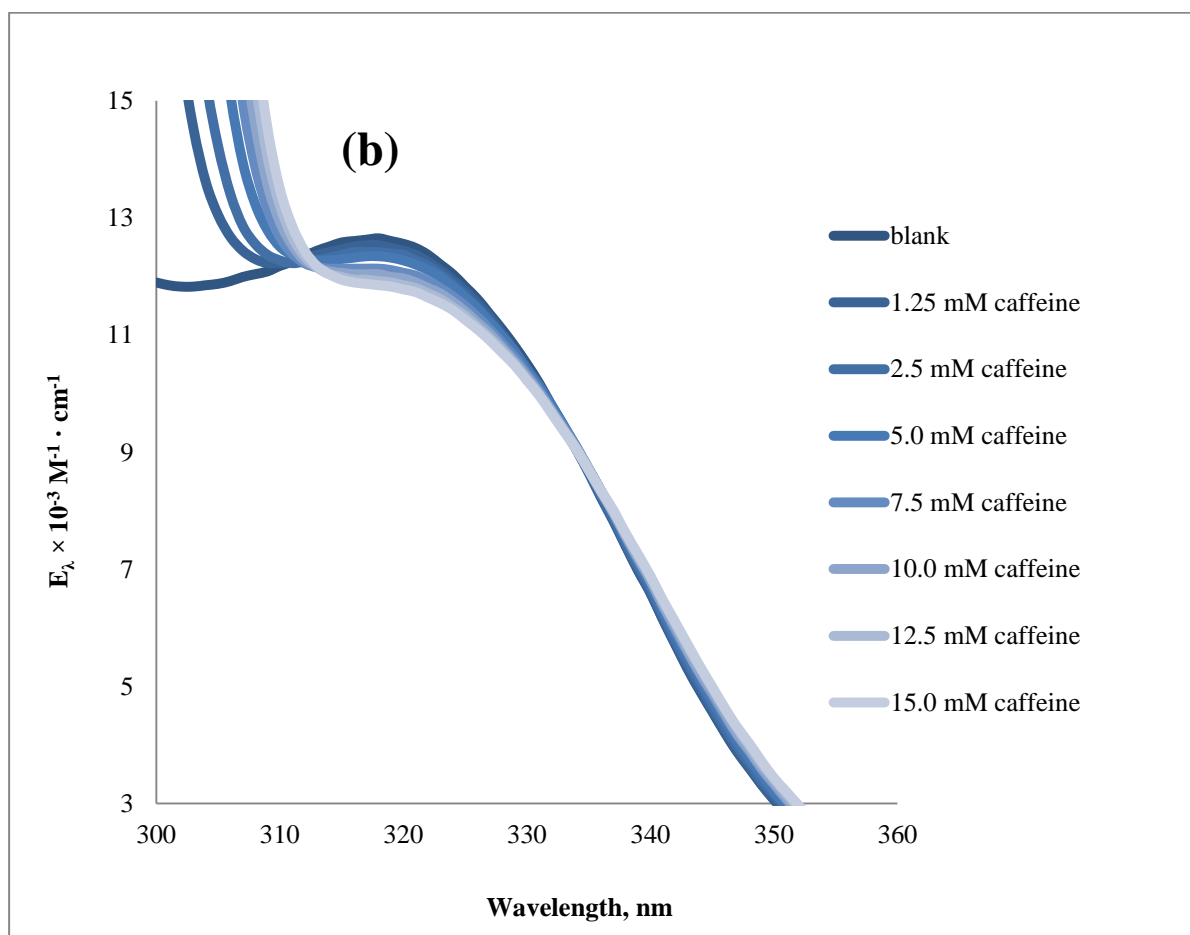
Figure 4. 36 Spectrophotometric titration of n-octyl caffeamide (initial concentration 0.042 mM) with caffeine (concentration ranging from 1.25 mM to 15.0 mM)

4.4.4 Foam fractionation efficiency

It was proved by the preliminary foaming test that the foam produced by n-octyl caffeamide was not enough for collection, under a comparatively mild condition. Therefore, certain amount of surfactant was added in each of the foaming experiments of caffeine and n-octyl caffeamide described in the following sections.

4.4.4.1 Influence of pH

The influence of pH value of the initial solution on the enrichment of caffeine and n-octyl caffeamide was investigated by varying the pH value from 3 to 7 in the foaming experiments. It was proved that compounds such as chlorogenic acid and caffeic acid that containing a caffeic acid based structure are not stable at a pH value above 7 (Friedman and Jurgens 2000). Our tests on n-octyl caffeamide were also observed the same phenomenon (results not showing here), thus the pH value above 7 was not adopted here. The other parameters were kept constant: saponin amount, 25 mg/100ml; caffeine and n-octyl caffeamide initial concentration, 1×10^{-5} M; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Fig. 4.37.

Figure 4. 37 pH-dependent enrichment ratio (ER) of caffeine and n-octyl caffeamide in their foam fractionation process

The results show that almost no enrichment was achieved for caffeine in the foaming experiments. The highest enrichment value was 1.27 at pH 3, which was proved to be significant statistically, compared with the ones at the other pH values. In contrast, n-octyl caffeamide exhibited very high separation efficiency: an enrichment ratio of 160.7 at pH 6. The huge difference of the enrichment ratio between caffeine and n-octyl caffeamide indicates that these two substances were not enriched in the form of complex, but separately.

4.4.4.2 Influence of saponin amount

The concentration of saponin was also varied in the foaming experiments to investigate its influence on the foaming efficiency of caffeine and n-octyl caffeamide. The other parameters were kept constant: pH original, 5.7; caffeine and n-octyl caffeamide initial concentration, 1×10^{-5} M; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Fig. 4.38.

As shown in Fig. 4.38, both the enrichment ratio of caffeine and n-octyl caffeamide was decreased with an increasing amount of saponin in the initial solution. For caffeine, the enrichment ratio was always around the value of 1, which means it was not enriched into the foam phase.
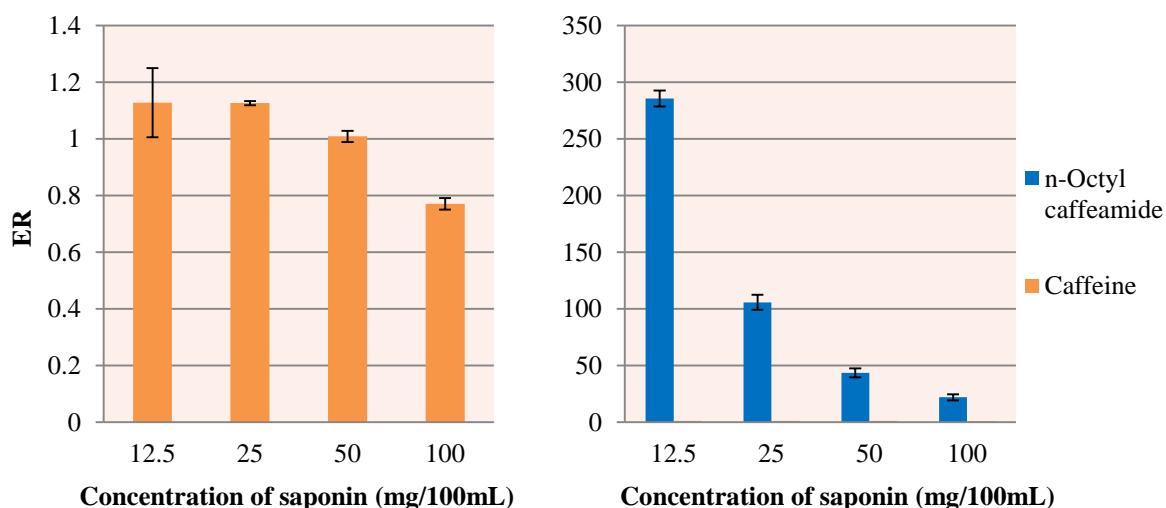
Figure 4. 38 Saponin amount-dependent enrichment ratio (ER) of caffeine and n-octyl caffeamide in their foam fractionation process

In contrast, n-octyl caffeamide was enriched into the foamate with a very high efficiency: 285.5 at the saponin concentration of 12.5 mg/100ml. Then it decreased dramatically when the concentration increased from 12.5 mg/100ml to 100 mg/100ml.

4.4.4.3   Influence of caffeine and n-octyl caffeamide ratio

Different caffeine and n-octyl caffeamide ratios were also applied in the foaming experiments, in order to evaluate their influence on the enrichment ratio of caffeine and n-octyl caffeamide. A higher concentration of n-octyl caffeamide (3.29×10^{-5} M) was used and was kept constant in all the experiments hereafter in this section, while the concentration of caffeine was varied to prepare a solution of different ratios. The other parameters were kept constant: pH original, 5.7; saponin amount, 25 mg/100ml; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Fig. 4.39.

Figure 4. 39 Caffeine/n-octyl caffeamide ratio-dependent enrichment ratio (ER) of caffeine and n-octyl caffeamide in their foam fractionation process

The results of caffeine indicated that no caffeine was enriched when different ratio of caffeine and n-octyl caffeamide was used, and also, its enrichment ratio was not influenced by a varying ratio. For n-octyl caffeamide, the enrichment at a caffeine/n-octyl caffeamide ratio 1:1 was almost twice of that at the other ratios. When the ratio is above 3 (included), the enrichment was not influenced by the ratio anymore, but was around the value 10. The results here imply us that the increasing amount of caffeine in the solution depressed the

enrichment of n-octyl caffeamide. Compared with the foam efficiency of n-octyl caffeamide at an initial concentration of $1×10^{-5}$ M, it was much lower here at a concentration of $3.29×10^{-5}$ M (105.6:19.5), which proves again that a lower initial concentration is favorable for foam fractionation of n-octyl caffeamide.

4.4.4.4   Influence of flow rate

The influence of the flow rate was also investigated by varying the flow rate from 12 ml/min to 30 ml/min in the foaming experiments of caffeine and n-octyl caffeamide binary aqueous solution. The other parameters were kept constant: pH original, 5.7; saponin amount, 25 mg/100ml; caffeine and n-octyl caffeamide concentration, $3.29×10^{-5}$ M; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Fig. 4.40.

For caffeine, still no enrichment was achieved during the foaming process, since the values of enrichment ratio are all around 1. In contrast, the enrichment ratio of n-octyl caffeamide exhibited a very significant decrease (from 19.5 to 3.9), when the flow rate increased from 12 ml/min to 30 ml/min, which proved that a low flow rate is favorable for the foam fractionation.

Figure 4. 40 Flow rate-dependent enrichment ratio (ER) of caffeine and n-octyl caffeamide in their foam fractionation process

4.4.4.5   Influence of column height

The foam experiments with a different height of column ranging from 9 cm to 38 cm were also conducted for the investigation of its influence on the foaming efficiency. The other

parameters were kept constant: pH original, 5.7; saponin amount, 25 mg/100ml; caffeine and n-octyl caffeamide concentration, 3.29×10^{-5} M; flow rate, 12 ml/min; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Fig. 4.41.
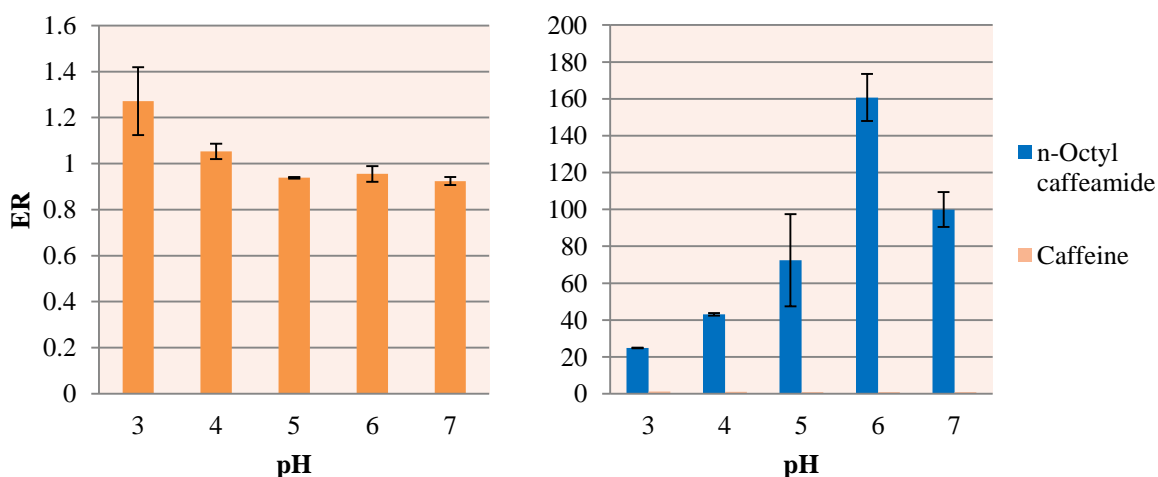

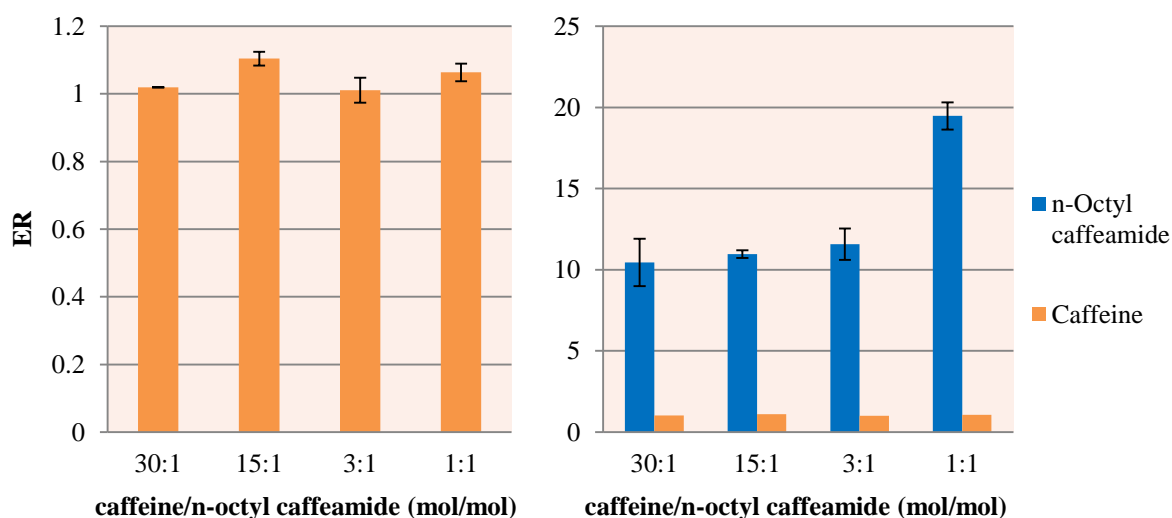**Figure 4. 41** Column height-dependent enrichment ratio (ER) of caffeine and n-octyl caffeamide in their foam fractionation process

The results show us that no enrichment was achieved for caffeine in this series of experiments. For n-octyl caffeamide, the results are consistent with the theory that long column is favorable for the foam fractionation: the enrichment ratio increased gradually and markedly from 3.8 to 19.5, when the column height was prolonged from 9 cm to 38 cm.

4.4.4.6   Influence of temperature

The influence of temperature on the foaming efficiency of caffeine and n-octyl caffeamide was also investigated by varying the temperature from 2 ℃ to 40 ℃. The other parameters were kept constant: pH original, 5.7; saponin amount, 25 mg/100ml; caffeine and n-octyl caffeamide concentration, 3.29×10^{-5} M; flow rate, 12 ml/min; initial solution volume, 40 ml; column height 38 cm. The results are shown in Fig. 4.42.

The results shown here in Fig. 4.42 indicate that caffeine was almost not enriched in all of experiments conducted in this section, and also temperature has no influence on the enrichment of caffeine here, since the enrichment ratios were proved to be no difference in statistic. Compared with caffeine, the enrichment of n-octyl caffeamide exhibited a significant increasing trend when the temperature was getting higher. The influence of

temperature on the enrichment is complicated, since it may influence many properties of the foam. The results here indicate that more molecules of n-octyl caffeamide absorbed at the gas-liquid interface when temperature increased, and its solubility also increased. As a result, better enrichment ratio was obtained.
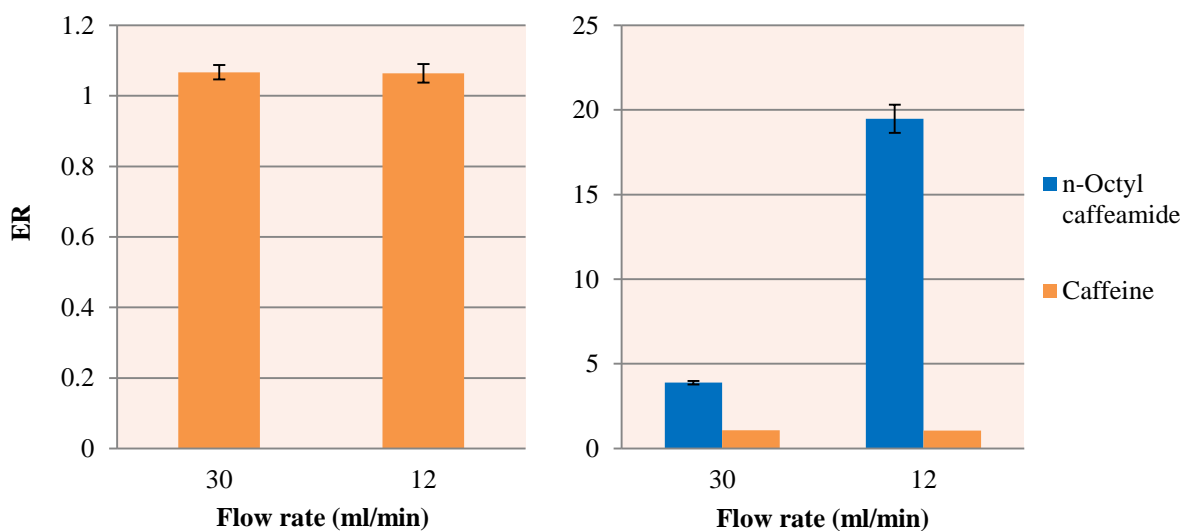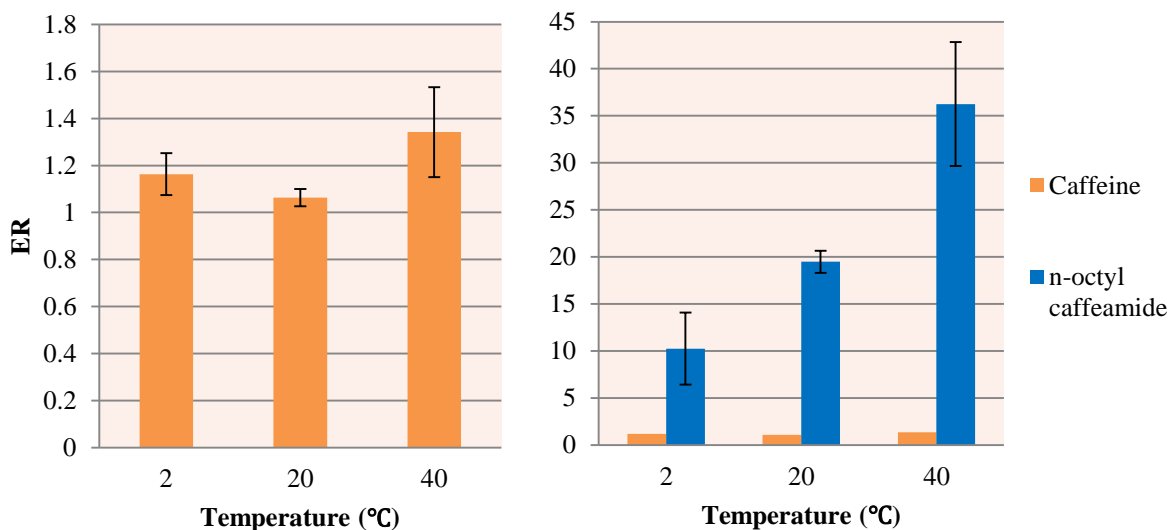

Figure 4. 42 Temperature-dependent enrichment ratio (ER) of caffeine and n-octyl caffeamide in their foam fractionation process

4.4.4.7   Influence of NaCl concentration

The influence of NaCl concentration on the foam efficiency of caffeine and n-octyl caffeamide was also investigated by conducting series of experiments with different amount of NaCl. The other parameters were kept constant: pH original, 5.7; saponin amount, 25 mg/100ml; caffeine and n-octyl caffeamide concentration, 3.29×10^{-5} M; flow rate, 12 ml/min; initial solution volume, 40 ml; column height 38 cm; temperature, 20 ℃. The results are shown in Fig. 4.43.

The ionic strength may enhance the absorption of the molecules at the gas-liquid interface, thus the enrichment was improved. Here the enrichment ratio of both caffeine and n-octyl caffeamide increased noticeable at a NaCl concentration of 5 g/100ml compared with the ones at a lower concentration of NaCl. The enrichment ratio for caffeine at the NaCl concentration of 5 g/100ml and 8 g/100ml were statistically the same, which means more amount of NaCl in the initial solution would not improve the foaming efficiency any further. For n-octyl caffeamide, it was the same situation.
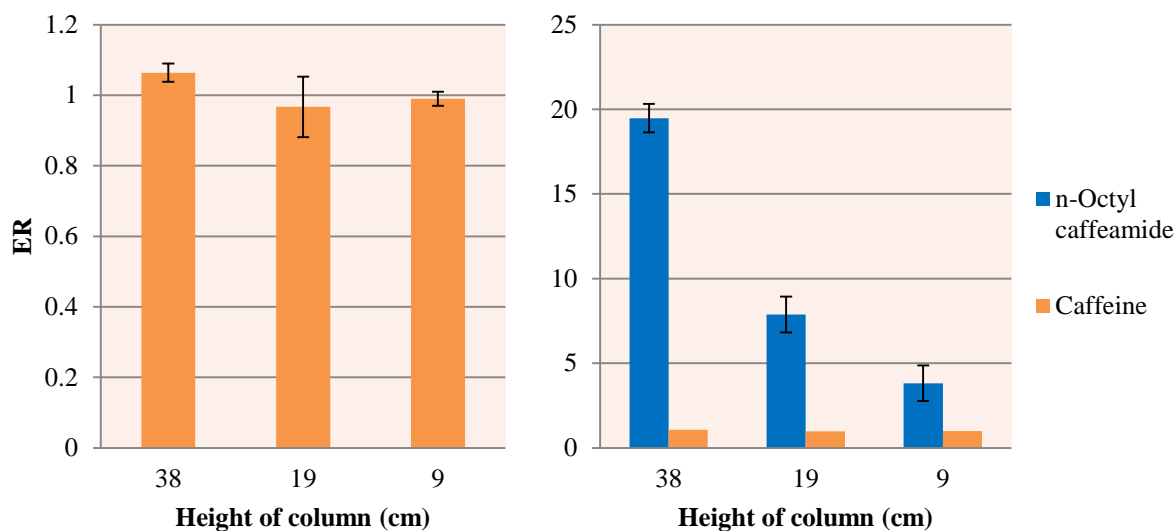
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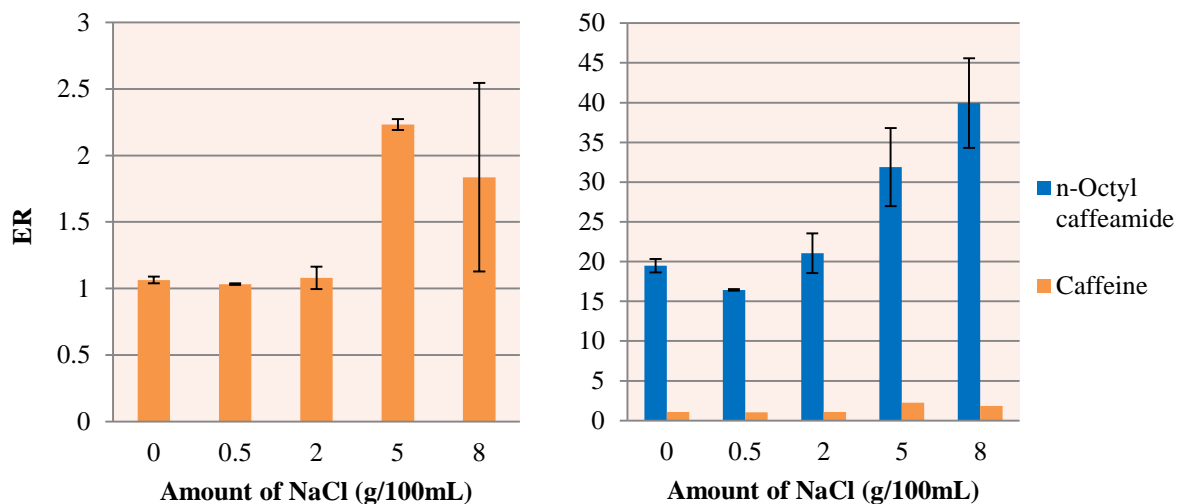
Figure 4. 43 NaCl amount-dependent enrichment ratio (ER) of caffeine and n-octyl caffeamide in their foam fractionation process

4.4.4.8 Over all evaluation of the foam fractionation of caffeine and n-octyl caffeamide binary aqueous solution

In all the parameters investigated for their influence on the foam efficiency of caffeine and n-octyl caffeamide, almost all of them had an influence on the enrichment ratio for n-octyl caffeamide, while only NaCl concentration had a slight influence on the enrichment of caffeine. Although some other experiments with a NaCl concentration of 5 g/100ml were conducted, the enrichment ratio for caffeine was not improved. Therefore, we believe that caffeine is not likely to be enriched with n-octyl caffeamide together as a complex, under the condition adopted here in our experiments. An example showing the huge difference in enrichment of caffeine and n-octyl caffeamide is exhibited in Figure 4.44. The change in size of the peaks in HPLC chromatograms from both caffeine and n-octyl caffeamide in initial, residual and foamate solution indicates the difference in enrichment behavior of these two compounds.

Similar with n-octyl caffeate, the replacement of the quinic acid moiety in chlorogenic acid made the product (n-octyl caffeamide) more hydrophobic and more easily to decomplex with caffeine because of the loss of some hydrogen bonds. Therefore, n-octyl caffeamide was enriched efficiently, but leaving caffeine behind in the bulk solution.
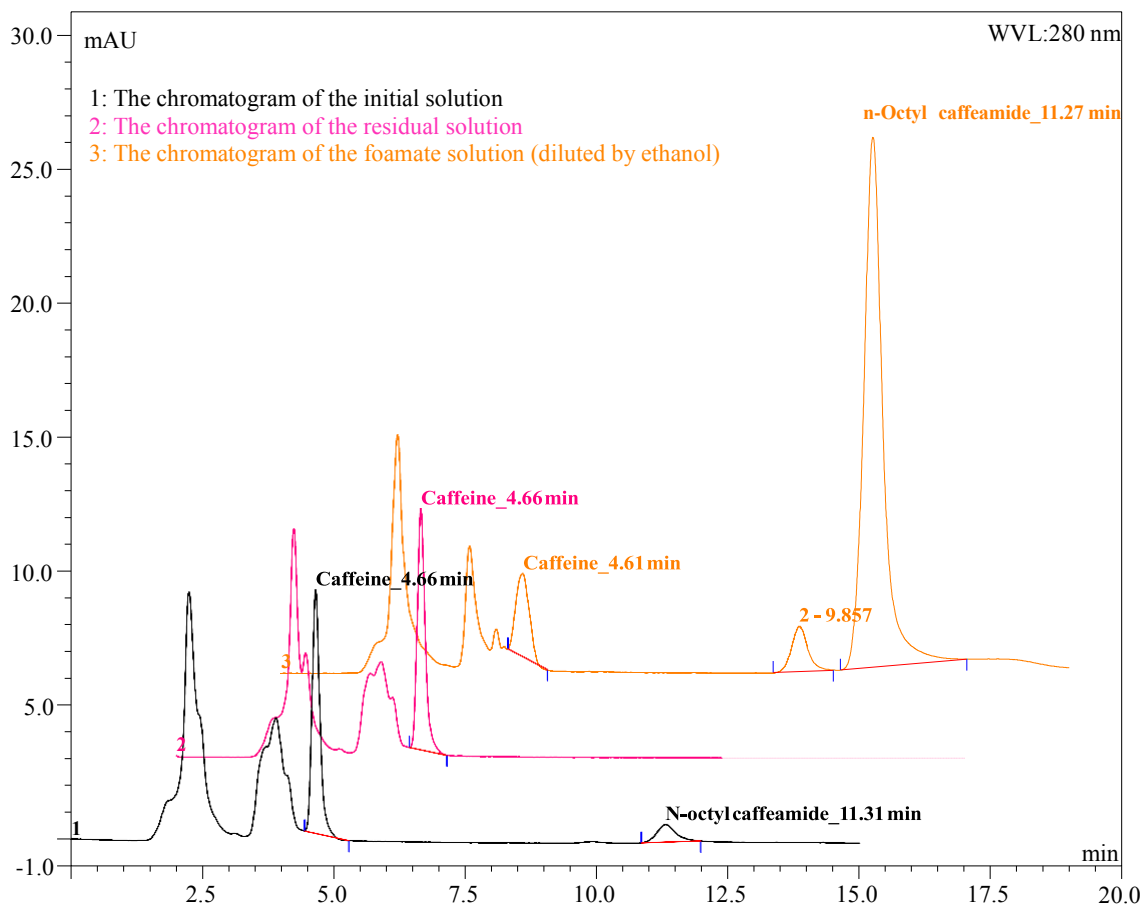
Figure 4. 44 HPLC analysis of the initial (in black), residual (in purple) and foamate (in orange) solution of caffeine and n-octyl caffeamide binary system. The parameters adopted are as follows: pH original, 5.7; saponin amount 25 mg/100ml; caffeine and n-octyl caffeamide initial concentration, 1×10^{-5} M; flow rate, 12 ml/min; height of column, 38 cm; initial solution volume, 40 ml; temperature, 20 ℃

4.5   Foam fractionation of caffeine and caffeic acid

4.5.1   Evaluation of the foamability and foam stability of caffeic acid

The aqueous solution of caffeic acid (The structure is shown in Figure 4.45) with a concentration from 10^{-7} M to 10^{-3} M was not able to produce any significant foam during the test, or collapsed immediately if any. The foamability and foam stability of caffeic acid at different pH values were also tested. Unfortunately, similar results were obtained, the foamability of caffeic acid was very poor, and almost no fluctuation was observed with the

variation of the pH value. Therefore, a small amount of saponin was needed before each of the foaming experiments.
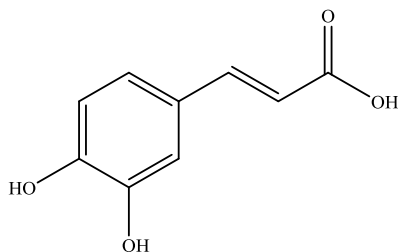
Figure 4. 45 The structure of caffeic acid

4.5.2 Evaluation of the complexation between caffeine and caffeic acid in aqueous solution
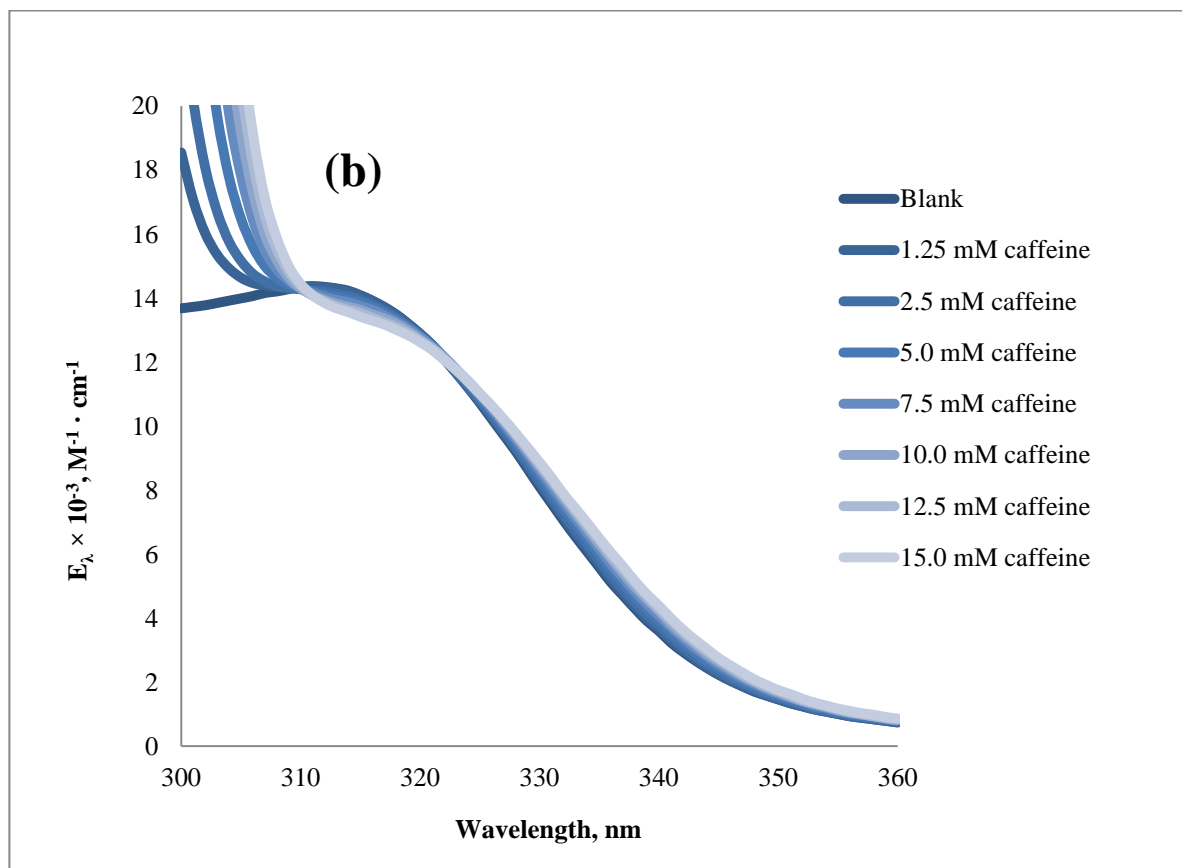
Figure 4. 46 Spectrophotometric titration of caffeic acid (initial concentration 0.042 mM) with caffeine (concentration ranging from 1.25 mM to 15.0 mM)

The absorption spectra of caffeic acid titrated with caffeine are presented in Fig. 4.46, in the form of molar absorption coefficient (E_λ). Wavelength ranging from 320 nm to 350 nm was chosen as the band to reflect the absorption changes of caffeic acid only, as caffeine has negligible absorption over 320 nm. The bathochromic shifts (or red shifts) are visible in these spectra, suggesting that aromatic chromophore interactions happened between caffeine and caffeic acid molecules, and a new absorbing component (complex of caffeine and caffeic acid) appeared in the mixture. The presence of an isosbestic point at around 322 nm in the spectra indicates that two components of caffeic acid were predominantly present in the mixture: monomer of caffeic acid and the caffeine-caffeic acid complex.

4.5.3 Foam fractionation efficiency

4.5.3.1 Influence of pH
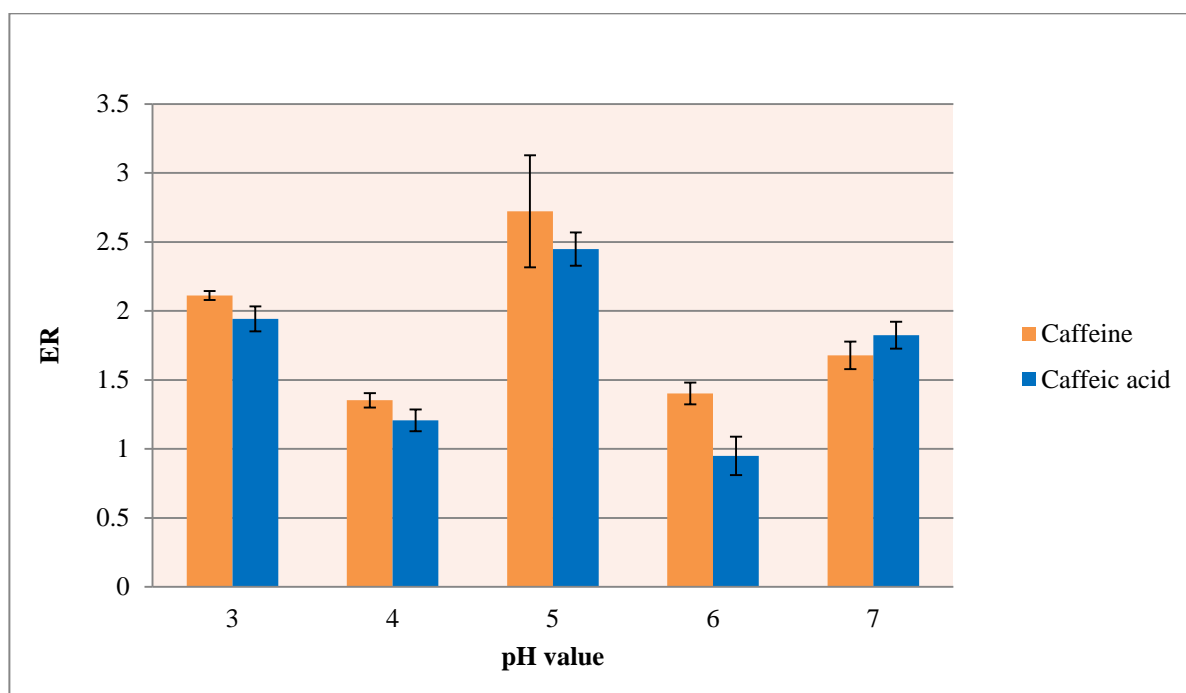
Figure 4. 47 pH-dependent enrichment ratio (ER) of caffeine and caffeic acid in their foam fractionation process

The influence of pH value on the foaming efficiency was investigated by varying the pH value of the initial solution from 3 to 7 during the foaming experiments. Caffeic acid would be degraded at a pH value above 7, and the degradation is time dependent and even nonreversible (Friedman and Jurgens 2000). Therefore, the pH value above 7 was not adopted here. The other parameters were kept constant: saponin amount, 25 mg/100ml;

caffeine and caffeic acid concentration, 1×10^{-5} M; flow rate, 12 ml/min; initial solution volume, 40 ml; column height 38 cm; temperature, 20 ℃. The results are shown in Fig. 4.47.

As the results here indicate that the highest enrichment ratio for caffeine was 2.72 obtained at a pH value of 5, and then followed by 2.11 and 1.82 at pH 3 and 7 respectively. For caffeic acid, the enrichment ratio at almost every pH point was quite close to the value of caffeine, and the value varied in a very similar way with that of caffeine, which may imply that caffeine and caffeic acid were enriched as a 1 to 1 complex into the foamate.

4.5.3.2 Influence of saponin amount

Saponin concentration was also varied in the foam experiments here, to investigate its influence on the separation efficiency. The other parameters were kept constant: pH original, 5.0; caffeine and caffeic acid concentration, 1×10^{-5} M; flow rate, 12 ml/min; initial solution volume, 40 ml; column height 38 cm; temperature, 20 ℃. The results were shown in Fig. 4.48.

Figure 4. 48 Saponin amount-dependent enrichment ratio (ER) of caffeine and caffeic acid in their foam fractionation process

As shown in Fig. 4.48, the values of enrichment ratio of caffeine at a saponin concentration of 12.5 mg/100ml and 25 mg/100ml are all around 2.5, which are higher than that of 50

mg/100ml. The difference of the enrichment ratio between the foaming experiments with a saponin concentration 12.5 mg/100ml and 25 mg/100ml were proved to be not significant, which means that a saponin concentration lower than 25 mg/100ml was not able to enhance the enrichment of caffeine any further. For caffeic acid, similar conclusion was obtained.

4.5.3.3 Influence of caffeine and caffeic acid concentration

The influence of the caffeine and caffeic acid ratio on the foaming efficiency of them both was also investigated by varying the ratio in the foaming experiments. Here, the concentration of the caffeic acid was all the same (1×10^{-5} M) in these series of experiments, but the caffeine amount was varied. And the other parameters were kept constant: pH original, 5.0; saponin amount, 25 mg/100ml; flow rate, 12 ml/min; initial solution volume, 40 ml; column height 38 cm; temperature, 20 ℃. The results are shown in Fig. 4.49.

Figure 4. 49 Caffeine/caffeic acid ratio-dependent enrichment ratio (ER) of caffeine and caffeic acid in their foam fractionation process

The highest enrichment ratio of both caffeine and caffeic acid was obtained at the caffeine and caffeic acid ratio of 1 to 1. For caffeine, a higher ratio to caffeic acid (5 to 1) increased the amount of both caffeine and the complex in the solution, and much more for free caffeine. As a result, the overall enrichment ratio of caffeine was suppressed (as the results indicate here). However, too low a ratio (0.2 to 1) may decrease the amount of the complex

of caffeine and caffeic acid too much or suppress the interactions between them, thus a lower efficiency of caffeine was obtained. For caffeic acid, the values of enrichment ratio at a ratio of 5 to 1 and 1 to 1 were proved to be the same statistically, which indicates that the increase of caffeine did not influence the enrichment of caffeic acid significantly. Meanwhile, the enrichment ratio of caffeic acid decreased when the ratio of caffeine was lower (0.2 to 1), in spite of the fact that the amount of caffeic acid was constant in all the experiments. This may also ascribe to the low amount of complex formed between caffeine and caffeic acid or suppressed interactions between them, because of the decreased amount of caffeine. Based on the results above, we may come to the conclusion that the complexation or the interactions between caffeine and caffeic acid would be beneficial for a higher enrichment of caffeine.

4.5.3.4   Influence of flow rate

The influence of flow rate on the foaming efficiency of caffeine and caffeic acid was also conducted. The values for different flow rate used here were 12 ml/min and 30 ml/min. The other parameters were kept constant: pH original, 5.0; saponin amount, 25 mg/100ml; caffeine and caffeic acid concentration, 1×10^{-5} M; initial solution volume, 40 ml; column height 38 cm; temperature, 20 ℃. The results are shown in Fig. 4.50.
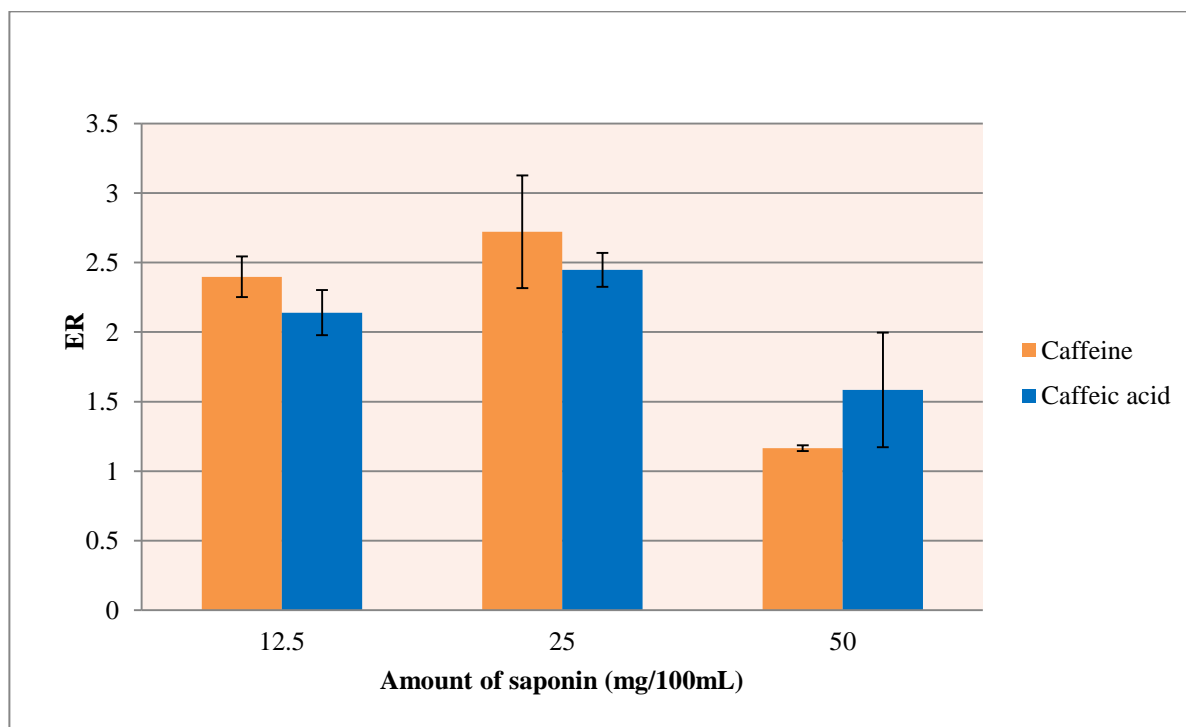

Figure 4. 50 Flow rate-dependent enrichment ratio (ER) of caffeine and caffeic acid in their foam fractionation process

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As expected, a low flow rate of 12 ml/min helped both caffeine and caffeic acid to gain a higher enrichment ratio of 2.72 and 2.45 for caffeine and caffeic acid respectively, compared with that of 1.12 and 0.94 obtained at a flow rate of 30 ml/min.

4.5.3.5 Influence of column height

The height of the column was also varied in this series of experiments, aiming to investigate its influence on the enrichment ratio of caffeine and caffeic acid. The other parameters were kept constant: pH original, 5.0; saponin amount, 25 mg/100ml; caffeine and caffeic acid concentration, 1×10^{-5} M; flow rate, 12 ml/min; initial solution volume, 40 ml; temperature, 20 °C. The results are shown in Fig. 4.51.

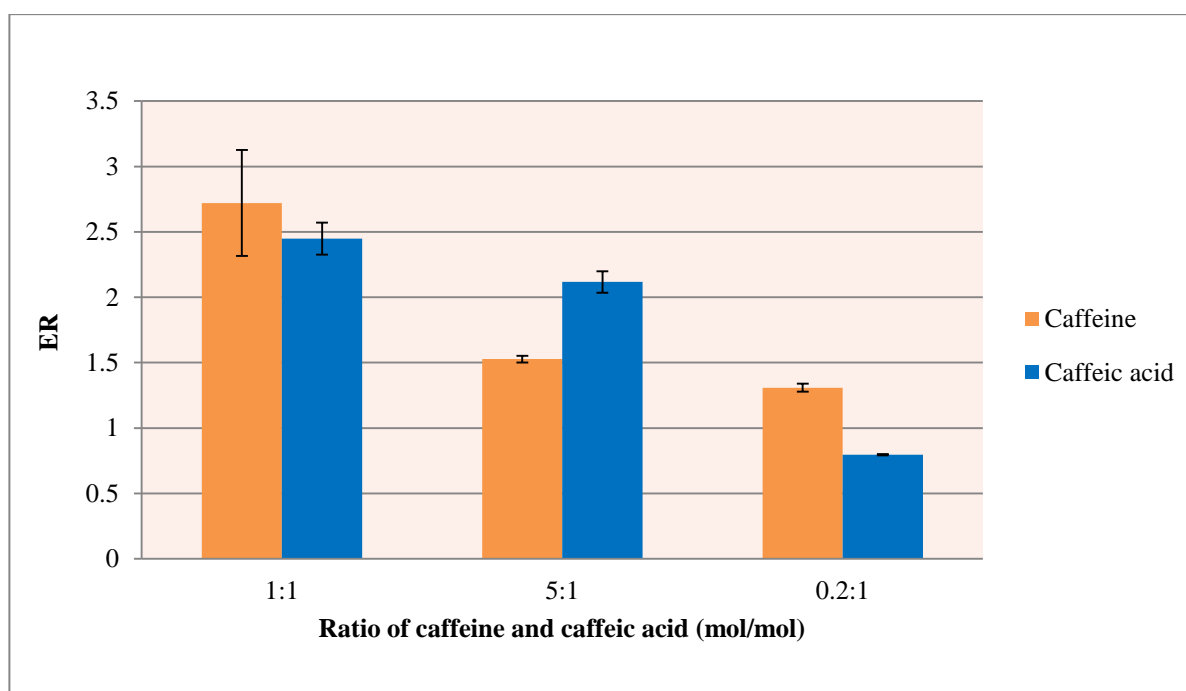**Figure 4. 51** Column height-dependent enrichment ratio (ER) of caffeine and caffeic acid in their foam fractionation process

A higher enrichment ratio was obtained for caffeine when a higher column (38 cm) was used in the foaming experiments, as shown in Fig. 4.51. Both the enrichment ratio of caffeine obtained with a column height of 9 cm and 19 cm were around 1, indicating that caffeine was poorly enriched with a lower column height. For caffeic acid, similar results were obtained.

4.5.3.6   Influence of temperature

The influence of temperature on the enrichment of caffeine and caffeic acid was also investigated. A temperature of 2 ℃, 20 ℃ and 40 ℃ were adopted here, and the other parameters were kept constant: pH original, 5.0; saponin amount, 25 mg/100ml; caffeine and caffeic acid concentration, 1×10^{-5} M; flow rate, 12 ml/min; column height 38 cm; initial solution volume, 40 ml. The results are shown in Fig. 4.52.

The results here indicate that a temperature of 20 ℃ was more favorable for the enrichment of caffeine from its binary aqueous solution. A temperature too low (2 ℃) or too high (40 ℃) all influenced the adsorption of caffeine onto the gas-liquid interface, thus decreased the foaming efficiency. For caffeic acid, similar results were obtained.
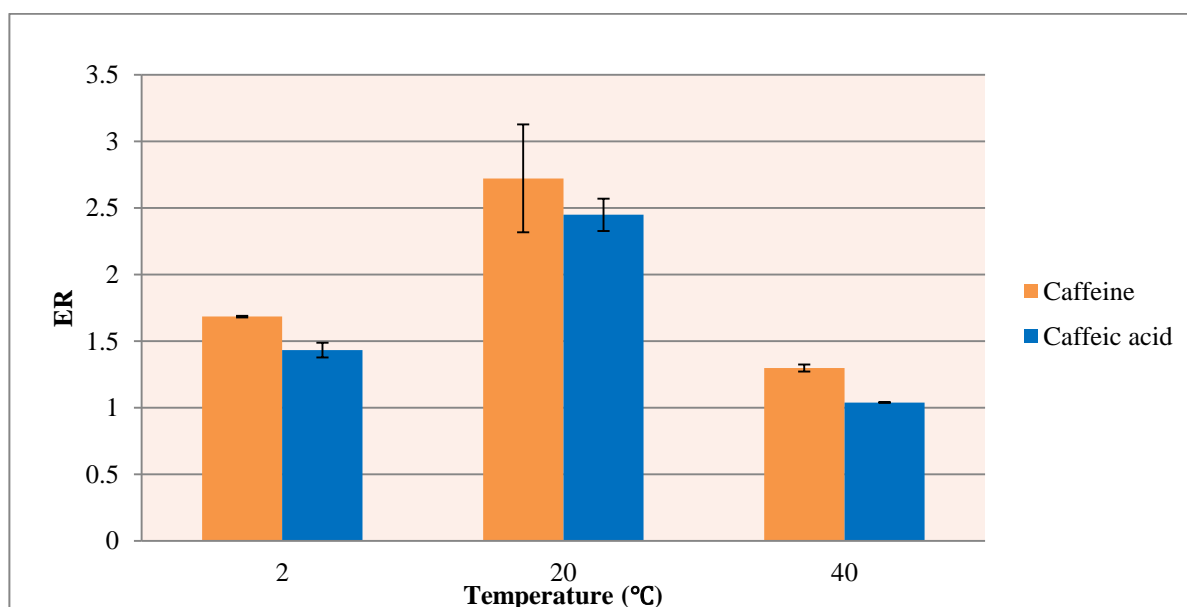
Figure 4. 52 Temperature-dependent enrichment ratio (ER) of caffeine and caffeic acid in their foam fractionation process

4.5.3.7   Influence of NaCl concentration

The amount of NaCl was also varied in the foaming experiments to evaluate its influence on the enrichment ratio of caffeine and caffeic acid from the aqueous solution. The other parameters were kept constant: pH original, 5.0; saponin amount, 25 mg/100ml; caffeine and caffeic acid concentration, 1×10^{-5} M; flow rate, 12 ml/min; initial solution volume, 40 ml; temperature, 20 ℃. The results are shown in Fig. 4.53.
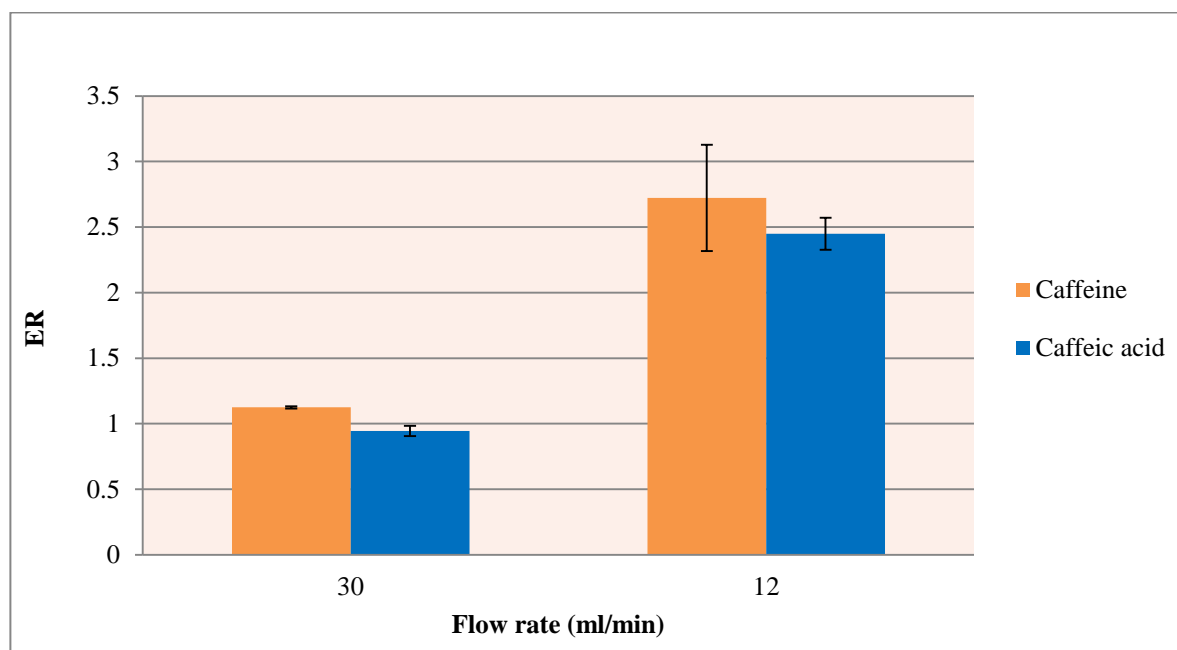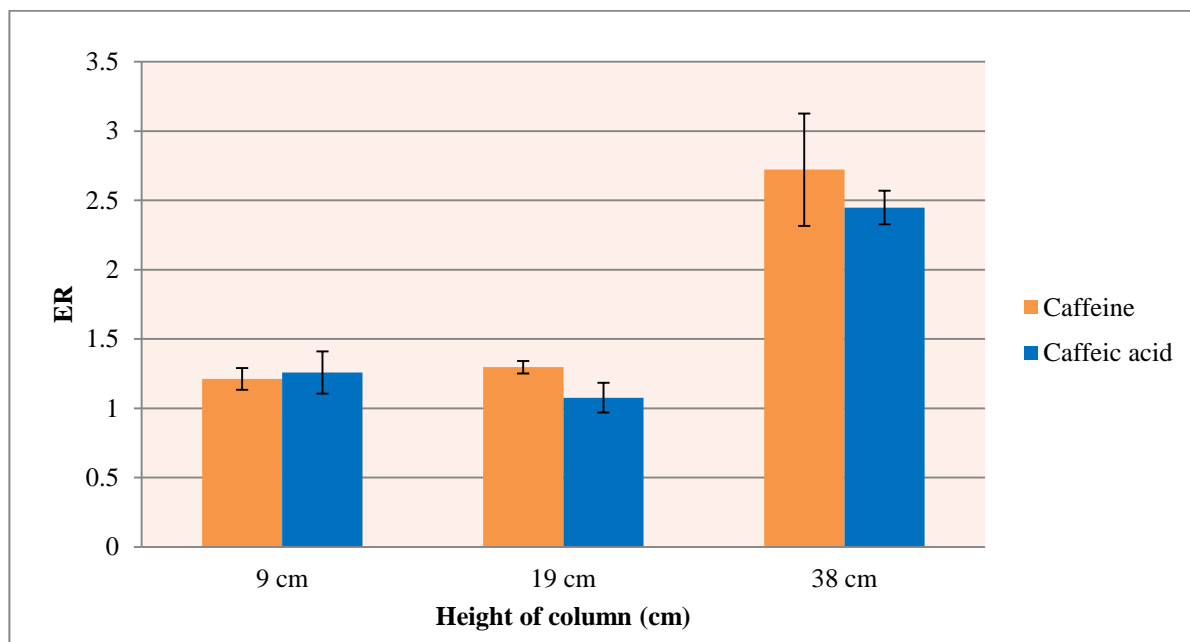
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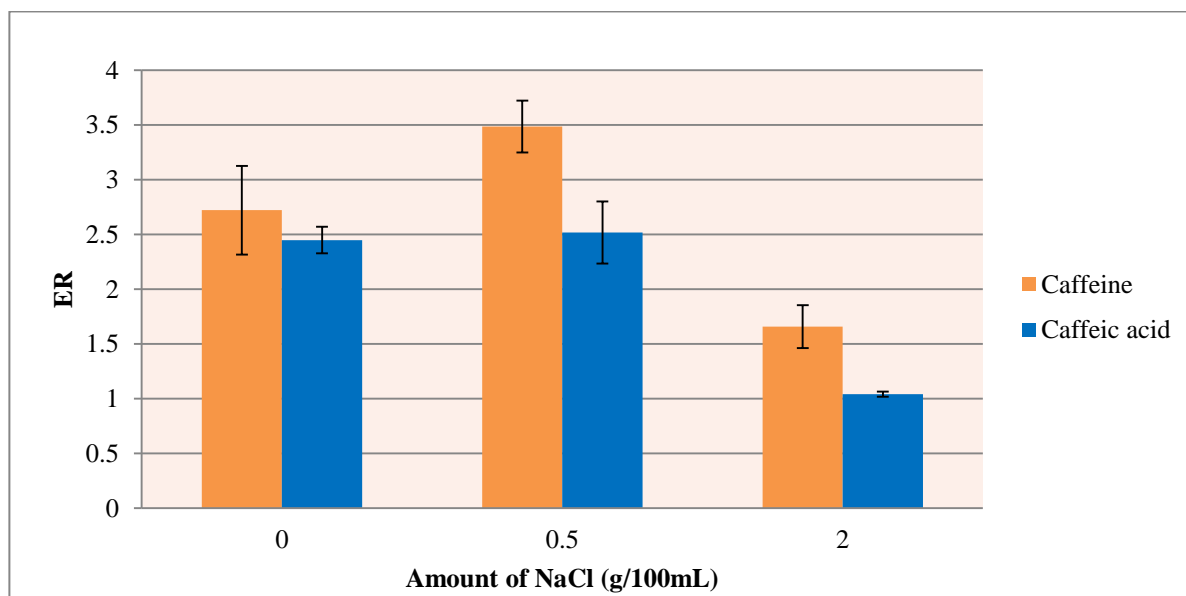
Figure 4. 53 NaCl amount-dependent enrichment ratio (ER) of caffeine and caffeic acid in their foam fractionation process

As indicated in Fig. 4.53, the enrichment ratio of caffeine was increased from 2.72 to 3.49 when 0.5 g/100ml NaCl was added into the initial solution, but decreased to 1.66 when more NaCl was added. A similar trend was observed for caffeic acid, except that the difference between the values obtained at a NaCl concentration of 0 g/100ml and 0.5 g/100ml was not significant statistically. Actually, a concentration of 5 g/100ml NaCl was also adopted in the experiments; however, no foam was collected since the amount of the foam was too limited and is collapsed easily. Here, the similar situation also happened to the foaming experiments with 2 g/100ml NaCl. The results here suggest that a low concentration of NaCl would help to promote the enrichment ratio by enhancing the adsorption of the molecules onto the gas-liquid interface; however, a too high concentration of NaCl would result in limited and easily collapsed foam.

4.5.3.8   Overall evaluation of the foam fractionation of caffeine and caffeic acid binary aqueous solution

Among the parameters here investigated, pH value, saponin concentration, caffeine/caffeic acid ratio, flow rate, NaCl amount, length of column and temperature were all proved to have a slight influence on the enrichment of caffeine and caffeic acid, in the foam fractionation of their binary aqueous solution. Meanwhile, the enrichment ratio for both

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caffeine and caffeic acid exhibited almost the same variation trend depending on each parameter. Therefore, we may conclude that caffeine and caffeic acid were able to be enriched as a complex by foam fractionation under the condition here adopted. In summary, the parameter values beneficial for a better enrichment ratio are as follows: pH 5; saponin amount, 25 mg/100ml; caffeine and caffeic acid concentration, 1×10^{-5} M; flow rate, 12 ml/min; column height, 38 cm; temperature, 20 ℃; and NaCl concentration, 0.5 g/100ml. As the result showing in section 4.5.3.7, an enrichment ratio of 3.49 was obtained for caffeine, by the combination of each of the parameter values with a better performance. Some other experiments were conducted further, for example using a column height of 60 cm, or even a lower flow rate, however, almost no further improvement of enrichment ratio (3.85) was obtained for caffeine.

4.6   Foam fractionation of green coffee sample

The above conducted investigations using chlorogenic acid, n-octyl caffeate, n-octyl caffeamide and caffeic acid as catchers to interact with caffeine in the parameters dependent foaming process unveiled that chlorogenic acid and caffeic acid are able to interact with caffeine and may be enriched together as a complex with a moderate efficiency from the aqueous solution of green coffee. Compared with caffeic acid, the foam fractionation of caffeine with chlorogenic acid was more efficient; therefore, foaming experiments using chlorogenic acid as a catcher for caffeine in green coffee aqueous solution were conducted.

Firstly, the amount of caffeine and chlorogenic acid in the green coffee beans was determined. As the results indicated that the amount were 11±0.5 mg/g and 12.5±0.7 mg/g for caffeine and chlorogenic acid, respectively. Since substantial amount of chlorogenic acid already existed in the coffee sample, no additional amount was added. Therefore, aqueous solution of green coffee with a concentration of 50 mg/250 mL was prepared as the initial solution, which equals to a concentration of 1.1×10^{-5} M and 0.7×10^{-5} M for caffeine and chlorogenic acid respectively. A certain amount of saponin was also added in each of the foaming experiments, since the green coffee solution was not able to produce adequate and stable foam. In order to evaluate the influence of each parameter on the enrichment ratio of caffeine, series of foaming experiments were conducted with varying the value of one parameter but keeping the others constant. The results are shown below (Figure 4.54 ~ 4.60):

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Figure 4. 54 pH-dependent enrichment ratio (ER) of caffeine and chlorogenic acid in the foam fractionation process of coffee. The other parameters were kept in constant: saponin amount, 50 mg/100ml; coffee concentration, 50 mg/250 ml; flow rate, 12 ml/min; column height, 38 cm; temperature, 20 ℃; initial solution volume, 40 ml
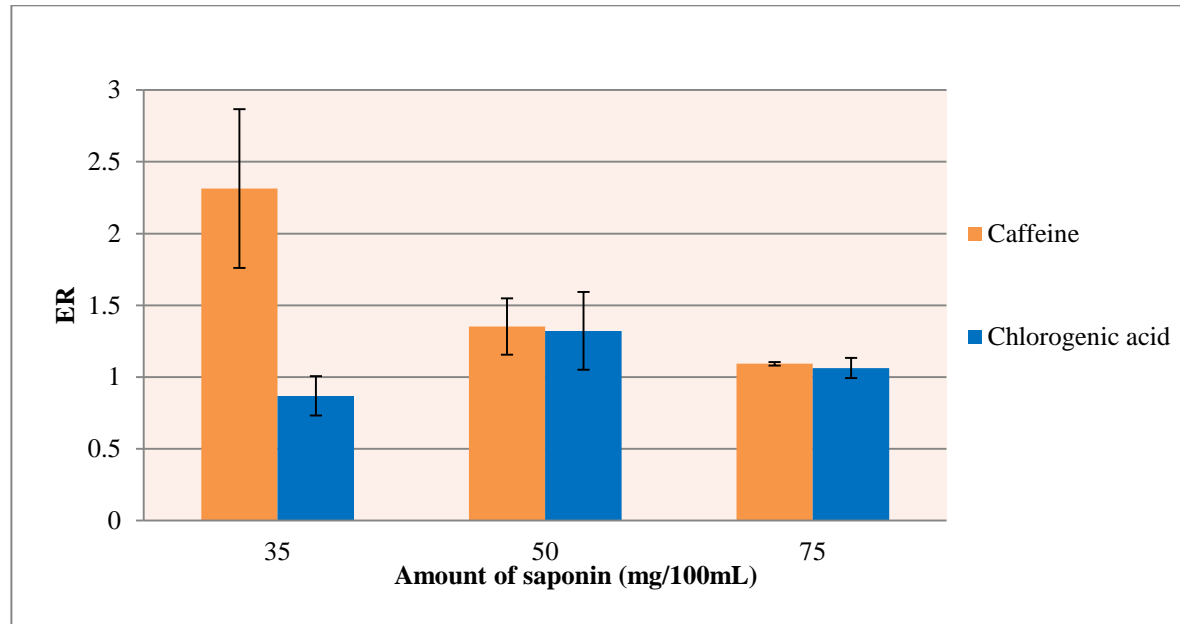
Figure 4. 55 Saponin amount-dependent enrichment ratio (ER) of caffeine and chlorogenic acid in the foam fractionation process of coffee. The other parameters were kept in constant: pH original, 5.4; coffee concentration, 50 mg/250 ml; flow rate, 12 ml/min; column height, 38 cm; temperature, 20 ℃; initial solution volume, 40 ml
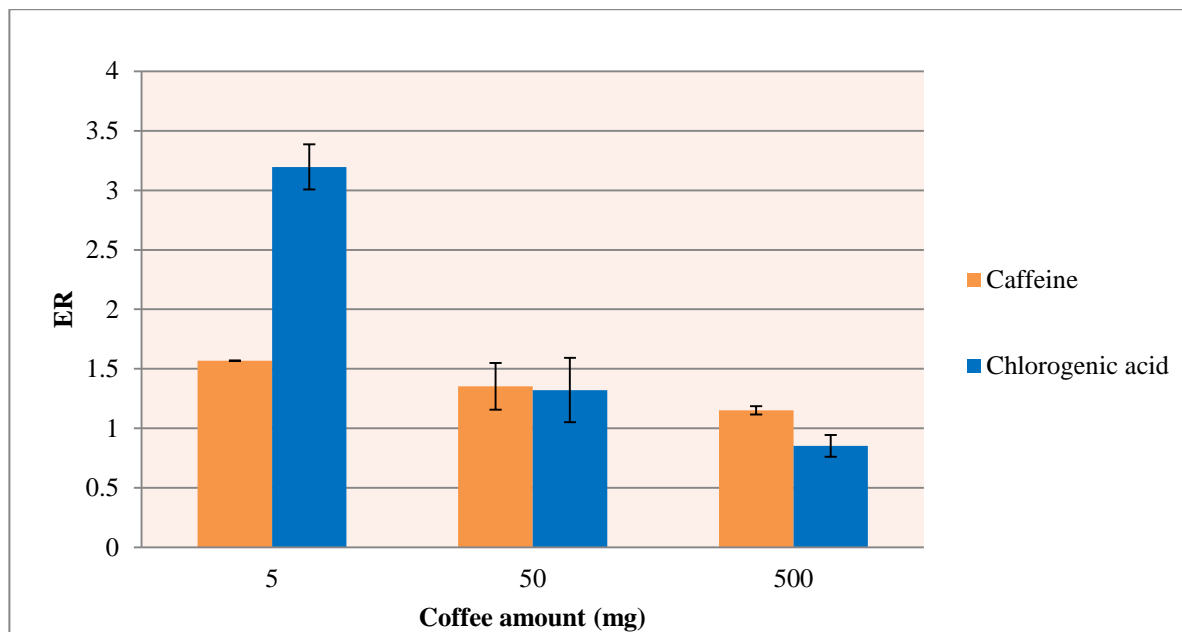
Figure 4. 56 Coffee amount-dependent enrichment ratio (ER) of caffeine and chlorogenic acid in the foam fractionation process of coffee. The other parameters were kept in constant: pH original, 5.4; saponin amount, 50 mg/100 ml; flow rate, 12 ml/min; column height, 38 cm; temperature, 20 ℃; initial solution volume, 40 ml
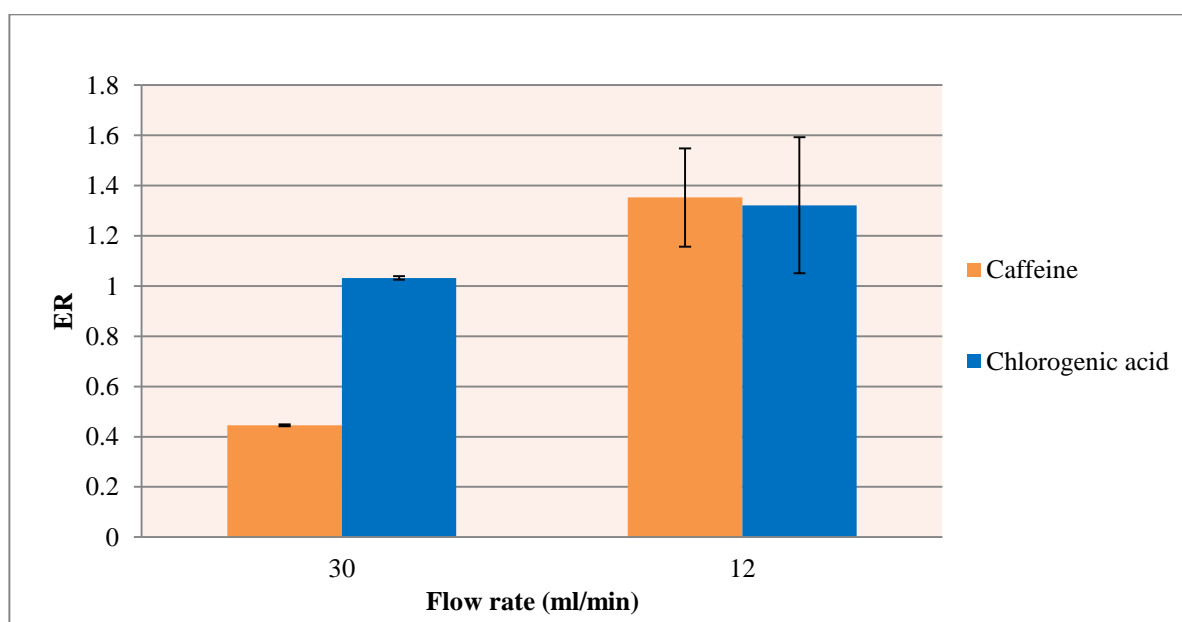
Figure 4. 57 Flow rate-dependent enrichment ratio (ER) of caffeine and chlorogenic acid in the foam fractionation process of coffee. The other parameters were kept in constant: pH original, 5.4; saponin amount, 50 mg/100 ml; coffee concentration, 50 mg/250 ml; column height, 38 cm; temperature, 20 ℃; initial solution volume, 40 ml
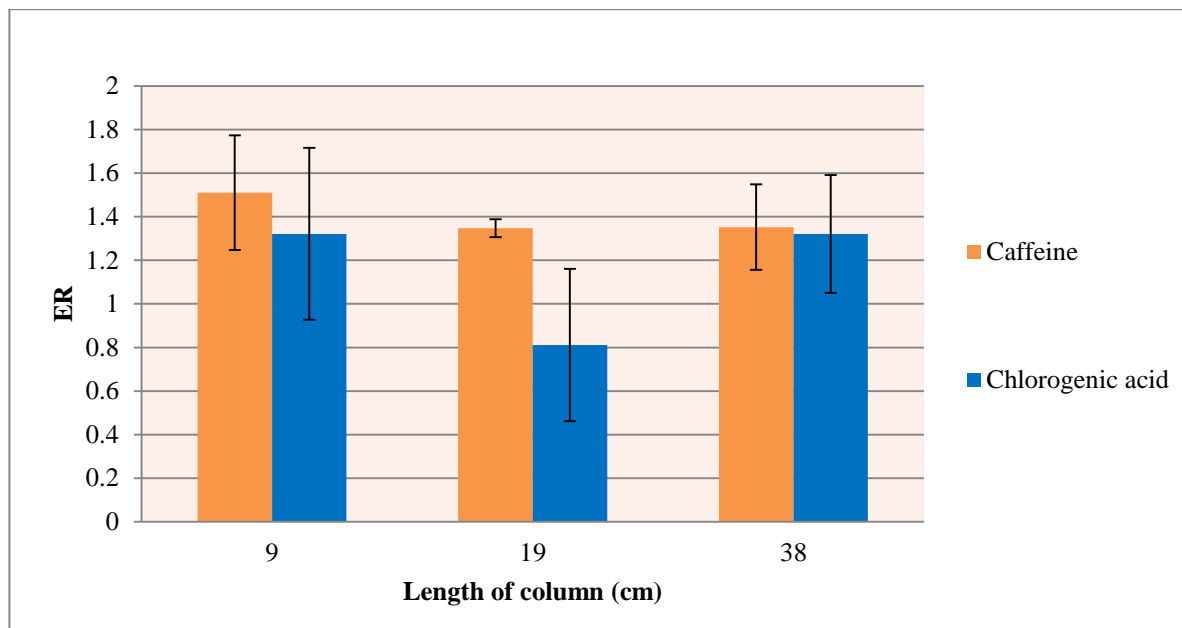
Figure 4. 58 Column height-dependent enrichment ratio (ER) of caffeine and chlorogenic acid in the foam fractionation process of coffee. The other parameters were kept in constant: pH original, 5.4; saponin amount, 50 mg/100 ml; coffee concentration, 50 mg/250 ml; flow rate, 12 ml/min; temperature, 20 ℃; initial solution volume, 40 ml
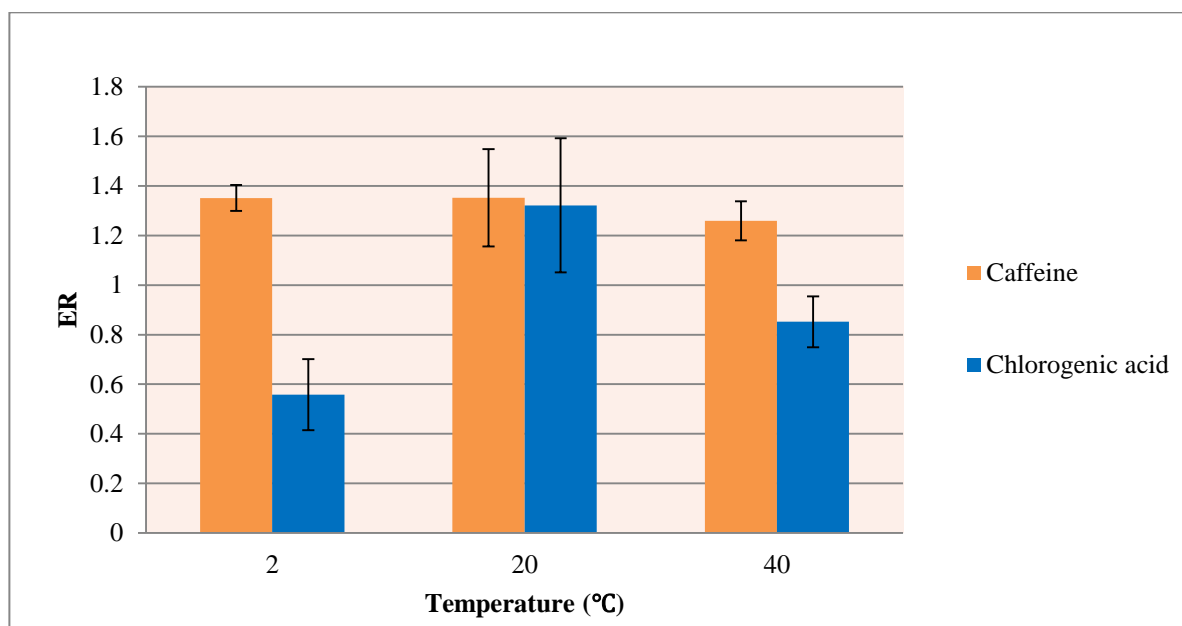
Figure 4. 59 Temperature-dependent enrichment ratio (ER) of caffeine and chlorogenic acid in the foam fractionation process of coffee. The other parameters were kept in constant: pH original, 5.4; saponin amount, 50 mg/100 ml; coffee concentration, 50 mg/250 ml; flow rate, 12 ml/min; column height, 38 cm; initial solution volume, 40 ml
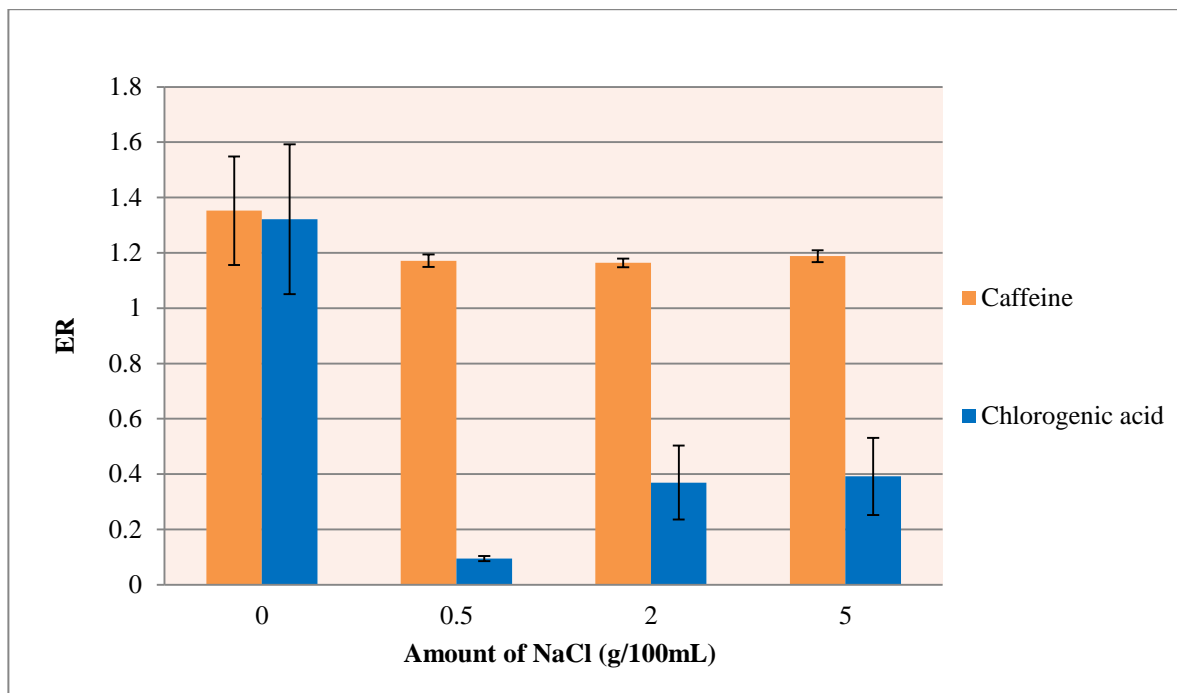
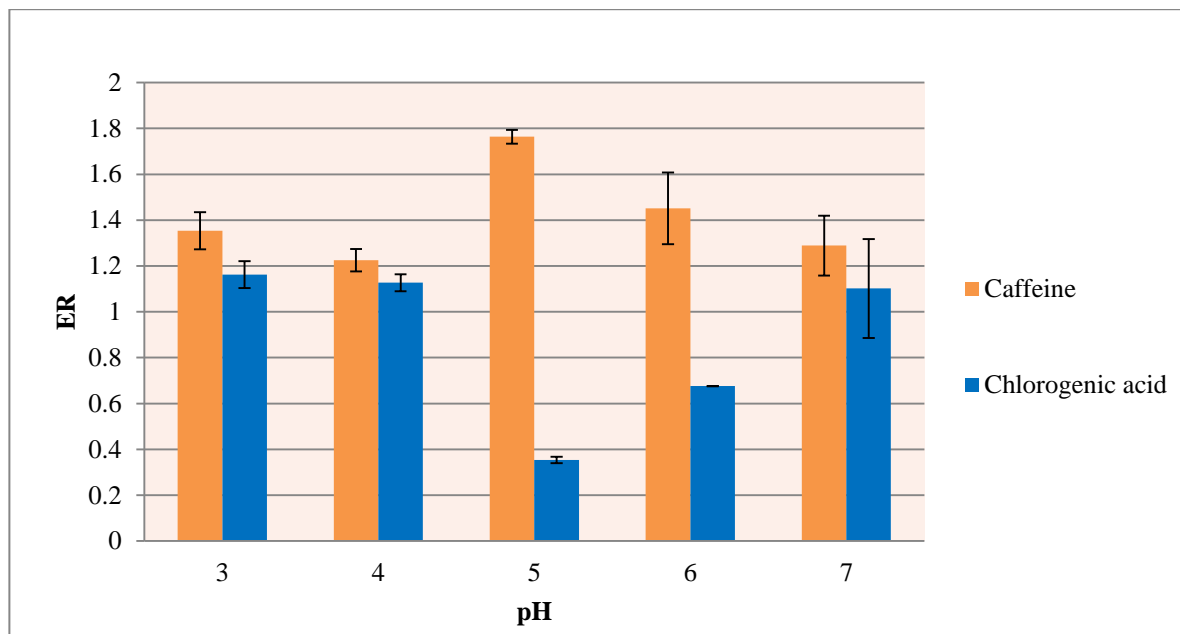**Figure 4. 60** NaCl amount-dependent enrichment ratio (ER) of caffeine and chlorogenic acid in the foam fractionation process of coffee. The other parameters were kept in constant: pH original, 5.4; saponin amount, 50 mg/100 ml; coffee concentration, 50 mg/250 ml; flow rate, 12 ml/min; column height, 38 cm; temperature, 20 ℃; initial solution volume, 40 ml

As the results indicate here, only pH value of the initial solution, saponin amount and gas flow rate showed a slight influence on the enrichment ratio of caffeine; a higher enrichment ratio of 1.76, 2.31 and 1.35 was observed at a pH value of 5, a saponin amount of 35 mg/100ml and a flow rate of 12 ml/min, respectively. For chlorogenic acid, the enrichment ratio was influence by almost every parameter; a higher enrichment ratio of 3.19 and 5.56 was obtained at a coffee concentration of 5 mg/250ml (equal to a chlorogenic acid concentration of 0.7×10^{-6} M) and a NaCl concentration of 0 mg/100ml, respectively. The difference in the enrichment behavior of caffeine and chlorogenic acid depending on varying parameters indicated that these two substances were not enriched simultaneously as a complex from the aqueous solution of green coffee, although the complex of caffeine and chlorogenic acid was proved to exist in coffee samples (D'Amelio, Fontanive et al. 2009). Moreover, almost no selectivity was observed in all the foam experiments conducted here for coffee: the composition of the gradients and their percentage in the initial solution, foamate and residual solution were not changed significantly. Therefore, no further experiments were carried on for coffee.

4.7 Comparison of the enrichment behavior of caffeine foamed with different catchers.

The enrichment ratios of caffeine foamed alone, with catchers and from crude coffee depending on the variation of each parameter are listed in Table 6.

As the results indicated in Table 6, the enrichment ratio of caffeine behaved significantly different when it was foamed alone, or with different catchers or from coffee crude samples directly.

For the foam experiments of caffeine alone, only three parameters, namely pH value, caffeine initial concentration, and flow rate had slight influence on the enrichment of caffeine, and also, only marginal enrichment was observed: 1.64 at pH 7 and 1.57 at pH 9, 1.27 at a caffeine concentration of 1×10^{-6} M, and 1.64 at the flow rate of 12 ml/min. Finally, an enrichment ratio of 3.01 was obtained for caffeine at the optimal value of each parameter. To increase the enrichment of caffeine, different catchers that are able to complex or interact with caffeine were foamed together.

When the complex of caffeine and chlorogenic acid was foamed directly from the aqueous solution, all the parameters were proved to have influence on the enrichment of caffeine. The optimal value of each parameter, at which a moderate enrichment for caffeine was obtained, was listed here: 3.94 at pH 3, 3.94 and 3.15 at a saponin concentration of 25 mg/100ml and 50 mg/100ml, 6.27 at a caffeine initial concentration of 1×10^{-6} M, 3.94 at a flow rate of 12 ml/min, 3.94 at a column height of 38 cm, 3.94 at a temperature of 20 ℃ and also 5.31 at a NaCl concentration of 5 g/100ml. After the optimization of all the parameters investigated, an enrichment ratio of 11.2 was obtained for caffeine.

For the foam experiments of caffeine with n-octyl caffeate and n-octyl caffeamide, almost no enrichment was observed for caffeine at each value of each parameter adopted, except a slight increase of the enrichment (2.23) at a NaCl concentration of 5 g/100ml in the foam experiments with n-octyl caffeamide.

Influence of the varying parameter on the enrichment of caffeine was also observed when it was foamed with caffeic acid; better enrichments were obtained at the corresponding optimal value of the parameters: 2.72 at pH 5, 2.40 and 2.72 at a saponin concentration of 12.5 mg/100ml and 25 mg/100ml respectively, 2.72 at a caffeine initial concentration of 1×10^{-5} M

(caffeine/caffeic acid = 1:1), 2.72 at a flow rate of 12 ml/min, 2.72 at a column height of 38 cm, 2.72 at a temperature of 20 °C, and 3.49 at a NaCl concentration of 0.5 mg/100ml. Finally, an enrichment ratio of 3.85 was observed for caffeine.

Table 6 Enrichment of caffeine when foamed alone, with different catchers and from crude coffee sample, depending on different operating parameters

	Caffeine alone	Chlorogenic acid	n-Octyl caffeate	n-Octyl caffeamide	Caffeic acid	Coffee
pH	7 (1.64) [a] 9 (1.57)	3 (3.94)	— [c]	—	5 (2.72)	5 (1.76)
Saponin amount (mg/100ml)	—	25 (3.94) [b] 50 (3.15)	—	—	12.5 (2.40) 25 (2.72)	35 (2.31)
Caffeine initial Concentration (M)	1×10^{-6} (1.27)	1×10^{-6} (6.27)	—	—	1×10^{-5} (2.72)	—
Flow rate (ml/min)	12 (1.64)	12 (3.94)	—	—	12 (2.72)	12 (1.35)
Height of column	—	38 (3.94)	—	—	38 (2.72)	—
Temperature (°C)	—	20 (3.94)	—	—	20 (2.72)	—
NaCl concentration (mg/100ml)	—	5 (5.31)	—	5 (2.23)	0.5 (3.49)	—
Total	(3.01)	(11.22)	N.D.	N.D.	(3.85)	N.D.

Note: [a] the value before the brackets is the value of the parameter, at which the foam experiment was conducted, and the value in the brackets is the enrichment ratio of caffeine obtained at the value of the corresponding parameter; [b] these values of enrichment ratio in the same cell were proved to be no difference statistically; [c] —, no enrichment was obtained for caffeine; N.D., not done; and the enrichment with a same value (such as 3.94 and 2.72) was obtained in the same foaming experiments, which was done first and used as a reference for the comparison later on.

For the foam experiments of coffee, only three parameters were proved to have an influence on the enrichment of caffeine in all the parameters investigated. A better enrichment of caffeine was obtained as follows: 1.76 at pH 5, 2.31 at saponin concentration of 35 mg/100ml, and 1.35 at a flow rate of 12 ml/min.

All the values exhibited above indicate that the different catchers had a different influence on the enrichment behavior of caffeine.

The complex formed by caffeine and chlorogenic acid was isolated almost one century ago by Gorter (Gorter 1907). This complex was described as a 1:1 hydrophobically bound π-molecular (Horman and Viani 1972), in which hydrogen bonding stabilizes the arrangement of different molecules (Horman and Viani 1972, Martin, Lilley et al. 1986 b) (also mentioned in section 4.3.4.8). In the scientific work of Horman and Viani (Horman and Viani 1972), series of substances including pyrocatechin, caffeate ion and chlorogenate were investigated for their ability to complex with caffeine in aqueous solution, and a corresponding association constant of 1.4, 12.2 and 16.9 was obtained (Figure 4.61 a, b and c), respectively. They ascribed the enhanced association ability of chlorogenate, compared with caffeate ion, to the binding from hydrogen bonding introduced by quinate moiety, which was only weakly complexed with caffeine, with an association constant of 0.8 (Figure 4.61 d).

Pyrocatechin, 1.4 Caffeate, 12.2

Chlorogenate, 16.9 Quinate, 0.8
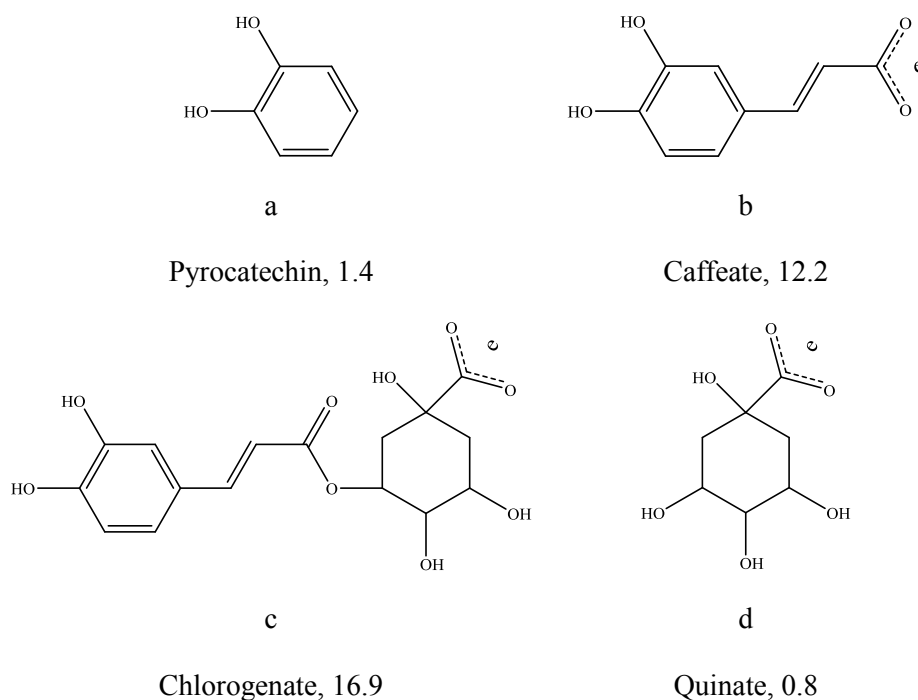
Figure 4. 61 Association constants for the formation of complexes between caffeine and compounds studied (Martin, Lilley et al. 1986 b)

Actually, the hydrogen bonds are both contributed by the hydroxyls from the caffeic acid moiety and the quinic acid moiety in chlorogenic acid, as shown in Figure 4.62 (Martin, Lilley et al. 1986 b).

Understandably, the absence of the quinic acid moiety in n-octyl caffeate, n-octyl caffeamide would certainly decrease the stability of the complex formed with caffeine or attenuate the interaction between them, thus enhance the occurrence of decomplexation of the complex in the foaming process. As a result, compared with chlorogenic acid, complexes formed between n-octyl caffeate, n-octyl caffeamide and caffeine would be more easily to be decomplexed during the foaming process. The strong hydrophobicity of the 8-carbon alkyl group in both n-octyl caffeate and n-octyl caffeamide makes them more prone to be adsorbed at the gas-liquid interface, but leaving the caffeine molecule behind in the bulk solution when the interactions between the catchers and caffeine are too weak. Moreover, the poor hydrophobicity of caffeine makes itself in absolute inferiority in the competition of the occupation of the adsorption site at the gas-liquid interface with its catchers, thus an even worse enrichment efficiency of caffeine was observed, compared with caffeine foamed alone; in contrast, a very efficient enrichment for the catchers was obtained. For caffeic acid, the hydrogen bonds contributed by the hydroxyls at the quinic acid moiety in chlorogenic acid may be replaced by the one contributed by the carboxylic hydroxyl from caffeic acid. Therefore, similar enrichment behavior of caffeine and caffeic acid was observed in their foam experiments.
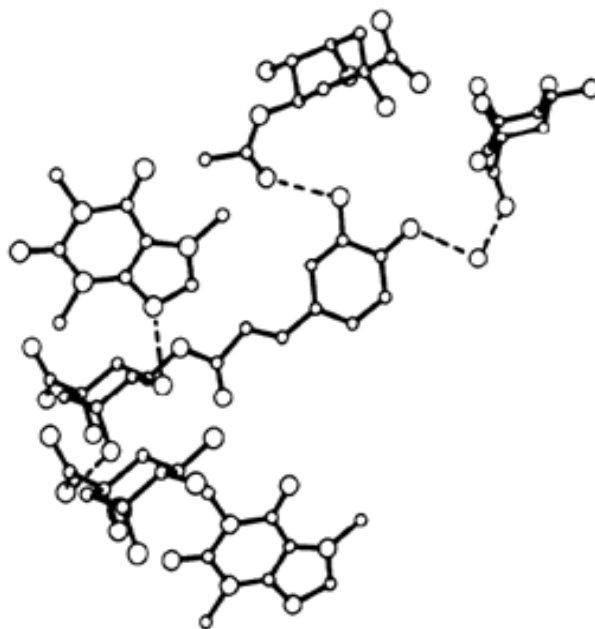
Figure 4. 62 The extensive hydrogen bonding network in caffeine-chlorogenic acid complex (Martin, Lilley et al. 1986 b)

Apparently, the complex formed between caffeine and chlorogenic acid is much more stable compared with the ones formed with the other catchers; therefore, similar enrichment behavior of them was observed in most situations in the foam experiments. However, only a moderate increase of the enrichment of caffeine was observed compared with that of caffeine foamed alone. This may partly ascribe to the low percentage (about 30% of the caffeine molecules) of the complex in the aqueous solution of caffeine and chlorogenic acid (D'Amelio, Fontanive et al. 2009), but what equally important could be the reason of the hydrophobicity of the complex, which is not so ideal for the foam fractionation. Therefore, n-octyl caffeate was synthesized, aiming to interact with caffeine to form a complex which is more easily to adsorb at the gas-liquid interface. Concerning the poor solubility of n-octyl caffeate in water, n-octyl caffeamide was synthesized.

As shown in Table 6, the pH value for a better enrichment for caffeine is different when it was foamed with different catchers. For caffeine foamed alone, a pH value of 7 may decrease the solubility of caffeine to its minimum in the aqueous solution, thus a better enrichment ratio was obtained. In comparison, the best enrichment ratio was obtained at a pH value of 3 for the foaming of the caffeine and chlorogenic acid complex. Probably, this could be ascribe to two reasons: first, the complex of caffeine and chlorogenic acid may have a poor solubility at pH 3, since the electron-donating N atom at position 9 in caffeine may be enveloped because of the stacking of the molecules in the complex; second, it was proved that the proportion of the complex in the aqueous solution is higher at a lower pH value (Sondheimer, Covitz et al. 1961). The similar explanation may also applicable to the situation of caffeic acid and coffee foam experiments, in which a better enrichment ratio for caffeine was obtained at a pH value of 5.

Since chlorogenic acid was proved to be the most efficient catcher to enhance the enrichment of caffeine in the foam fractionation presented in this study, and moreover, the complex exists naturally in the coffee sample, the foam experiments of green coffee sample were therefore conducted, aiming to enrich caffeine in the form of complex between caffeine and chlorogenic acid. However, the situation in practice was much more complicated than what we imagined. Caffeine is known to interact with a lot of polyphenolic molecules (as mentioned in section 2.3.2), thus all of these interactions may disturb the foam fractionation of caffeine from the crude coffee sample. Since the complexation between caffeine and chlorogenic acid is governed by π stacking interaction and stabilized by hydrogen bonding, the complex could be partly decomposed by the introduction of the gas-liquid interface in

the foaming process, and then may re-complex with chlorogenic acid molecules or other molecules exist in the coffee sample, which would have a very complicate and unpredictable effect on the enrichment of caffeine. Moreover, the other relative hydrophobic substances will also influence the separation process; even a lot of insoluble substances have already been removed by filtration before foaming.

For the other parameters, similar results were obtained. For example, a lower concentration (1×10^{-6} M) of caffeine in the initial solution was favorable for the foam fractionation; a lower flow rate (12 ml/min) was also beneficial for the enrichment, since longer time was provided for the collapse and drainage, during which separation happens; a higher column (38 cm) had a similar effect with the lower flow rate for caffeine enrichment; too low (0 ℃) or too high (40 ℃) a temperature all influenced the stability of the foam; the adding of NaCl enhanced the adsorption of the caffeine molecules on the gas-liquid interface and hence increased the enrichment of caffeine.

As a separation technique of long time ago developed but newly came back to the view of public, foam fractionation is environmental friendly and low cost. However, it is not commonly used up to now. One reason is the lack of sufficient understanding of the complicated principles governing the separation process, which makes it difficult for prediction. Another reason is the numerous parameters that may influence the separation efficiency in the foaming process, such as pH value, temperature, initial concentration, column height, ionic strength. All of the parameters need to be varied and then optimized, since all of them may influence the results. And also, the separation parameters is not universally applicable, but should be specialized for each of the substances which are going to be separated, even the same substances but from different matrix. In the present research here, beside the difficulties mentioned above, a specific catcher, which is not only surface active but also selectively and firmly bond with the target molecules, is needed.

5. Conclusions

Foam fractionation represents a method of eco-friendly and low cost for the separation of natural products. The substances that are able to be separated using foam fractionation should be hydrophobic or surface active, or able to form a complex which is hydrophobic or surface active. Caffeine, a polar substance with hydrophobic portions but able to interact with a lot of other substances, was subject to foam fractionation in the present study. Here, some conclusions are drawn as follows:

- As a polar substance, extremely poor enrichment ratios were obtained for caffeine in all the parameter-dependent foam fractionation experiments conducted using its aqueous solution in the present study.

- When foamed with chlorogenic acid, which forms a π stacking based and hydrogen bonding stabilized complex with caffeine in aqueous solution, a moderate increase of the enrichment of caffeine was observed in the foam fractionation experiments.

- The hydrogen bonding contributed by the quinic acid moiety in chlorogenic acid is very important for the stability of the complex in the foaming process. Decrease or absence of these hydrogen bonds in the interaction with caffeic acid, n-octyl caffeate and n-octyl caffeamide, resulted in attenuated enhancement of the enrichment or even no enrichment ratio for caffeine.

- As different complexes were formed or different interactions happened during the foam experiments of caffeine with chlorogenic acid, n-octyl caffeate, n-octyl caffeamide and caffeic acid, pH values for a better enrichment of caffeine were different.

- The foam fractionation of caffeine from green coffee sample was much more complicated, because of the disturbances from not only many substances which are able to interact with caffeine molecules, but also the other substances which are equally polar.

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- As foam fractionation experiments, some general conclusions were also obtained: a lower concentration of the molecule, a lower flow rate, a longer column, and the increase of ionic strength would be normally beneficial for the enrichment of the target molecules.

6. Summary

Compared with the traditional solvent extraction methods and chromatographic techniques, foam fractionation technique consumes no or very limited amount of organic solvents, and also is low cost because of the simplicity of the operation devices and their maintenance. Based on the difference in the adsorption ability of the substances at the gas-liquid interface provided by foam, substances with a different surface activity would be preferentially and selectively enriched in the foamate in the process of foam fractionation. Up to now, the researches on foam fractionation techniques have been explored not only in the field of protein and enzyme separation, but also in the field of bioactive natural products separation.

Caffeine, a substance naturally produced by many plants, is now the most widely consumed drug by human being. Besides coffee and tea, it is also found in common soft drinks, such as cola, as well as the products containing cocoa and chocolate, and also plenty of medications and dietary supplements. In industry, caffeine is acquired as a byproduct of coffee decaffeination, which is normally complicated and energy consuming. Therefore, the enrichment of caffeine using foam fractionation technique would be meaningful: to decrease the caffeine amount in coffee and provide raw caffeine material for downstream food and medicine industry, with a more sustainable cost. Further, the foam fractionation of caffeine assisted by molecular interactions would be somehow a prediction for the application of this technique in the field of the separation of natural products which are able to interact with other compounds and form surface active complexes.

After the summation of the theoretical background of the foam fractionation technique and the introduction of the materials and methods used in this research, the foam fractionation of caffeine from aqueous solution containing different catchers was presented.

Firstly, the aqueous solution of caffeine was foamed, with series of variation of each parameter. However, it was proved that the foam fractionation of caffeine was inefficient. Therefore, the complexation of caffeine with other catchers to form a hydrophobic or surface active complex was necessary.

Four caffeic acid structure based substances, namely chlorogenic acid, n-octyl caffeate, n-octyl caffeamide and caffeic acid, were selected as catchers to be foamed with caffeine in aqueous solution. Chlorogenic acid and caffeic acid were purchased, while n-octyl caffeate

and n-octyl caffeamide were synthesized and their structures were confirmed by MS and NMR experiments. Caffeine-chlorogenic acid complex was readily prepared, however, the complex with the other three catchers were not able to be obtained in this research. The foamability and foam stability of the complex or catchers were evaluated, and the results indicated that saponin was needed for the foam experiments. The complexation between caffeine and four catchers in aqueous solution was proved by the observation of the bathochromic effects of molar extinction coefficient of the catchers in spectrophometric titration with caffeine. Foam fractionation experiments were conducted with variation of each parameter, in order to evaluate their influence on the enrichment ratio of caffeine.

A moderate increase of the enrichment was observed for caffeine in the foaming experiments of caffeine-chlorogenic acid complex compared with that of caffeine alone. Compared with chlorogenic acid, the 8-C alkyl chain in n-octyl caffeate and n-octyl caffeamide enabled them to be much more surface active. However, the results indicate that no enrichment of caffeine was obtained when it was foamed with these two catchers. Compared with caffeine, both of these two catchers were enriched very efficiently into the foamate. For caffeic acid, the results showed that caffeine was slightly enriched in the foam experiments. The similarity in the enrichment behavior between caffeine and its catchers, chlorogenic acid and caffeic acid, depending on the variation of each parameter, indicates that caffeine was enriched into the foamate almost simultaneously with these two catchers, which may ascribe to the complexation or interaction between them.

Finally, the aqueous solution of green coffee sample was foamed directly, since chlorogenic acid was proved to be most effective in all the catchers tested, and also caffeine and chlorogenic acid are readily contained in the coffee sample in a ratio of almost 1 to 1. All the parameters were also varied to investigate their influence on the enrichment of caffeine. Unfortunately, only slight enrichment was obtained for caffeine.

In conclusion, polar substances such as caffeine can be enriched by foam fractionation technique, assisted by molecular interaction based complexation, from a relatively uncomplicated aqueous system. However, a catcher which is more surface active and is also able to form a more stable complex selectively with the target molecules, is essential for a successful separation with higher efficiency from real complicated biological matrixes.

7. Zusammenfassung

Verglichen mit den herkömmlichen Lösungsmittelextraktionsverfahren und chromatographischen Techniken verbraucht die Zerschäumungstechnik (oder auch Schaumfraktionierung genannt) keine oder nur sehr begrenzte Mengen an organischen Lösungsmitteln. Darüber hinaus ist sie auch kostengünstig aufgrund der einfachen Betriebseinheiten und ihrer simplen Wartung. Basierend auf der unterschiedlichen Adsorptionsfähigkeit der Substanzen an der Gas – Flüssigkeits – Grenzfläche im Schaum werden Stoffe mit unterschiedlicher Oberflächenaktivität bevorzugt und selektiv im Konzentrat der Zerschäumung angereichert. Bis jetzt waren die Zerschäumungsversuche nicht nur auf das Gebiet der Protein - und Enzymtrennung begrenzt, sondern wurden auch auf das Gebiet der bioaktiven Naturstofftrennung ausgeweitet.

Koffein, eine Substanz die auf natürliche Weise von vielen Pflanzen produziert wird, ist derzeit die am häufigsten vom Menschen konsumierte Droge. Neben Kaffee und Tee ist sie auch in Softdrinks wie Coca Cola, Kakao und Schokoladeprodukten enthalten. Daneben kommt sie auch in vielen Medikamenten und Nahrungsergänzungsmitteln vor. In der Industrie wird Koffein als ein Nebenprodukt der üblicherweise komplizierten und energieaufwendigen Entkoffeinierung von Kaffee gewonnnen. Daher wäre die Anreicherung von Koffein mit Hilfe der Schaumfraktionierungstechnik sinnvoll: Man könnte auf einfache Weise bei geringen Kosten die Koffeinmengen im Kaffee reduzieren und reines Koffein für die Nahrungsmittel- und Pharmaindustrie gewinnen. Außerdem würde die erfolgreiche Zerschäumung von Koffein - unterstützt durch molekulare Wechselwirkungen - eine Vorhersage über die Anwendungsmöglichkeiten dieser Technik auf dem Gebiet der Trennung von natürlichen Produkten ermöglichen, die in der Lage sind, mit anderen Verbindungen Wechselwirkungen einzugehen und dabei oberflächenaktive Komplexe bilden zu können.

Nach der Zusammenfassung des theoretischen Hintergrundes der Zerschäumungstechnik und der Beschreibung der in dieser Arbeit verwendeten Materialien und Methoden wird die Schaumfraktionierung von Koffein aus einer wässrigen Lösung, die unterschiedliche Catchern enthält, dargestellt.

Zunächst wurde die wässrige Koffeinlösung mit einer Reihe von Variationen der einzelnen Parameter geschäumt. Es konnte dabei bewiesen werden, dass die Schaumfraktionierung

von Koffein ineffizient war. Daher war die Komplexierung von Koffein mit anderen Catchern notwendig, um einen hydrophoben oder oberflächenaktiven Komplex zu erhalten.

Vier koffeinsäurestrukturbasierte Substanzen, nämlich Chlorogensäure, n-Octyl caffeate, n-Octyl caffeamide und Kaffeesäure, wurden als Catchern ausgewählt, um mit Koffein in wässriger Lösung geschäumt zu werden. Chlorogensäure und Kaffeesäure wurden dafür käuflich erworben, während n-Octyl caffeate und n-Octyl caffeamide synthetisiert wurden. Ihre Strukturen konnten durch MS und NMR Experimente bestätigt werden. Der Koffein-Chlorogensäure-Komplex konnte leicht hergestellt werden, wohingegen der Komplex mit den anderen drei Catchern leider nicht in dieser Arbeit gelang. Die Schäumbarkeit und Schaumstabilität des Komplexes oder der Catchern wurden bewertet. Die Ergebnisse zeigten, dass Saponin für die Schäumungsexperimente notwendig war. Die Komplexbildung zwischen Koffein und den vier Catchern in wässriger Lösung wurde durch Beobachtung der bathochromen Effekte des molaren Extinktionskoeffizienten der Catchern in spektrophotometrischer Titration mit Koffein nachgewiesen. Die Schaumfraktionierungsversuche wurden unter Variation der einzelnen Parameter durchgeführt, um deren Einfluss auf das Anreicherungsverhältnis von Koffein zu beurteilen.

Eine moderate Erhöhung der Anreicherung für Koffein wurde beobachtet in den Zerschäumungsexperimenten des Koffein-Chlorogensäure-Komplexes verglichen mit Koffein allein. Verglichen mit Chlorogensäure ermöglichte die 8-C Alkylkette in n-Octyl caffeate und n-Octyl caffeamide diesen Substanzen eine höhere Oberflächenaktivität. Jedoch zeigten die Ergebnisse, dass keine Anreicherung von Koffein erfolgte, wenn es mit diesen beiden Catchern aufgeschäumt wurde. Verglichen mit Koffein wurden diese beiden Catchern sehr effizient im Konzentrat angereichert. Für Kaffeesäure zeigten die Ergebnisse, dass das Koffein leicht in den Zerschäumungsexperimenten angereichert wurde. Die Ähnlichkeit im Anreicherungsverhalten zwischen Koffein und seinen beiden Catchern Chlorogensäure und Kaffeesäure war abhängig von der Variation der einzelnen Zerschäumungsparameter. Dies ist ein Hinweis darauf, dass Koffein im Konzentrat gleichzeitig mit den beiden Catchern angereichert wurde, was möglicherweise der Komplexierung oder Wechselwirkung zwischen ihnen zuzuschreiben ist.

Schließlich wurde die wässrige Lösung einer grünen Rohkaffeeprobe direkt geschäumt, da sich Chlorogensäure als am wirksamsten von allen getesteten Catchern erwies. Dabei wurden ebenfalls Koffein und Chlorogensäure leicht in einem Verhältnis von nahezu 1 zu 1

erhalten. Alle Parameter wurden außerdem variiert, um ihren Einfluss auf die Anreicherung von Koffein zu untersuchen. Leider wurde nur eine geringe Anreicherung für Koffein erzielt.

Abschließend kann gesagt werden, dass polare Substanzen wie Koffein durch Zerschäumung in einer relativ unkomplizierten wässrigen Lösung angereichert werden können, unterstützt durch molekulare Wechselwirkung basierend auf Komplexierung. Die Anwesenheit eines Catchern ist jedoch unabdingbar, der oberflächenaktiver und zudem in der Lage ist, einen stabileren Komplex selektiv mit den Zielmolekülen zu bilden, um eine erfolgreiche Trennung mit höherer Effizienz bei echten, wirklich komplizierten biologischen Matrices zu erreichen.

8. Literature

Adam, K. P. (1995). "Caffeic Acid-Derivatives in Fronds of the Lady Fern (Athyrium-Filix-Femina)." Phytochemistry **40**(5): 1577-1578.

Ahmad, I., S. Ahmed, M. A. Sheraz, M. Aminuddin and F. H. Vaid (2009). "Effect of caffeine complexation on the photolysis of riboflavin in aqueous solution: a kinetic study." Chem Pharm Bull (Tokyo) **57**(12): 1363-1370.

Ahmad, S. I. (1975). "Laws of Foam Formation and Foam Fractionation. I. The Effect of Different Operating Parameters on the Foam Fractionation of Albumin from a Solution Containing Organic and Inorganic Materials." Separation Science **10**(6): 673-688.

Andrews, G. and F. Schutz (1945). "Differential adsorption of pepsin and rennin on foam." Biochem J **39**(5): li.

Andrews, K. W., A. Schweitzer, C. Zhao, J. M. Holden, J. M. Roseland, M. Brandt, J. T. Dwyer, M. F. Picciano, L. G. Saldanha, K. D. Fisher, E. Yetley, J. M. Betz and L. Douglass (2007). "The caffeine contents of dietary supplements commonly purchased in the US: analysis of 53 products with caffeine-containing ingredients." Analytical and Bioanalytical Chemistry **389**(1): 231-239.

Antonov, L., G. Gergov, V. Petrov, M. Kubista and J. Nygren (1999). "UV-Vis spectroscopic and chemometric study on the aggregation of ionic dyes in water." Talanta **49**(1): 99-106.

Arab, L. and J. B. Blumberg (2008). "Introduction to the proceedings of the Fourth International Scientific Symposium on Tea and Human Health." Journal of Nutrition **138**(8): 1526-1528.

Ashihara, H. and A. Crozier (2001). "Caffeine: a well known but little mentioned compound in plant science." Trends in Plant Science **6**(9): 407-413.

Backleh-Sohrt, M., P. Ekici, G. Leupold and H. Parlar (2005). "Efficiency of foam fractionation for the enrichment of nonpolar compounds from aqueous extracts of plant materials." <u>J Nat Prod</u> **68**(9): 1386-1389.

Backleh, M., P. Ekici, G. Leupold and H. Parlar (2003). "Quantitative elimination of Flavokavines A and B from Kava Kava (Piper methysticum G. Forst) by isoelectric focused adsorptive bubble separation." <u>Naturwissenschaften</u> **90**(8): 366-369.

Backleh, M., G. Leupold and H. Parlar (2003). "Rapid quantitative enrichment of carnosic acid from rosemary (Rosmarinus officinalis L.) by isoelectric focused adsorptive bubble chromatography." <u>J Agric Food Chem</u> **51**(5): 1297-1301.

Banerjee, R., R. Agnihotri and B. C. Bhattacharyya (1993). "Purification of Alkaline Protease of Rhizopus-Oryzae by Foam Fractionation." <u>Bioprocess Engineering</u> **9**(6): 245-248.

Barone, J. J. and H. R. Roberts (1996). "Caffeine consumption." <u>Food and Chemical Toxicology</u> **34**(1): 119-129.

Bhattacharjee, S., R. Kumar and K. S. Gandhi (2001). "Modelling of protein mixture separation in a batch foam column." <u>Chemical Engineering Science</u> **56**(19): 5499-5510.

Bhattacharya, P., S. K. Ghosal and K. Sen (1991). "Effect of Physicochemical Parameters on the Separation of Proteins from Human Placental Extract by Using a Continuous Foam Fractionating Column." <u>Separation Science and Technology</u> **26**(10-11): 1279-1293.

Brasch, D. J., N. N. L. Ngeh and K. R. Robilliard (1983). "The Foam Separation of Some Polysaccharide Mixtures." <u>Carbohydrate Research</u> **116**(1): 1-19.

Brown, L., G. Narsimhan and P. C. Wankat (1990). "Foam fractionation of globular proteins." <u>Biotechnol Bioeng</u> **36**(9): 947-959.

Brown, L., G. Narsimhan and P. C. Wankat (1990). "Foam fractionation of globular proteins Biotechnology and Bioengineering Volume 36, Issue 9." Biotechnology and Bioengineering **36**(9): 947-959.

Bucar, F., A. Wube and M. Schmid (2013). "Natural product isolation--how to get from biological material to pure compounds." Nat Prod Rep **30**(4): 525-545.

Burghoff, B. (2012). "Foam fractionation applications." J Biotechnol **161**(2): 126-137.

Cai, Y., S. H. Gaffney, T. H. Lilley, D. Magnolato, R. Martin, C. M. Spencer and E. Haslam (1990). "Polyphenol interactions. Part 4. Model studies with caffeine and cyclodextrins." Journal of the Chemical Society, Perkin Transactions 2(12): 2197.

Chan, E. W. C., Y. Y. Lim, S. K. Ling, S. P. Tan, K. K. Lim and M. G. H. Khoo (2009). "Caffeoylquinic acids from leaves of Etlingera species (Zingiberaceae)." LWT - Food Science and Technology **42**(5): 1026-1030.

Charlton, A. J., A. L. Davis, D. P. Jones, J. R. Lewis, A. P. Davies, E. Haslam and M. P. Williamson (2000). "The self-association of the black tea polyphenol theaflavin and its complexation with caffeine." Journal of the Chemical Society-Perkin Transactions 2(2): 317-322.

Charm, S. E., J. Morningstar, C. C. Matteo and B. Paltiel (1966). "The separation and purification of enzymes through foaming." Analytical Biochemistry **15**(3): 498-508.

Chen, C.-Y., S. C. Baker and R. C. Darton (2006). "Batch production of biosurfactant with foam fractionation." Journal of Chemical Technology & Biotechnology **81**(12): 1923-1931.

Chen, C.-Y., S. C. Baker and R. C. Darton (2006). "Continuous production of biosurfactant with foam fractionation." Journal of Chemical Technology & Biotechnology **81**(12): 1915-1922.

Chiu, H.-L. and S.-D. Huang (1991). "Adsorptive Bubble Separation of Heptachlor and Hydroxychlordene." Separation Science and Technology **26**(1): 73-83.

Chou, T. M. and N. L. Benowitz (1994). "Caffeine and Coffee - Effects on Health and Cardiovascular-Disease." Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology **109**(2): 173-189.

Clarke, R. J. (2003). "COFFEE | Decaffeination." 1506-1511.

Clarkson, J. R., Z. F. Cui and R. C. Darton (2000). "Effect of solution conditions on protein damage in foam." Biochemical Engineering Journal **4**(2): 107-114.

Crofcheck, C., M. Loiselle, J. Weekley, I. Maiti, S. Pattanaik, P. M. Bummer and M. Jayt (2003). "Histidine tagged protein recovery from tobacco extract by foam fractionation." Biotechnology Progress **19**(2): 680-682.

Crofcheck, C., I. Maiti, S. Pattanaik and M. Jay (2004). "Effect of ion and surfactant choice on the recovery of a histidine-tagged protein from tobacco extract using foam fractionation." Applied Biochemistry and Biotechnology **119**(1): 79-91.

D'Amelio, N., L. Fontanive, F. Uggeri, F. Suggi-Liverani and L. Navarini (2009). "NMR Reinvestigation of the Caffeine–Chlorogenate Complex in Aqueous Solution and in Coffee Brews." Food Biophysics **4**(4): 321-330.

Davis, D. A., H. C. Lynch and J. Varley (2001). "The application of foaming for the recovery of Surfactin from B. subtilis ATCC 21332 cultures." Enzyme Microb Technol **28**(4-5): 346-354.

DeLucena, S. L., E. A. Miranda and C. C. Santana (1996). "The effect of external reflux on the foam fractionation of proteins." Applied Biochemistry and Biotechnology **57-8**: 57-65.

Eastoe, J. and J. S. Dalton (2000). "Dynamic surface tension and adsorption mechanisms of surfactants at the air–water interface." Advances in Colloid and Interface Science **85**(2-3): 103-144.

Ekici, P., M. Backleh-Sohrt and H. Parlar (2005). "High efficiency enrichment of total and single whey proteins by pH controlled foam fractionation." Int J Food Sci Nutr **56**(3): 223-229.

Elworthy, P. H. and K. J. Mysels (1966). "The surface tension of sodium dodecylsulfate solutions and the phase separation model of micelle formation." Journal of Colloid and Interface Science **21**(3): 331-347.

Etzenhouser, B., C. Hansch, S. Kapur and C. D. Selassie (2001). "Mechanism of toxicity of esters of caffeic and dihydrocaffeic acids." Bioorg Med Chem **9**(1): 199-209.

Friedman, M. and H. S. Jurgens (2000). "Effect of pH on the stability of plant phenolic compounds." J Agric Food Chem **48**(6): 2101-2110.

Fu, Y.-C., P.-Y. Huang and C.-J. Chu (2005). "Use of continuous bubble separation process for separating and recovering starch and mucilage from yam (Dioscorea pseudojaponica yamamoto)." LWT - Food Science and Technology **38**(7): 735-744.

Fujioka, K. and T. Shibamoto (2008). "Chlorogenic acid and caffeine contents in various commercial brewed coffees." Food Chemistry **106**(1): 217-221.

Gattuso, G., G. Manfredi and S. Sammartano (2011). "Quantitative study on the non-covalent interactions between ATP and caffeine, theophylline and theobromine in aqueous solution." Fluid Phase Equilibria **308**(1-2): 47-54.

Gerken, B., A. Nicolai, D. Linke, H. Zorn, R. Berger and H. Parlar (2006). "Effective enrichment and recovery of laccase C using continuous foam fractionation." Separation and Purification Technology **49**(3): 291-294.

Gerken, B. M., C. Wattenbach, D. Linke, H. Zorn, R. G. Berger and H. Parlar (2005). "Tweezing-adsorptive bubble separation. Analytical method for the selective and high enrichment of metalloenzymes." Anal Chem **77**(19): 6113-6117.

Gibbs, J. W. (1928). The collected works of J. Willard Gibbs. New York,, Longmans, Green.

Gorter, K. (1907). "Beitrage zur Kenntnis des Kaffees." Ann. Chem. **359**: 217.

Gould, R. F. (1968). "Molecular Association in Biological and Related Systems, Copyright, Advances in Chemistry Series, FOREWORD." **84**: i-vi.

Grieves, R. B. and R. C. Aronica (1966). "Foam fractionation of phenol." Nature **210**(5039): 901-903.

Grieves, R. B., W. Charewicz and S. M. Brien (1974). "The separation of phenol from dilute, alkaline aqueous solution by solvent extraction, solvent sublation, and foam fractionation." Analytica Chimica Acta **73**(2): 293-300.

Haller, D., P. Ekici, A. Friess and H. Parlar (2010). "High enrichment of MMP-9 and carboxypeptidase A by tweezing adsorptive bubble separation (TABS)." Appl Biochem Biotechnol **162**(6): 1547-1557.

Hayashi, N., T. Ujihara and K. Kohata (2004). "Binding energy of tea catechin/caffeine complexes in water evaluated by titration experiments with H-1-NMR." Bioscience Biotechnology and Biochemistry **68**(12): 2512-2518.

Heckman, M. A., J. Weil and E. G. de Mejia (2010). "Caffeine (1, 3, 7-trimethylxanthine) in Foods: A Comprehensive Review on Consumption, Functionality, Safety, and Regulatory Matters." Journal of Food Science **75**(3): R77-R87.

Holmstro.B (1968). "Foam Concentration of Streptokinase from Crude Culture Filtrates." Biotechnology and Bioengineering **10**(4): 551-&.

Horman, I. and R. Viani (1972). "The Nature and Conformation of the Caffeine-Chlorogenate Complex of Coffee." Journal of Food Science **37**(6): 925-927.

Jaikang, C., C. Chaiyasut, P. Narongchai, K. Niwatananun, S. Narongchai and W. Kusirisin (2011). "Inhibitory effects of caffeic acid ester analogues on free radicals and human liver microsome CYP1A2 activities." Med Chem **7**(2): 99-105.

Jayaprakasam, B., M. Vanisree, Y. Zhang, D. L. Dewitt and M. G. Nair (2006). "Impact of alkyl esters of caffeic and ferulic acids on tumor cell proliferation, cyclooxygenase enzyme, and lipid peroxidation." J Agric Food Chem **54**(15): 5375-5381.

Jobstl, E., J. P. Fairclough, A. P. Davies and M. P. Williamson (2005). "Creaming in black tea." J Agric Food Chem **53**(20): 7997-8002.

Kan, L. S., P. N. Borer, D. M. Cheng and P. O. Ts'o (1980). "1H- and 13C-NMR studies on caffeine and its interaction with nucleic acids." Biopolymers **19**(9): 1641-1654.

Kapuscinski, J. and M. Kimmel (1993). "Thermodynamical model of mixed aggregation of intercalators with caffeine in aqueous solution." Biophys Chem **46**(2): 153-163.

Karger, B. L. and D. G. Devivo (1968). "General Survey of Adsorptive Bubble Separation Processes." Separation Science **3**(5): 393-424.

Koehler, S. A., S. Hilgenfeldt and H. A. Stone (2000). "A Generalized View of Foam Drainage: Experiment and Theory." Langmuir **16**(15): 6327-6341.

Koehler, S. A., Sascha Hilgenfeldt and H. A. Stone (2000). "A generalized view of foam drainage- experiment and theory." Langmuir **16**(15): 6327-6341.

Lalchev, Z., L. Dimitrova, P. Tzvetkova and D. Exerowa (1982). "Foam separation of DNA and proteins from solutions Biotechnology and Bioengineering Volume 24, Issue 10." Biotechnology and Bioengineering **24**(10): 2253-2262.

Lemlich, R. (1968). "Adsorptive Bubble Separation Methods - Foam Fractionation and Allied Techniques." Industrial and Engineering Chemistry **60**(10): 16-&.

Lemlich, R. (1972). Adsorptive Bubble Separation Techniques, Academic Press.

Lemlich, R. and E. Lavi (1961). "Foam Fractionation with Reflux." Science **134**(347): 191-&.

Li, C., Y. Li, R. Yuan and W. Lv (2013). "Study of the microcharacter of ultrastable aqueous foam stabilized by a kind of flexible connecting bipolar-headed surfactant with existence of magnesium ion." Langmuir **29**(18): 5418-5427.

Li, R., Z. L. Wu, Y. J. Wang and L. L. Li (2013). "Separation of total saponins from the pericarp of Sapindus mukorossi Gaerten. by foam fractionation." Industrial Crops and Products **51**: 163-170.

Linke, D., M. Nimtz, R. G. Berger and H. Zorn (2009). "Separation of Extracellular Esterases from Pellet Cultures of the Basidiomycete Pleurotus sapidus by Foam Fractionation." Journal of the American Oil Chemists' Society **86**(5): 437-444.

Linke, D., H. Zorn, B. Gerken, H. Parlar and R. G. Berger (2007). "Laccase isolation by foam fractionation—New prospects of an old process." Enzyme and Microbial Technology **40**(2): 273-277.

Liu, S., C. Elmer, N. H. Low and M. T. Nickerson (2010). "Effect of pH on the functional behaviour of pea protein isolate–gum Arabic complexes." Food Research International **43**(2): 489-495.

Liu, W., H. X. Zhang, Z. L. Wu, Y. J. Wang and L. J. Wang (2013). "Recovery of isoflavone aglycones from soy whey wastewater using foam fractionation and acidic hydrolysis." J Agric Food Chem **61**(30): 7366-7372.

Lockwood, C. E., P. M. Bummer and M. Jay (1997). "Purification of proteins using foam fractionation." Pharmaceutical Research **14**(11): 1511-1515.

Loha, V., A. Prokop, L. Du and R. D. Tanner (1999). "Preserving the activity of cellulase in a batch foam fractionation process." Appl Biochem Biotechnol **77-79**: 701-712.

Ma, J. G., Z. L. Xiu, D. J. Zhang and L. Y. Jia (2002). "Concentration and separation of glycyrrhizic acid by foam separation." Journal of Chemical Technology and Biotechnology **77**(6): 720-724.

Martin, R., T. H. Lilley, N. A. Bailey, C. P. Falshaw, E. Haslam, D. Magnolato and M. J. Begley (1986 a). "Polyphenol-caffeine complexation." Journal of the Chemical Society, Chemical Communications(2): 105.

Martin, R., T. H. Lilley, C. P. Falshaw, E. Haslam, M. J. Begley and D. Magnolato (1986 b). "The caffeine—potassium chlorogenate molecular complex." Phytochemistry **26**(1): 273-279.

Maruyama, H., H. Seki, A. Suzuki and N. Inoue (2007). "Batch foam separation of a soluble protein." Water Res **41**(3): 710-718.

Merz, J., G. Schembecker, S. Riemer, M. Nimtz and H. Zorn (2009). "Purification and identification of a novel cutinase from Coprinopsis cinerea by adsorptive bubble separation." Separation and Purification Technology **69**(1): 57-62.

Morishita, H., H. Iwahashi, N. Osaka and R. Kido (1984). "Chromatographic separation and identification of naturally occurring chlorogenic acids by 1H nuclear magnetic resonance spectroscopy and mass spectrometry." J Chromatogr **315**: 253-260.

Nagaoka, T., A. H. Banskota, Y. Tezuka, I. Saiki and S. Kadota (2002). "Selective antiproliferative activity of caffeic acid phenethyl ester analogues on highly liver-metastatic murine colon 26-L5 carcinoma cell line." Bioorg Med Chem **10**(10): 3351-3359.

Nakatani, N., S.-i. Kayano, H. Kikuzaki, K. Sumino, K. Katagiri and T. Mitani (2000). "Identification, Quantitative Determination, and Antioxidative Activities of Chlorogenic Acid Isomers in Prune (PrunusdomesticaL.)." Journal of Agricultural and Food Chemistry **48**(11): 5512-5516.

Nicolai, A., A. Friess and H. Parlar (2008). "Efficient enrichment of alpha,beta-unsaturated bovine insulins-(C12)n using tweezing adsorptive bubble separation (TABS) with bovine serum albumin." J Sep Sci **31**(12): 2310-2317.

Noel, J., A. Prokop and R. D. Tanner (2002). "Foam fractionation of a dilute solution of bovine lactoferrin." Appl Biochem Biotechnol **98-100**: 395-402.

Nomura, E., A. Kashiwada, A. Hosoda, K. Nakamura, H. Morishita, T. Tsuno and H. Taniguchi (2003). "Synthesis of amide compounds of ferulic acid, and their stimulatory effects on insulin secretion in vitro." Bioorganic & Medicinal Chemistry **11**(17): 3807-3813.

Nurminen, M. L., L. Niittynen, R. Korpela and H. Vapaatalo (1999). "Coffee, caffeine and blood pressure: a critical review." Eur J Clin Nutr **53**(11): 831-839.

Ogawa, N. and H. Ueki (2007). "Clinical importance of caffeine dependence and abuse." Psychiatry and Clinical Neurosciences **61**(3): 263-268.

Ostwald, W. and W. Mischke (1940a). "Untersuchungen über Zerschäumung mit besonderer Rücksicht auf Fragen der angewandten Chemie. I." Kolloid-Zeitschrift **90**(1): 17-25.

Ostwald, W. and W. Mischke (1940b). "Untersuchungen über Zerschäumung mit besonderer Rücksicht auf Fragen der angewandten Chemie. III." Kolloid-Zeitschrift **90**(2): 205-215.

Ostwald, W. and A. Siehr (1936). "Über Zerschäumungsanalyse." Kolloid-Zeitschrift **76**(1): 33-46.

Peker, H., M. P. Srinivasan, J. M. Smith and B. J. McCoy (1992). "Caffeine extraction rates from coffee beans with supercritical carbon dioxide." AIChE Journal **38**(5): 761-770.

Pinfold, T. A. (1970). "Adsorptive Bubble Separation Methods." <u>Separation Science</u> **5**(4): 379-384.

Robertson, G. H. (1970). <u>Foam fractionation of rare-earth elements</u> Ph D in Chemical Engineering, Univ. of California, Berkeley.

Roleira, F. M., C. Siquet, E. Orru, E. M. Garrido, J. Garrido, N. Milhazes, G. Podda, F. Paiva-Martins, S. Reis, R. A. Carvalho, E. J. Silva and F. Borges (2010). "Lipophilic phenolic antioxidants: correlation between antioxidant profile, partition coefficients and redox properties." <u>Bioorg Med Chem</u> **18**(16): 5816-5825.

Saleh, Z., R. Stanley and M. Nigam (2006). "Extraction of Polyphenolics from Apple Juice by Foam Fractionation." <u>International Journal of Food Engineering</u> **2**(2).

Sarachat, T., O. Pornsunthorntawee, S. Chavadej and R. Rujiravanit (2010). "Purification and concentration of a rhamnolipid biosurfactant produced by Pseudomonas aeruginosa SP4 using foam fractionation." <u>Bioresour Technol</u> **101**(1): 324-330.

Sattar, E. A., H. Glasl, A. Nahrstedt, S. H. Hilal, A. Y. Zaki and S. M. H. Elzalabani (1990). "Hydroxycinnamic Acid-Amides from Iochroma-Cyaneum." <u>Phytochemistry</u> **29**(12): 3931-3933.

Schnepf, R. W. and E. L. Gaden (1959). "Foam Fractionation of Proteins - Concentration of Aqueous Solutions of Bovine Serum Albumin." <u>Journal of Biochemical and Microbiological Technology and Engineering</u> **1**(1): 1-11.

Sett, S., S. Sinha-Ray and A. L. Yarin (2013). "Gravitational drainage of foam films." <u>Langmuir</u> **29**(16): 4934-4947.

Shea, A. P., C. L. Crofcheck, F. A. Payne and Y. L. Xiong (2009). "Foam fractionation of α-lactalbumin and β-lactoglobulin from a whey solution." <u>Asia-Pacific Journal of Chemical Engineering</u> **4**(2): 191-203.

Shefter, E. (1968). "Structural studies on complexes. II. Crystal and molecular structure of a 1:1 caffeine and 5-chlorosalicyclic acid complex." J Pharm Sci **57**(7): 1163-1168.

Shi, S., Y. Zhao, H. Zhou, Y. Zhang, X. Jiang and K. Huang (2008). "Identification of antioxidants from Taraxacum mongolicum by high-performance liquid chromatography-diode array detection-radical-scavenging detection-electrospray ionization mass spectrometry and nuclear magnetic resonance experiments." J Chromatogr A **1209**(1-2): 145-152.

Sitkowski, J., L. Stefaniak, L. Nicol, M. L. Martin, G. J. Martin and G. A. Webb (1995). "Complete assignments of the 1H, 13C and 15N NMR spectra of caffeine." Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy **51**(5): 839-841.

Smit, H. J. and P. J. Rogers (2002). "Effects of 'energy' drinks on mood and mental performance: critical methodology." Food Quality and Preference **13**(5): 317-326.

Somasundaran, P. (1972). "Foam Separation Methods." Separation & Purification Reviews **1**(1): 117-198.

Sondheimer, E., F. Covitz and M. J. Marquisee (1961). "Association of naturally occurring compounds, the chlorogenic acid-caffeine complex." Archives of Biochemistry and Biophysics **93**(1): 63-71.

Sondheimer, E., Szymansk.Cd and J. W. Corse (1961). "Coffee Constituents - Isolation of Chlorogenic Acid and Its Isomers from Coffee." Journal of Agricultural and Food Chemistry **9**(2): 146-&.

Spencer, C. M., Y. Cai, R. Martin, S. H. Gaffney, P. N. Goulding, D. Magnolato, T. H. Lilley and E. Haslam (1988). "Polyphenol complexation—some thoughts and observations." Phytochemistry **27**(8): 2397-2409.

Stevenson, P. and G. J. Jameson (2007). "Modelling continuous foam fractionation with reflux." Chemical Engineering and Processing: Process Intensification **46**(12): 1286-1291.

Sticher, O. (2008). "Natural product isolation." Nat Prod Rep **25**(3): 517-554.

Sugiura, M., Y. Naito, Y. Yamaura, C. Fukaya and K. Yokoyama (1989). "Inhibitory activities and inhibition specificities of caffeic acid derivatives and related compounds toward 5-lipoxygenase." Chem Pharm Bull (Tokyo) **37**(4): 1039-1043.

Trabelsi, N., S. Oueslati, R. Ksouri, M. Nassra, A. Marchal, S. Krisa, C. Abdelly, J. M. Merillon and P. Waffo-Teguo (2014). "The antioxidant properties of new dimer and two monomers of phenolic acid amides isolated from Limoniastrum guyonianum." Food Chem **146**: 466-471.

Uraizee, F. and G. Narsimhan (1990). "Foam fractionation of proteins and enzymes. II. Performance and modelling." Enzyme Microb Technol **12**(4): 315-316.

Uraizee, F. and G. Narsimhan (1996). "Effects of kinetics of adsorption and coalescence on continuous foam concentration of proteins: Comparison of experimental results with model predictions." Biotechnology and Bioengineering **51**(4): 384-398.

Uwai, K., Y. Osanai, T. Imaizumi, S. Kanno, M. Takeshita and M. Ishikawa (2008). "Inhibitory effect of the alkyl side chain of caffeic acid analogues on lipopolysaccharide-induced nitric oxide production in RAW264.7 macrophages." Bioorg Med Chem **16**(16): 7795-7803.

Vitasari, D., P. Grassia and P. Martin (2013). "Surfactant transport onto a foam lamella." Chemical Engineering Science **102**: 405-423.

Westerterp-Plantenga, M., K. Diepvens, A. M. C. P. Joosen, S. Berube-Parent and A. Tremblay (2006). "Metabolic effects of spices, teas, and caffeine." Physiology & Behavior **89**(1): 85-91.

Woziwodzka, A., A. Gwizdek-Wisniewska and J. Piosik (2011). "Caffeine, pentoxifylline and theophylline form stacking complexes with IQ-type heterocyclic aromatic amines." Bioorg Chem **39**(1): 10-17.

Xiang, M. X., H. W. Su, J. Y. Hu and Y. J. Yan (2011). "Isolation, identification and determination of methyl caffeate, ethyl caffeate and other phenolic compounds from Polygonum amplexicaule var. sinense." Journal of Medicinal Plants Research **5**(9): 1685-1691.

Xie, W., S. J. Neethling and J. J. Cilliers (2004). "A novel approach for estimating the average bubble size for foams flowing in vertical columns." Chemical Engineering Science **59**(1): 81-86.

Yan, J., Z. Wu, Y. Zhao and C. Jiang (2011). "Separation of tea saponin by two-stage foam fractionation." Separation and Purification Technology **80**(2): 300-305.

Yingyongnarongkul, B. E., N. Apiratikul, N. Aroonrerk and A. Suksamrarn (2006). "Solid-phase synthesis and antibacterial activity of hydroxycinnamic acid amides and analogues against methicillin-resistant Staphylococcus aureus and vancomycin-resistant S. aureus." Bioorg Med Chem Lett **16**(22): 5870-5873.

Zdunek, M., J. Piosik and J. Kapuscinski (2000). "Thermodynamical model of mixed aggregation of ligands with caffeine in aqueous solution. Part II." Biophys Chem **84**(1): 77-85.

Ostwald, W., 1918. Verfahren zum Verdampfen von Flüssigkeiten, Patent No. 327976, patented 30 November 1918, released 16 October 1920.

Appendix

Appendix A: NMR spectra of caffeine-chlorogenic acid complex
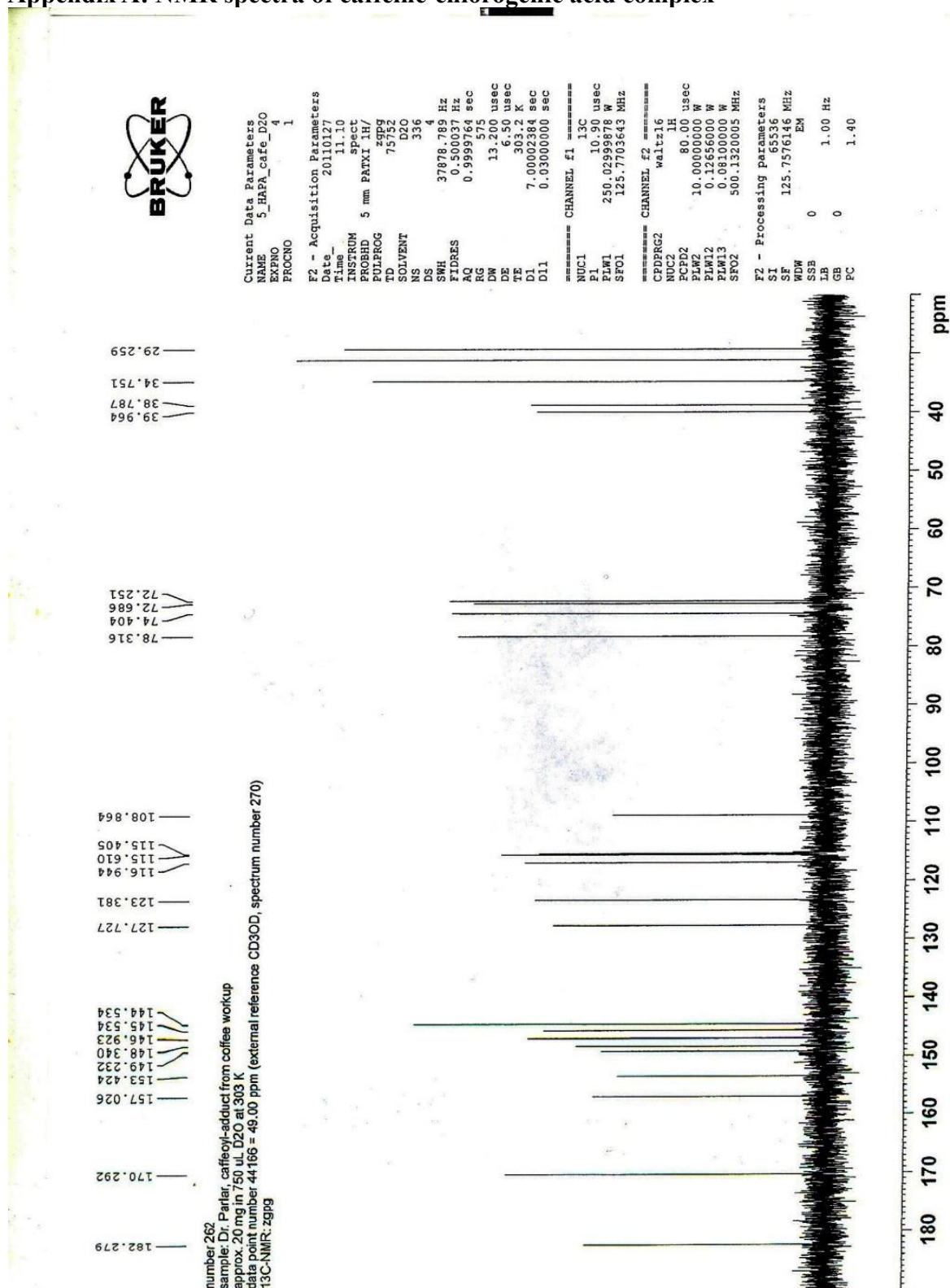
Figure A-1 ^{13}C NMR spectrum of caffeine-chlorogenic acid complex (125 MHz, D$_2$O)
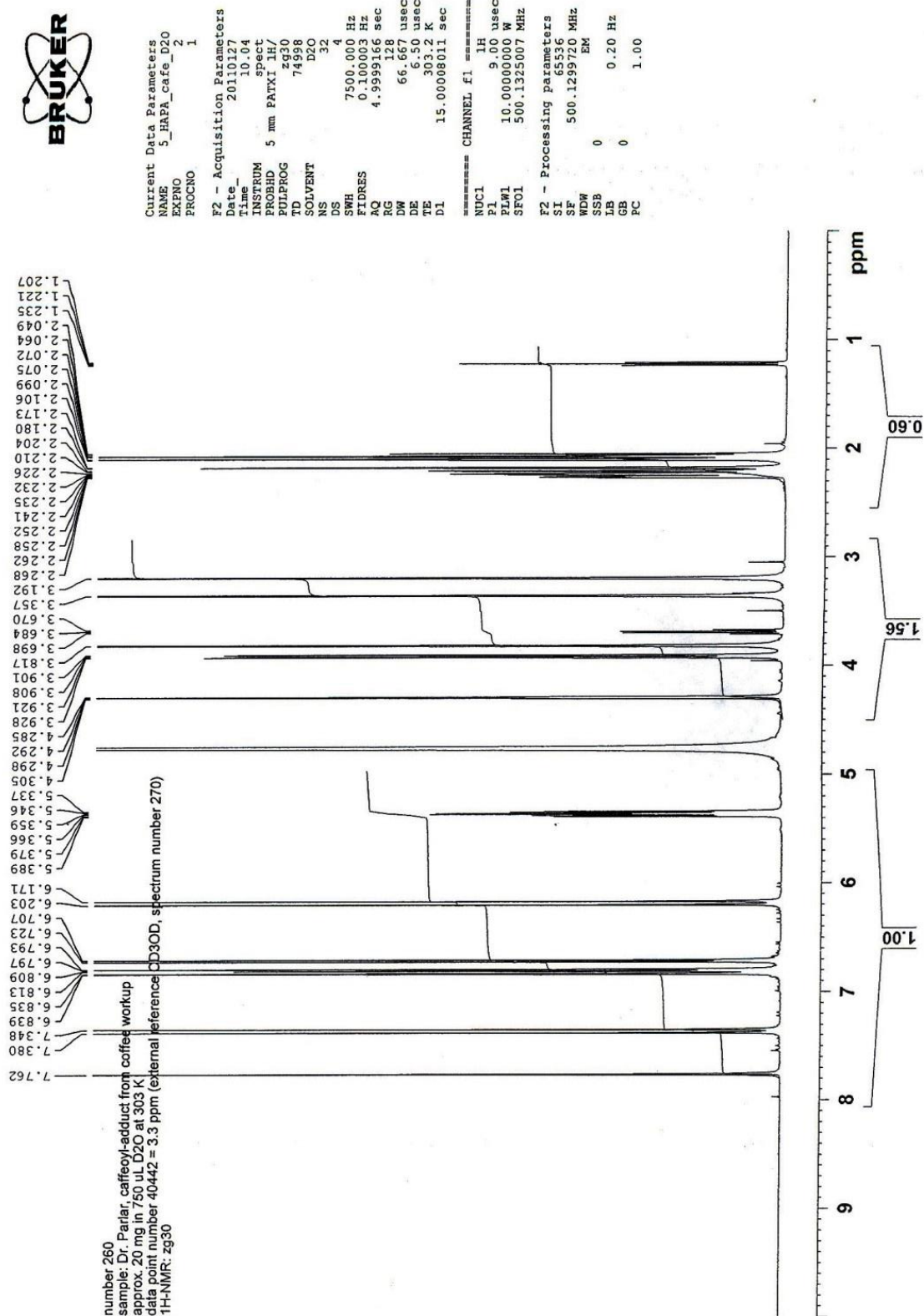
Figure A-2 ^{1}H NMR spectrum of caffeine-chlorogenic acid complex (500 MHz, D$_2$O)

Figure A-3 HMBC spectrum of caffeine-chlorogenic acid complex (in D$_2$O)
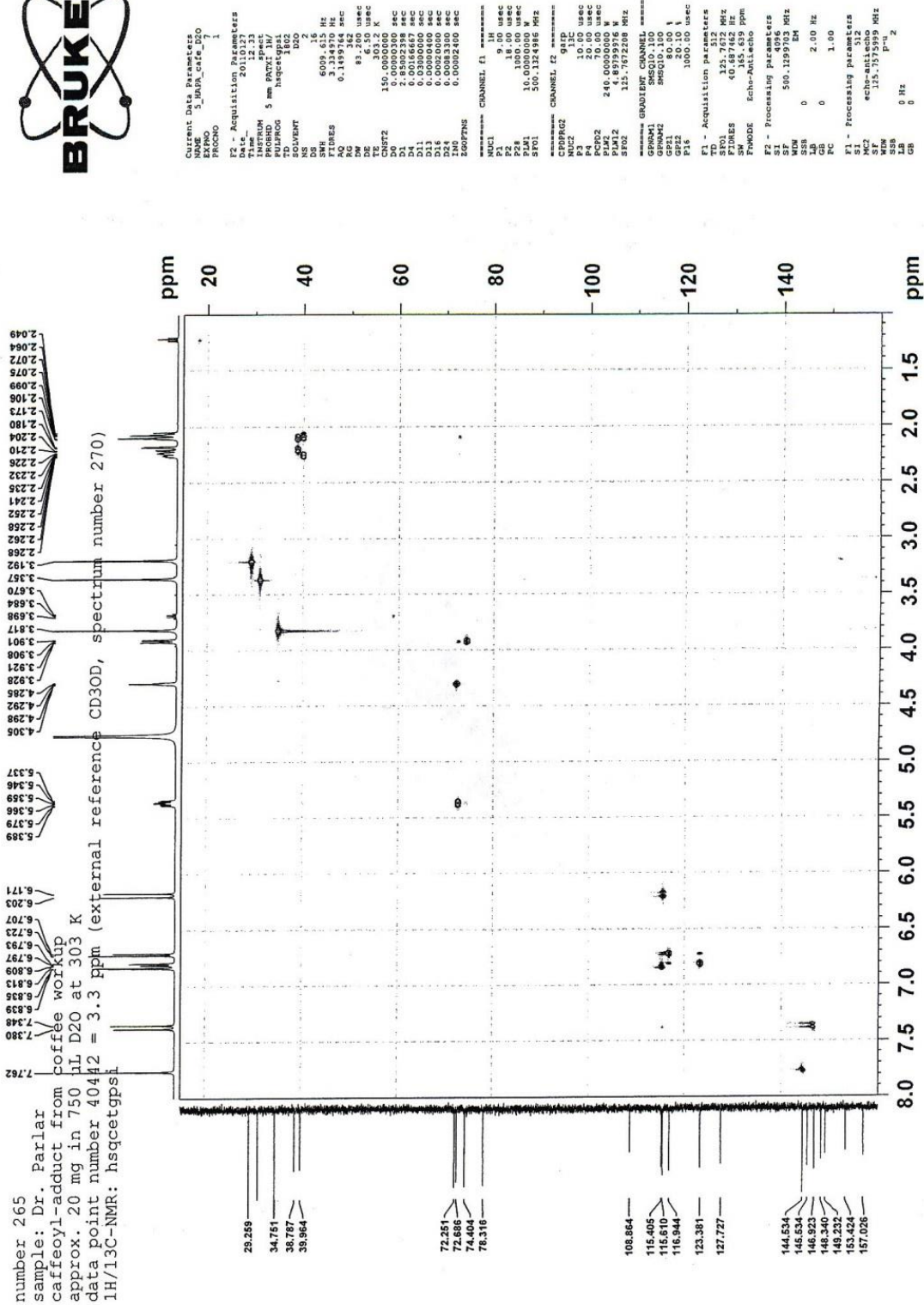
Figure A-4 HSQC spectrum of caffeine-chlorogenic acid complex (in D_2O)
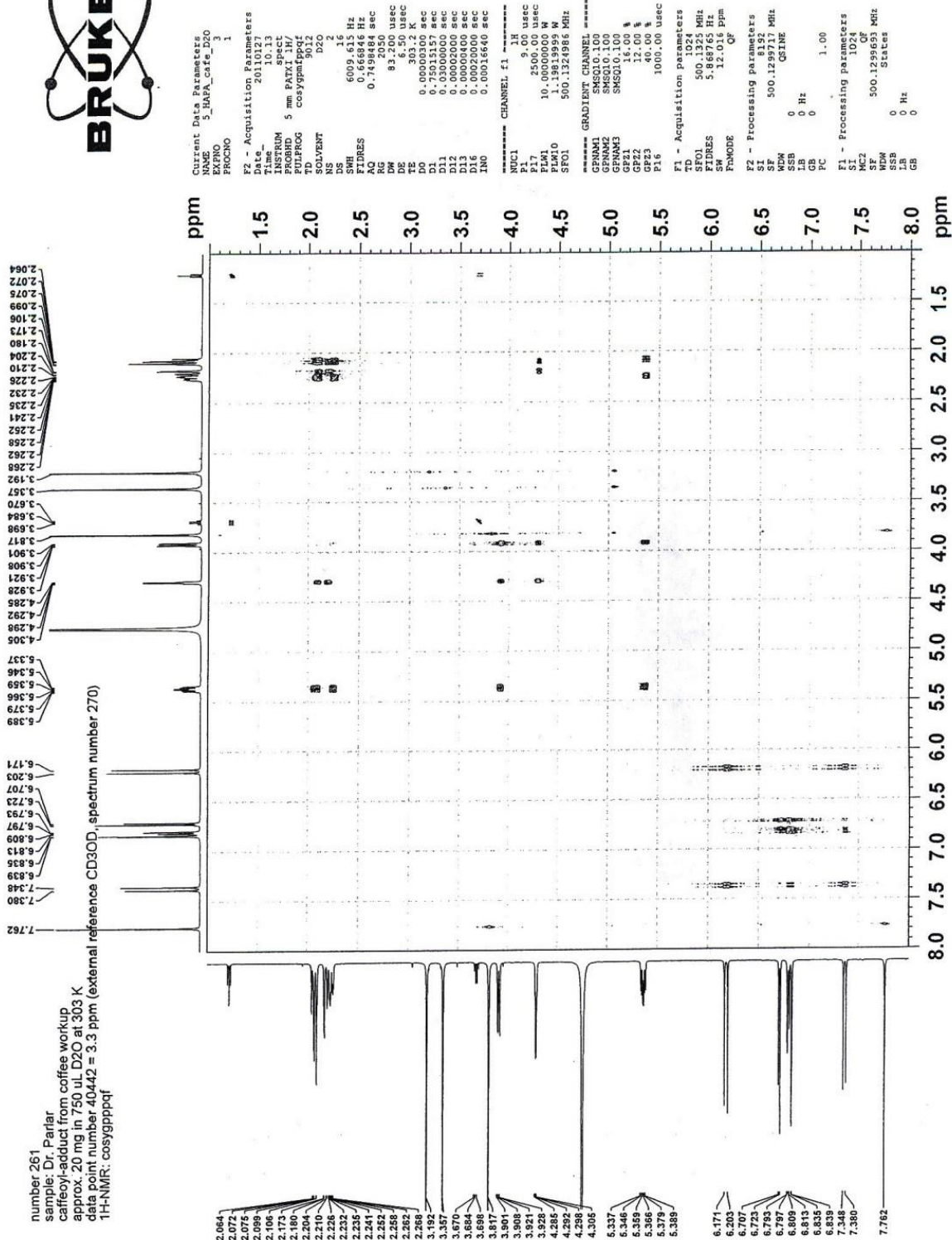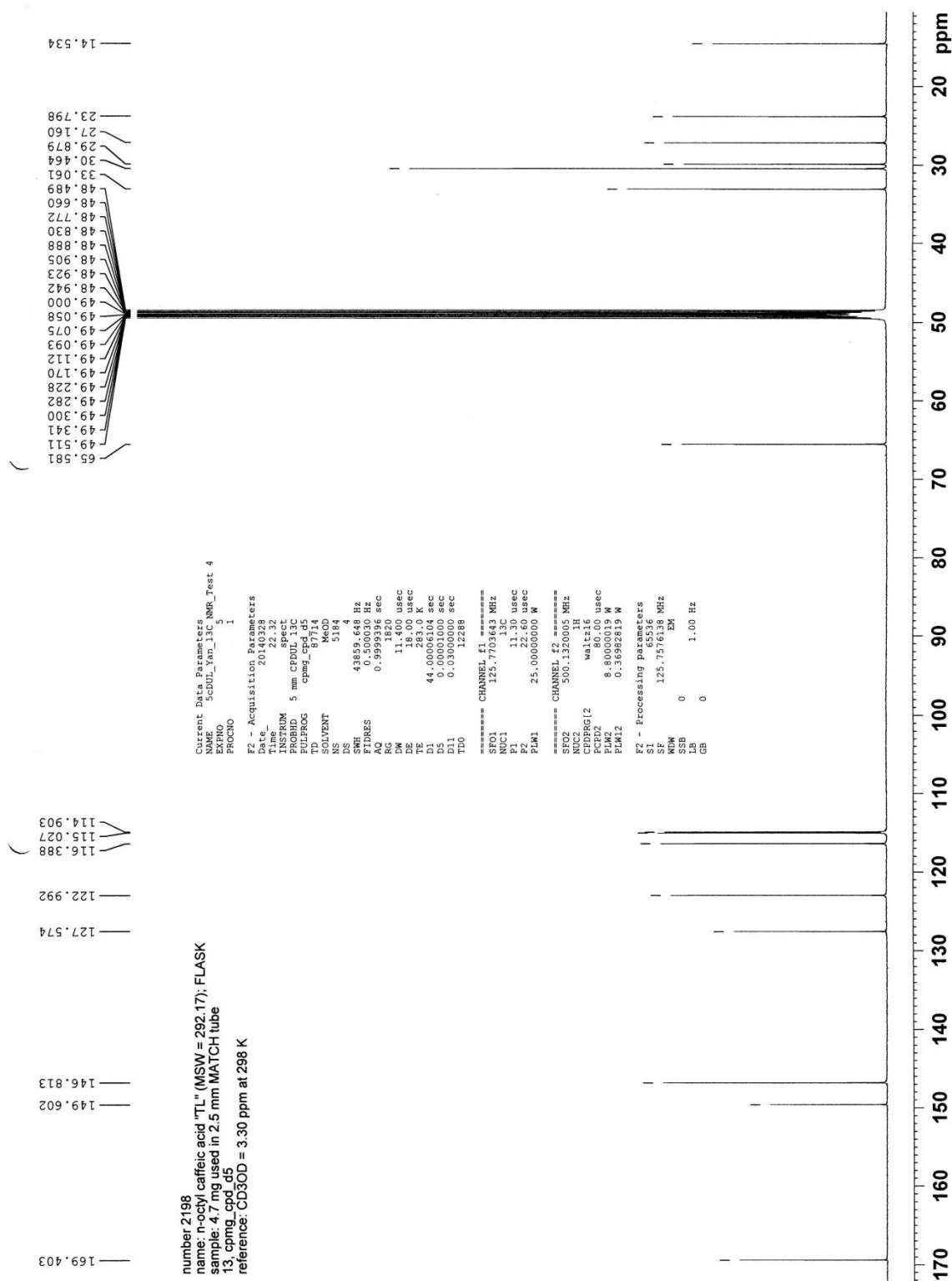
Figure A-5 ^{1}H-^{1}H COSY spectrum of caffeine-chlorogenic acid complex (500 MHz, D$_2$O)
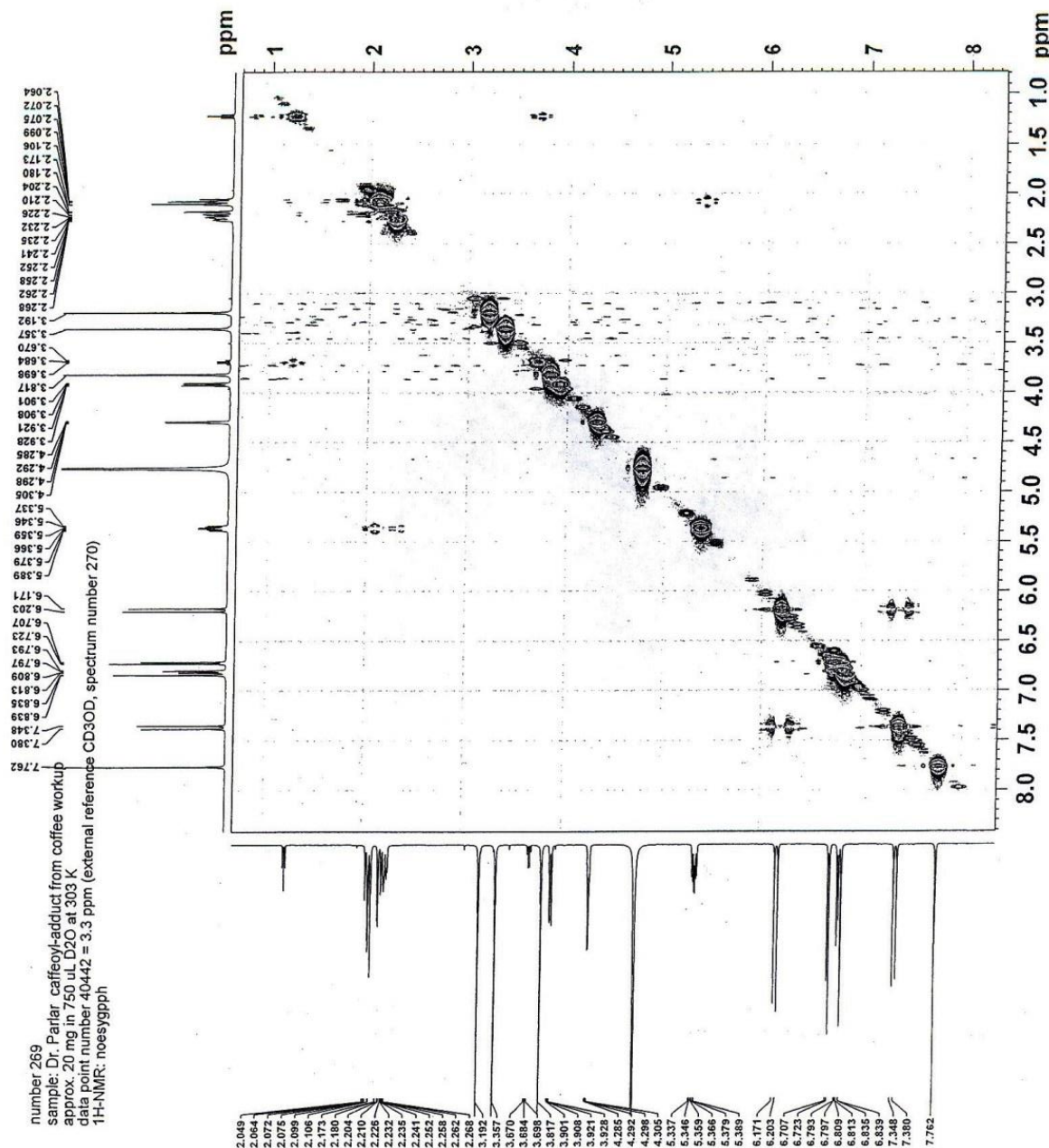
Figure A-6 NOESY spectrum of caffeine-chlorogenic acid complex (500 MHz, D₂O)

Appendix B: NMR and MS spectra of n-octyl caffeate

Figure B-1 ^{13}C NMR spectrum of n-octyl caffeate (125 MHz, MeOD)
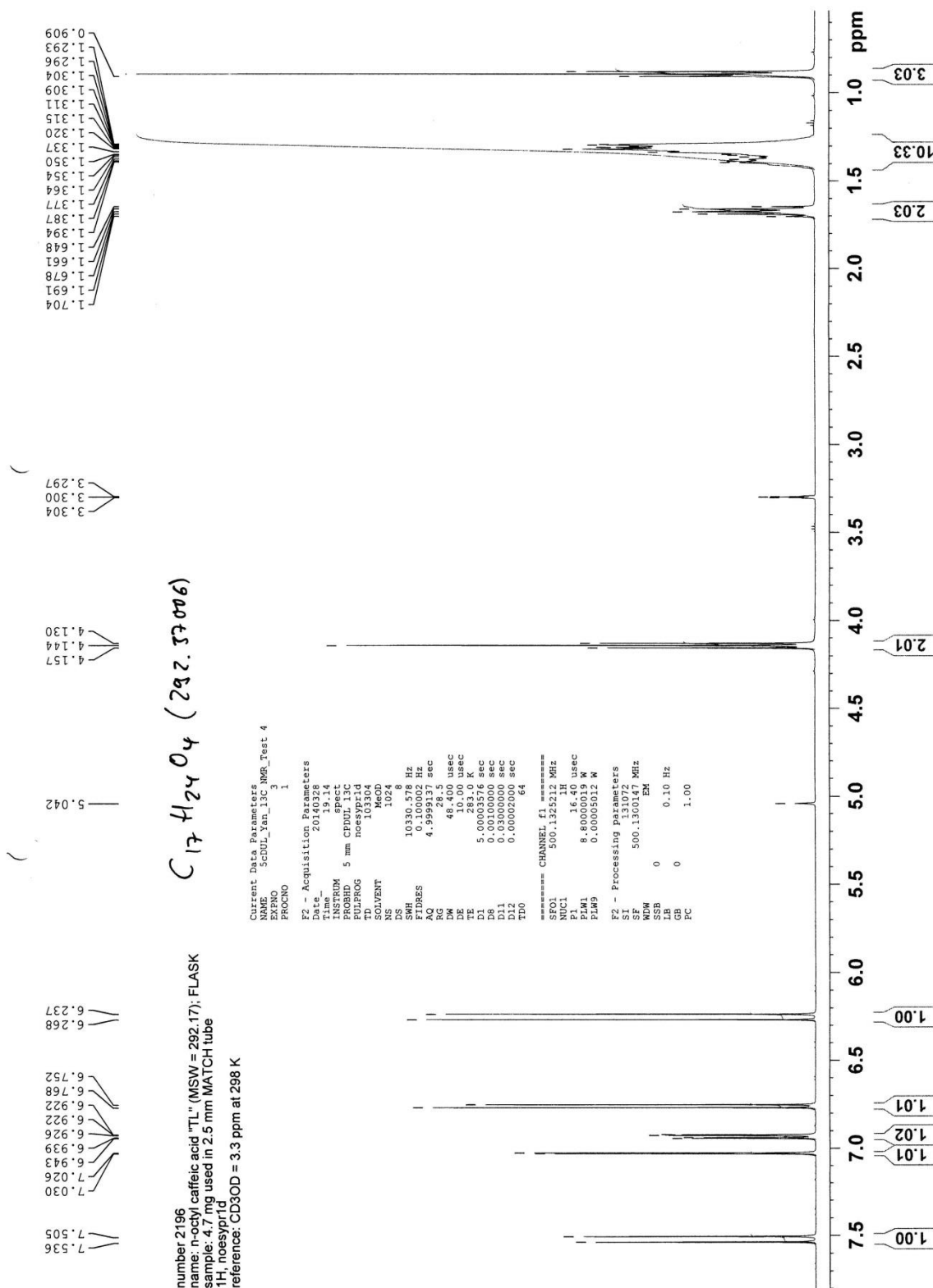
Figure B-2 ^{1}H NMR spectrum of n-octyl caffeate (500 MHz, MeOD)
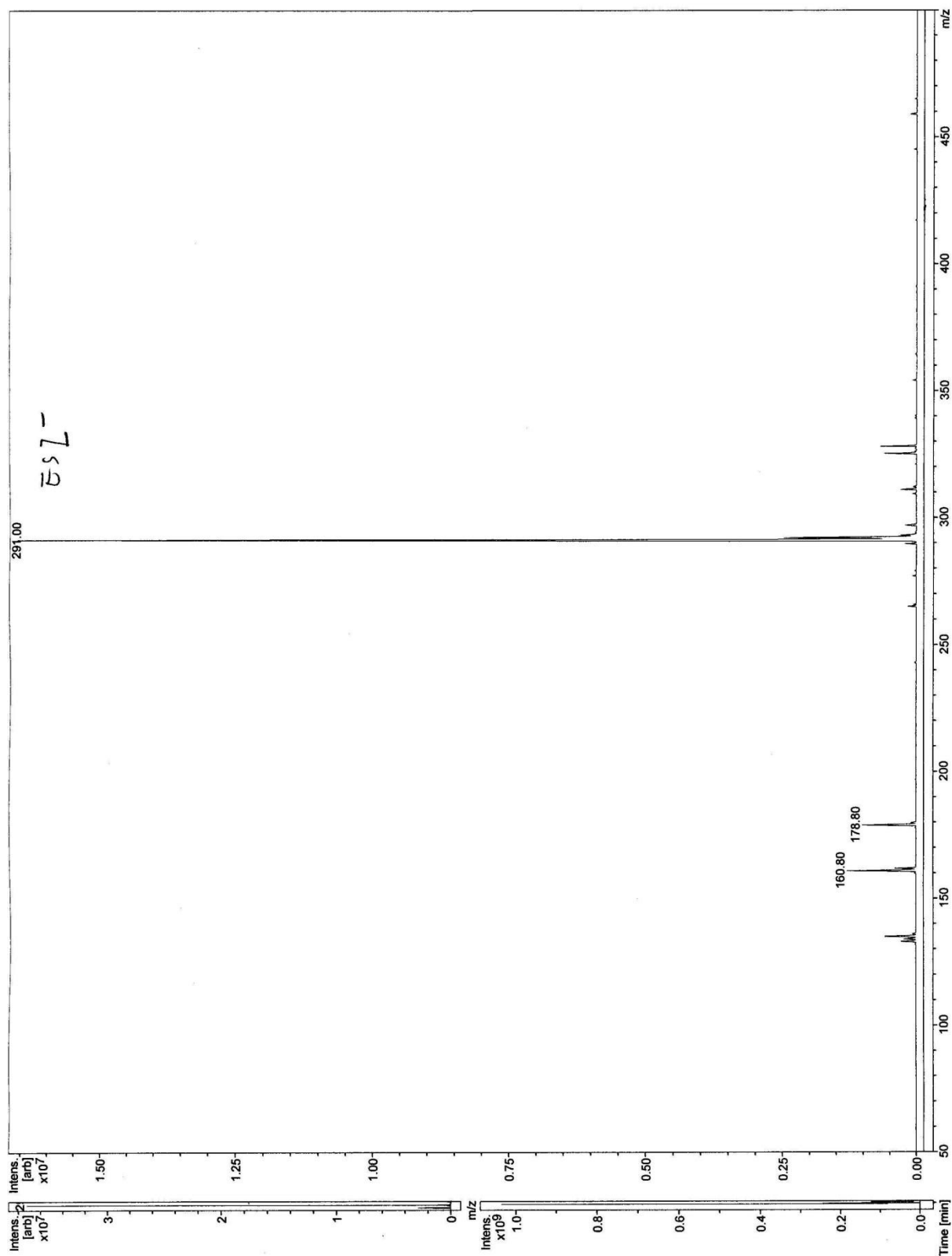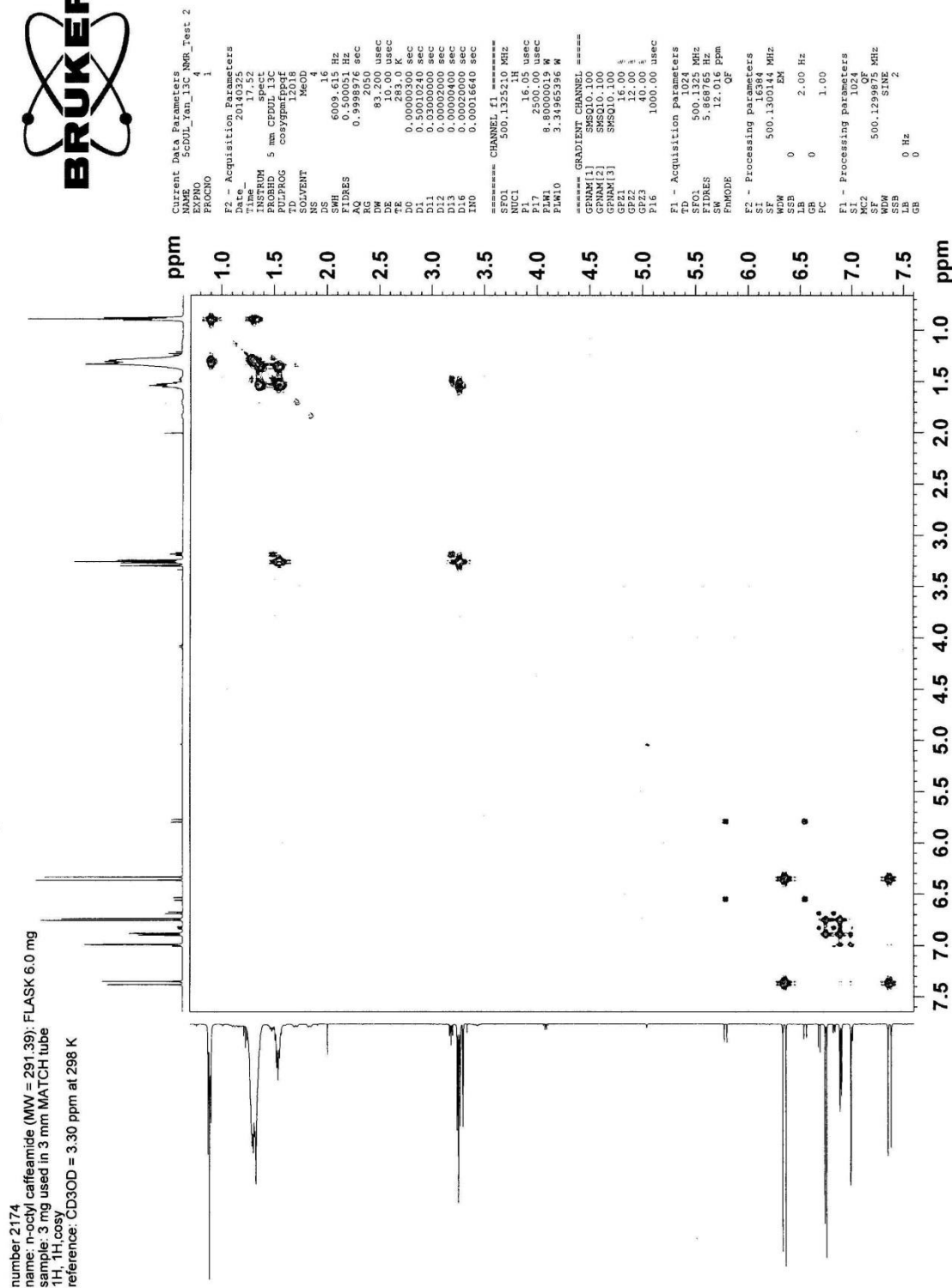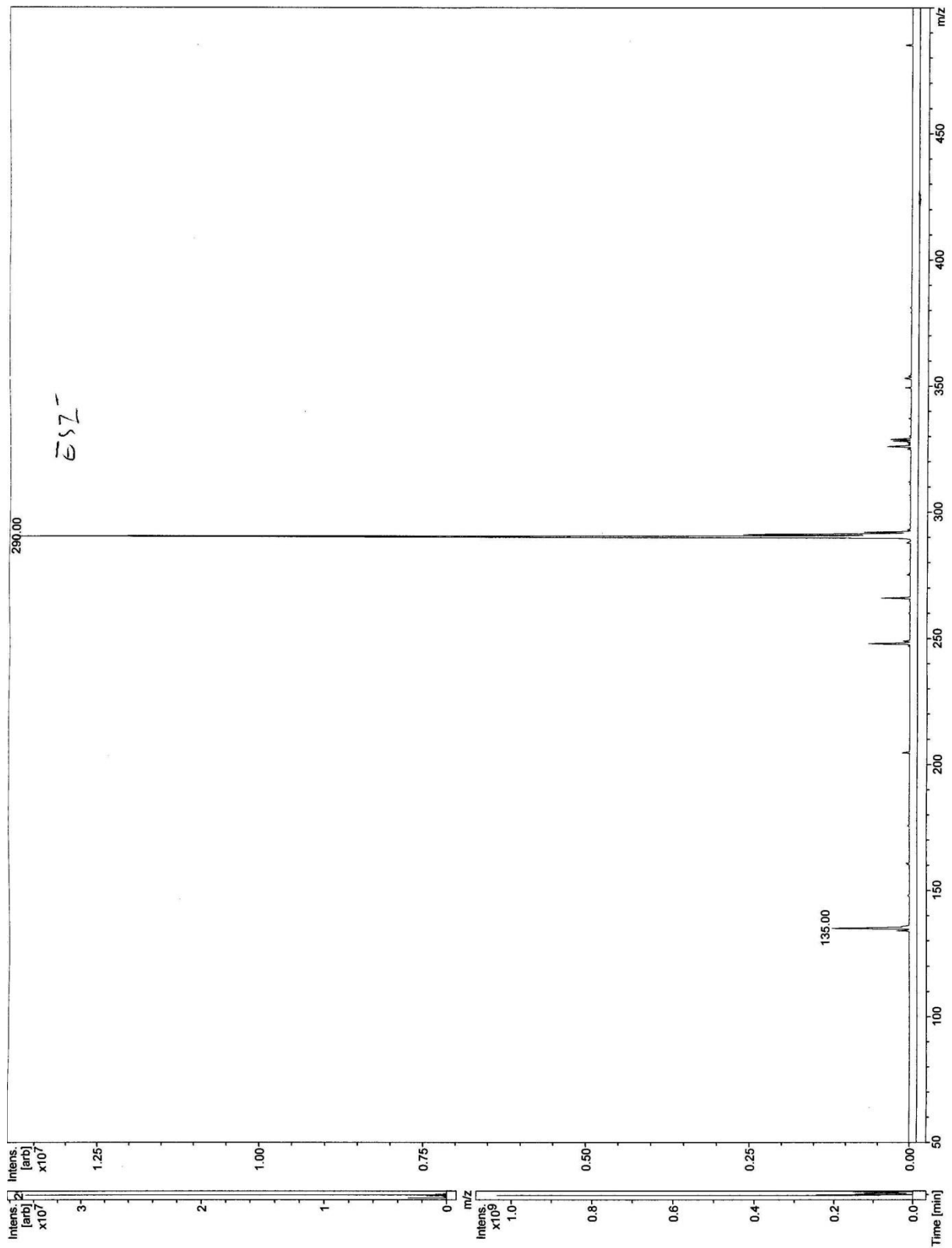
Figure B-3 ESI-MS spectrum of n-octyl caffeate in negative mode
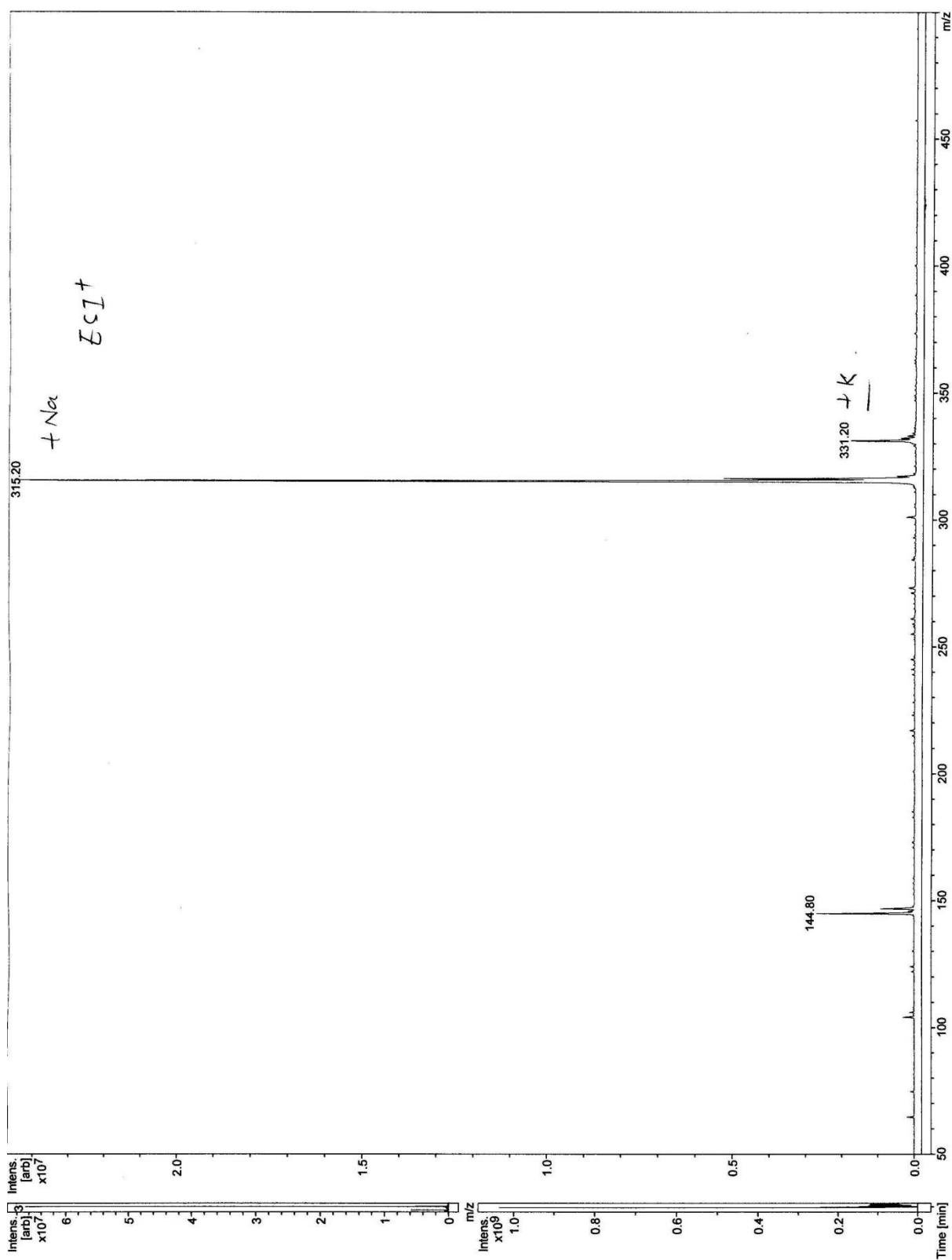
Figure B-4 ESI-MS spectrum of n-octyl caffeate in positive mode
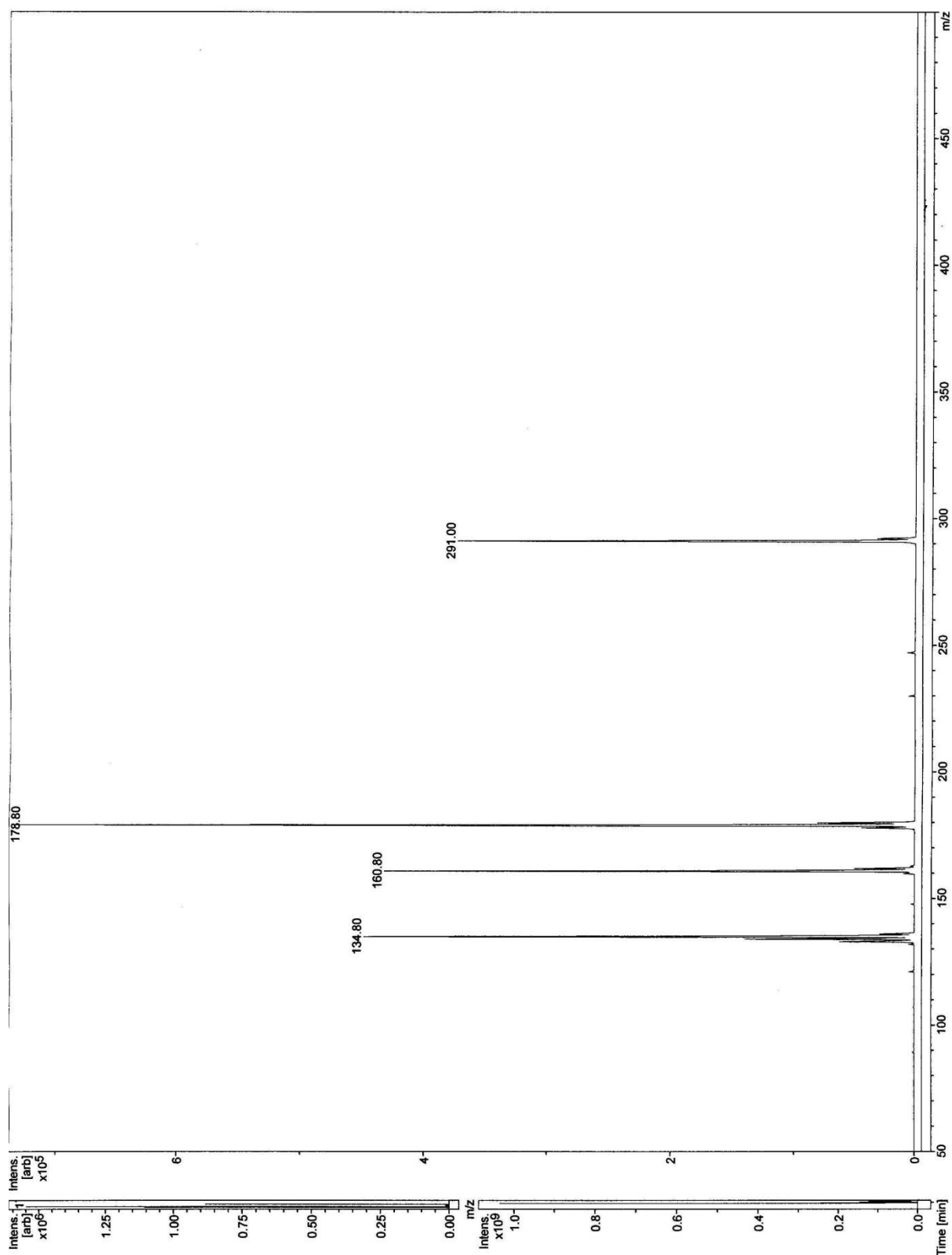
Figure B-5 ESI-MS/MS spectrum of n-octyl caffeate in negative mode
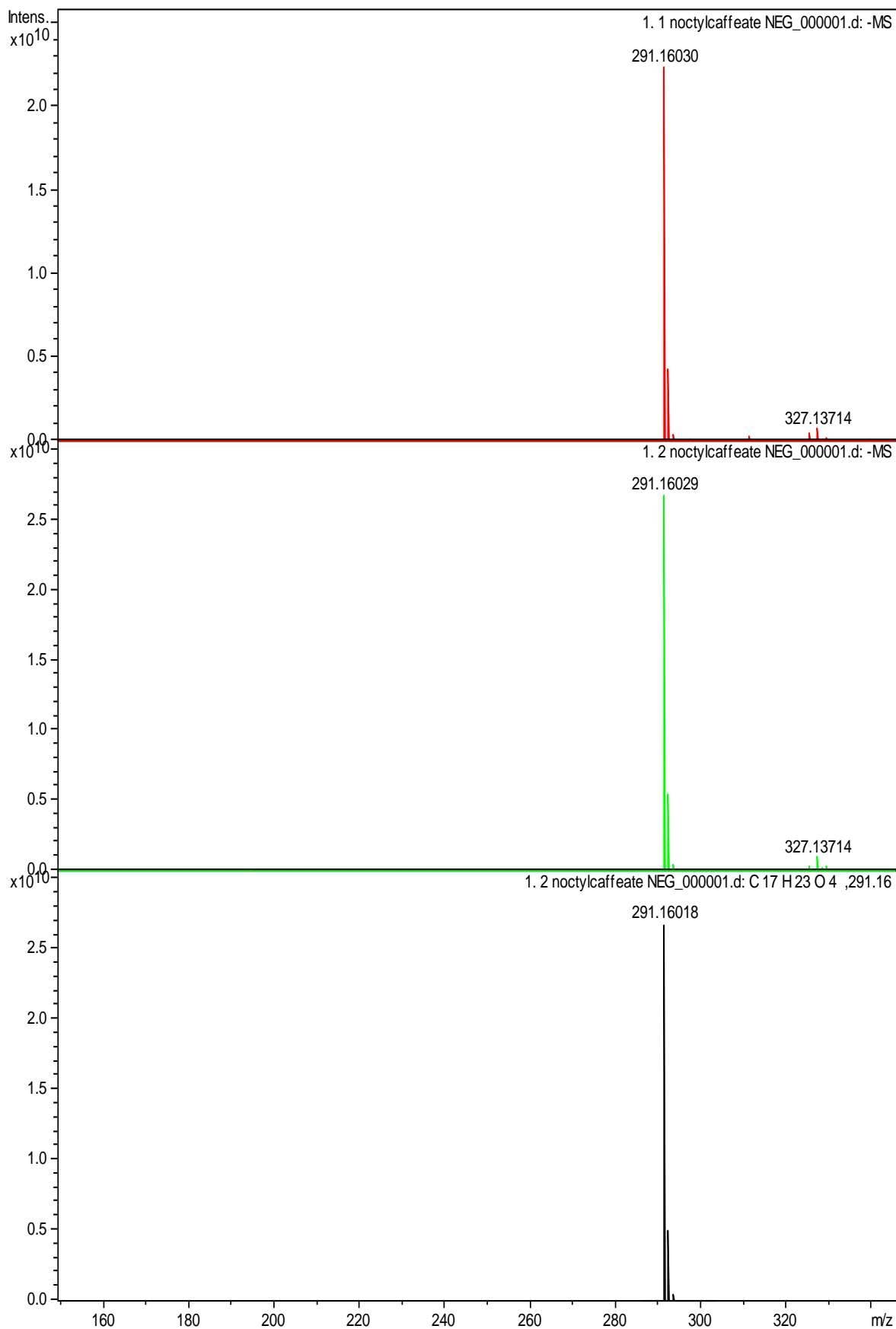
Figure B-6 FT-ICR mass spectrum of n-octyl caffeate in negative mode

Appendix C: NMR and MS spectra of n-octyl caffeamide
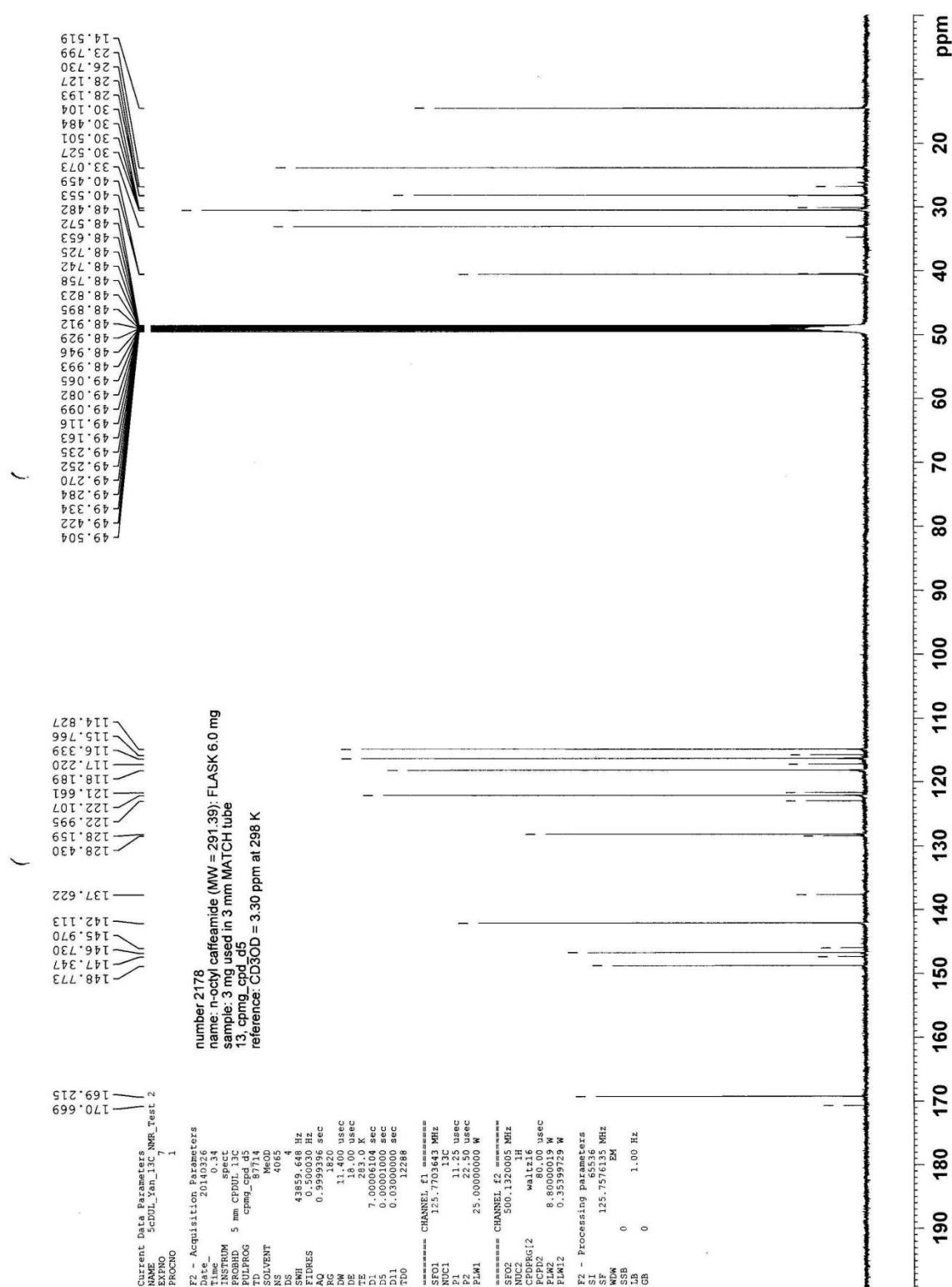
Figure C-1 ^{13}C NMR spectrum of n-octyl caffeamide (125 MHz, MeOD)
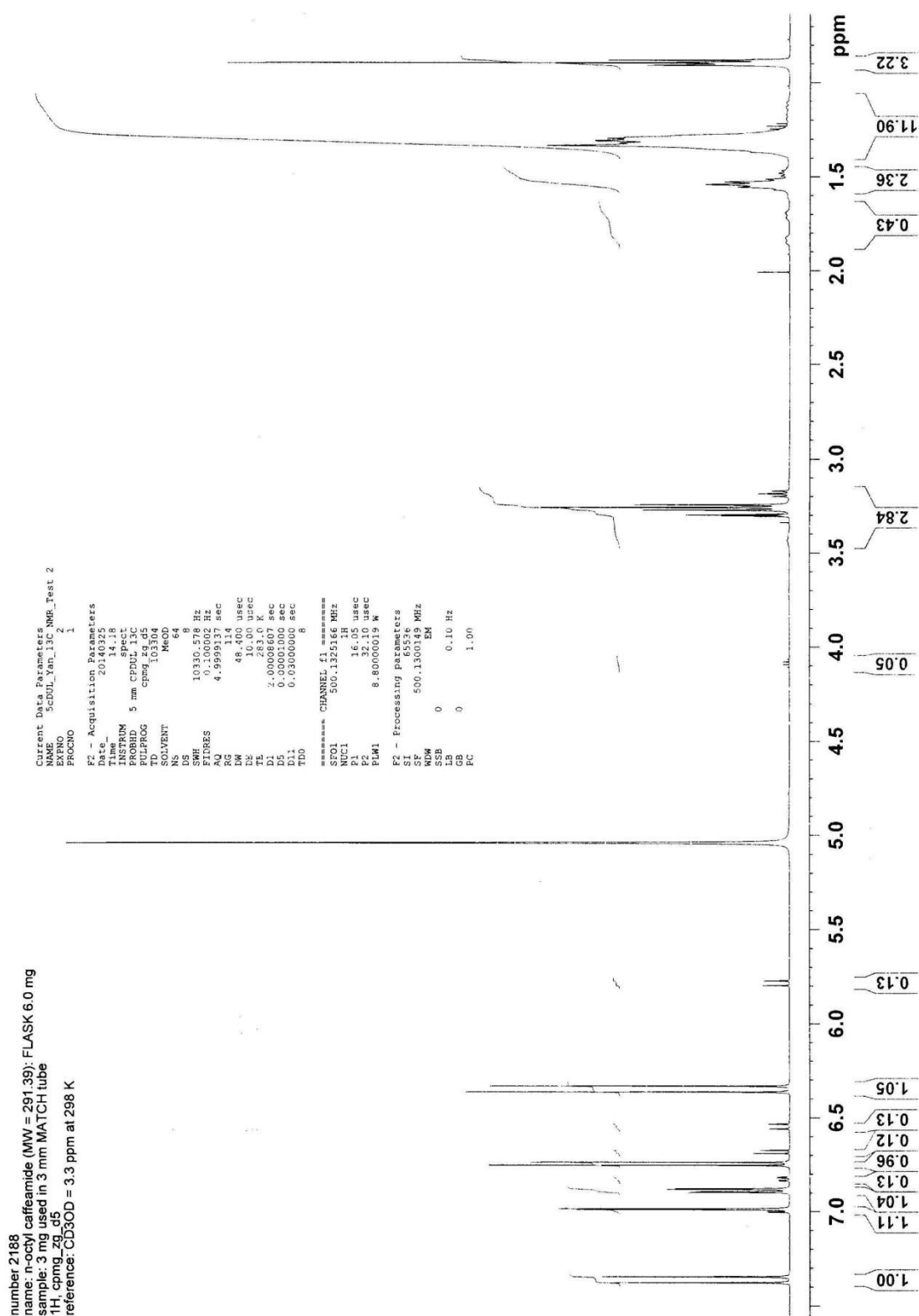
Figure C-2 ^{1}H NMR spectrum of n-octyl caffeamide (500 MHz, MeOD)

Figure C-3 HSQC spectrum of n-octyl caffeamide (in MeOD)

Figure C-4 ^{1}H -^{1}H COSY spectrum of n-octyl caffeamide (500 MHz, MeOD)

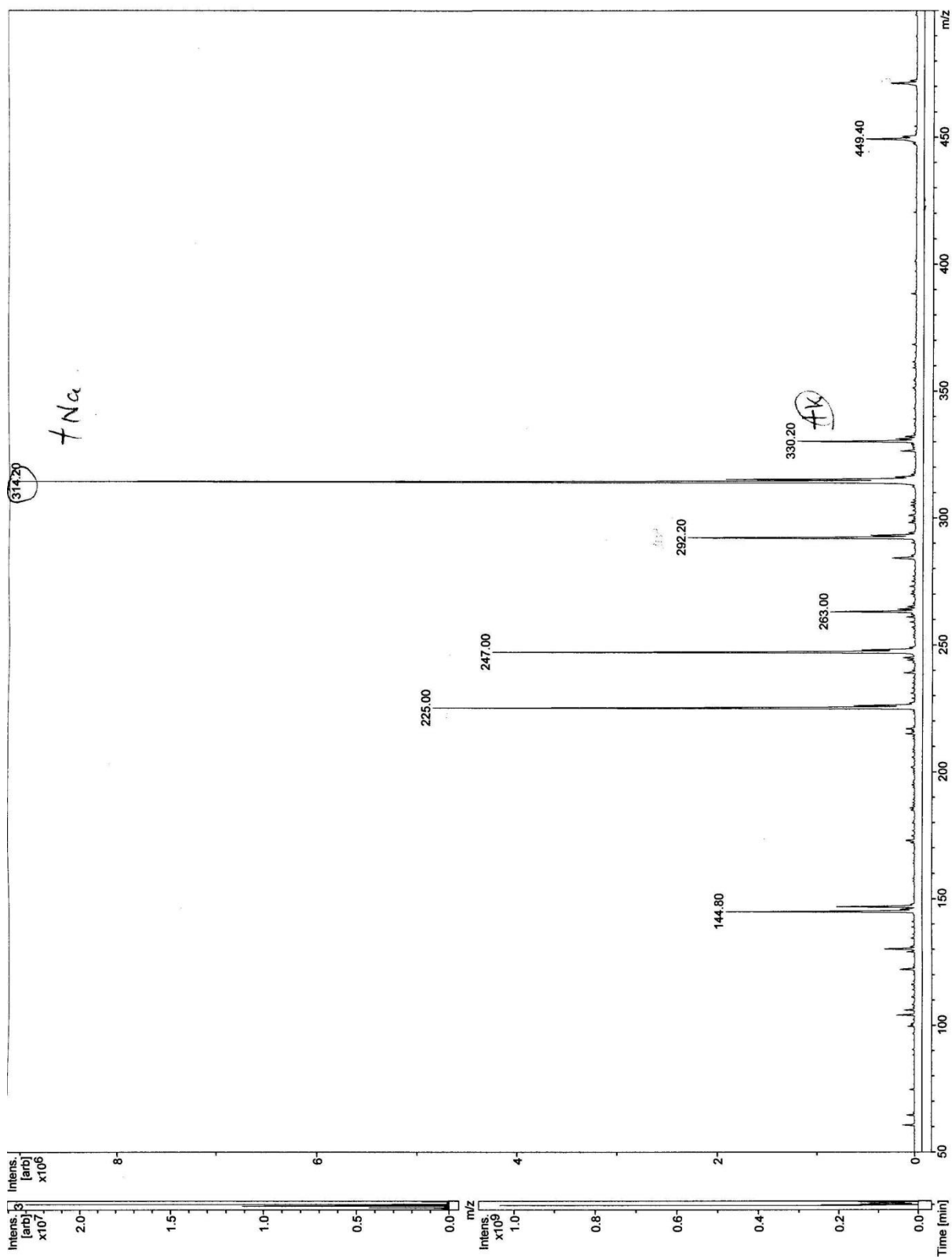**Figure C-5** ESI-MS spectrum of n-octyl caffeamide in negative mode

Figure C-6 ESI-MS spectrum of n-octyl caffeamide in positive mode
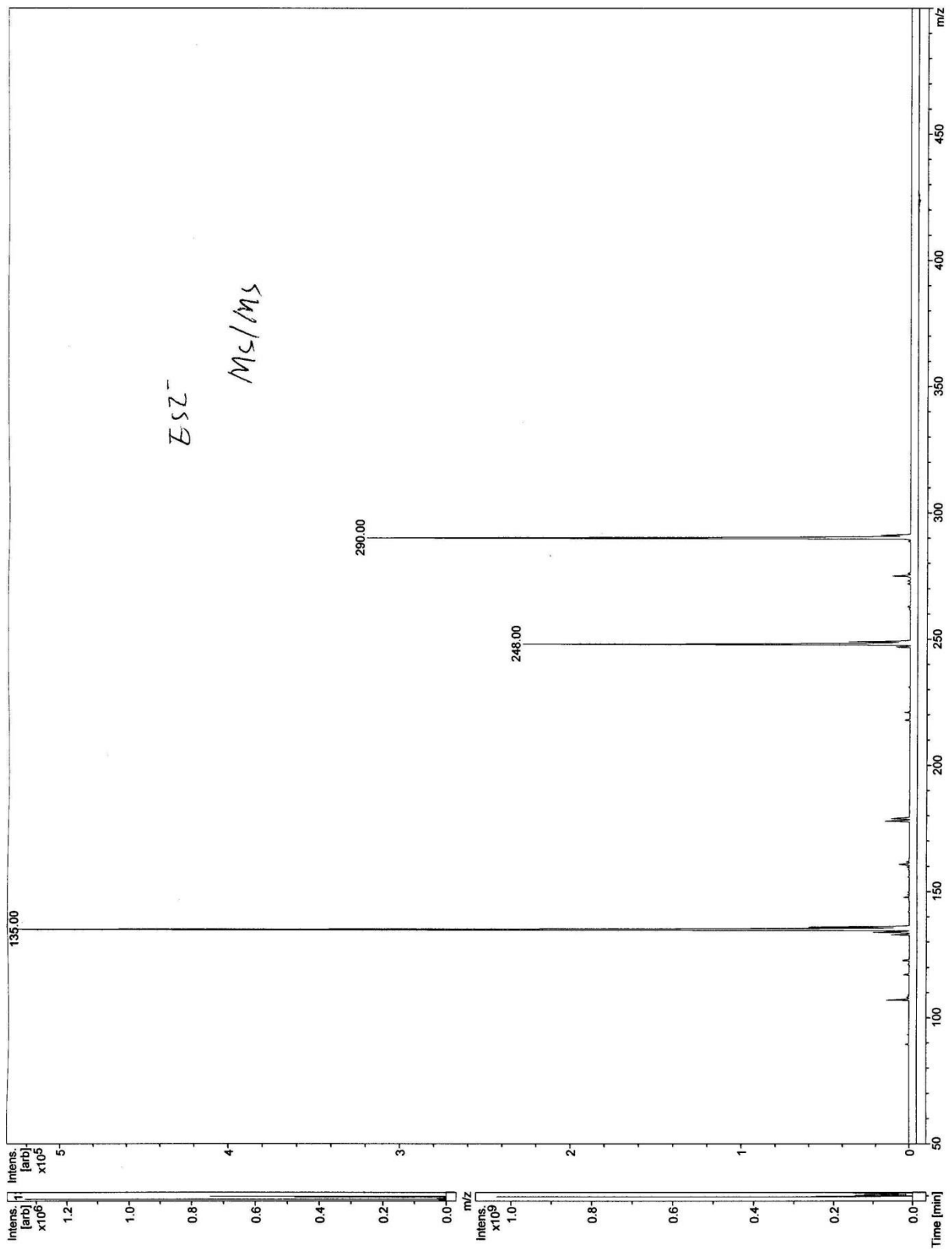
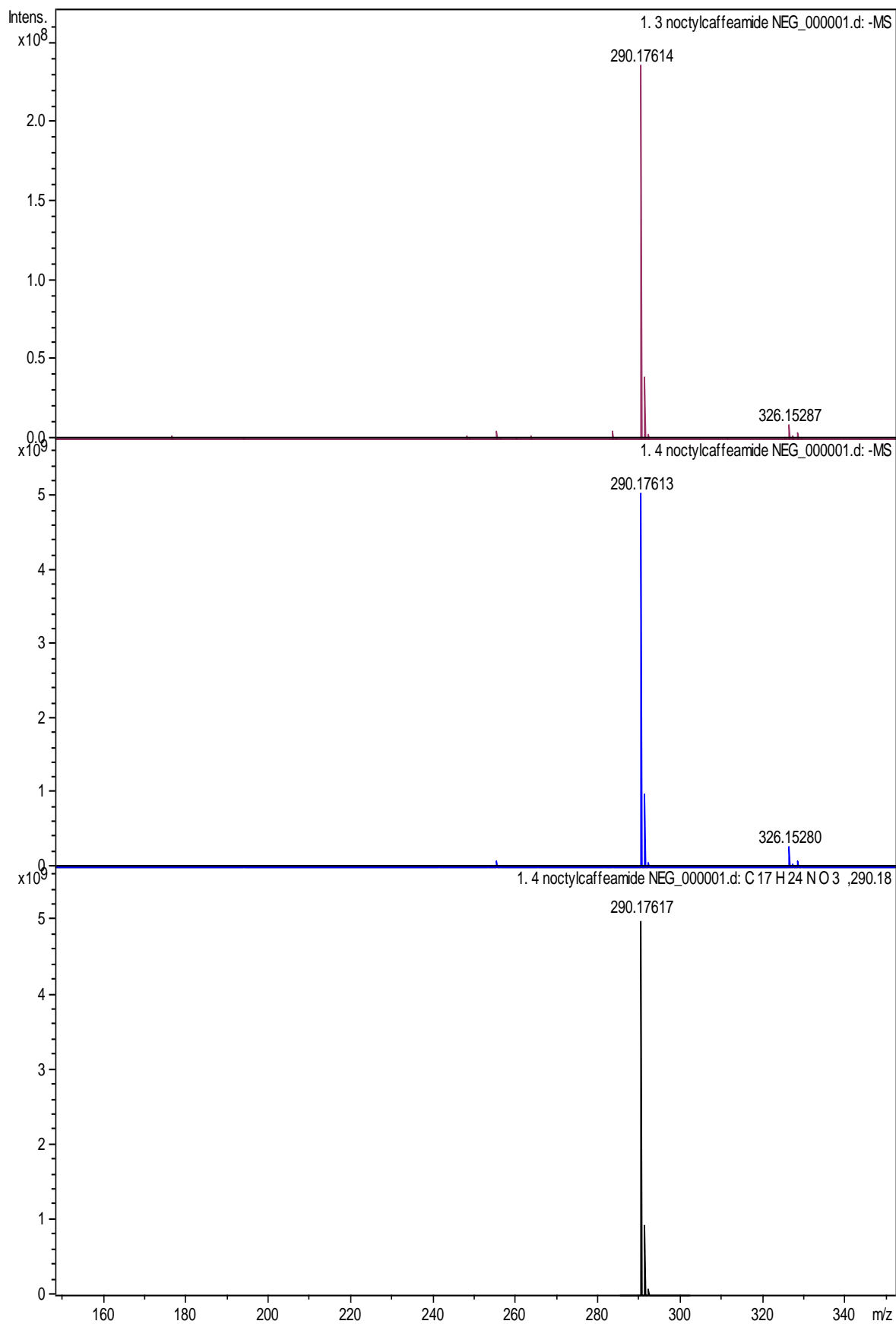**Figure C-7** ESI-MS/MS spectrum of n-octyl caffeamide in negative mode

Figure C-8 FT-ICR mass spectrum of n-octyl caffeamide in negative mode