Characterization of the Volumetric Properties of Five Bioactive Peptides, Liposomes and their Interactions

by

Luis Enrique Maya Desdier

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Food Science

University of Manitoba

Winnipeg, Manitoba

Acknowledgements

I would like to thank:

To God.

To my beloved wife for her help and support.

To Dr Martin Scanlon for his support, patience and guidance during my master studies.

To Glanbia Nutrionials for supplying with the dairy peptides for this study.

To Dr. Rotimi Aluko and Abraham Girgih for providing the hemp seed peptides for this study.

To the committee members Dr. Rotimi Aluko and Dr. Curtis Rempel.

To the people of the Food Science Department, academics, support staff and students for their help and all the good moments we shared.

To NSERC (Canada) for financial support through Dr. Martin Scanlon.

To the University of Manitoba for financial support by the International Graduate Student Scholarship (IGSES)**.**

"May you live all the days of your life" (Jonathan Swift)

Dedication

To my beloved wife for all her support, patience and encouragement to follow and make my dreams come true.

To my parents who will always be with me.

To Carlos, Ariadna and Karlita for all their love and support.

Abstract

The thermodynamic properties of bioactive peptides determine how they interact with cellular assemblies. Ultrasonic velocity and density measurements were used to analyse the volumetric properties in aqueous solution of 3 different materials: KCl, bioactive peptides (from hemp seed and dairy proteins), and liposomes (cell membrane models), as well as the interaction between peptides and liposomes. Serial dilutions of the three different materials showed linear relationships between density and concentration and between ultrasonic velocity and concentration. The apparent specific volume and apparent specific compressibility in solution of all materials showed concentration dependence as a result of increased electrostriction as solutions were diluted. The experimental ultrasonic velocities of liposome-dairy peptide mixes were higher than the theoretical additive value, due to interactions between liposomes and peptides. My research demonstrates the benefits of precise volumetric assessments in biological assays.

Table of Contents

List of Figures

List of Tables

1. Introduction

Proteins have a very important role in human nutrition; primarily, they are the source of amino acids needed in the diet (Korhonen & Pihlanto, 2006). Beyond the nutritional benefit of proteins, they can also perform physiological functions and are the source of an undetermined number of bioactive peptides. These peptides are not active within the protein structure, but after they are released from the protein structure by different processes, like human digestion, ripening of food products or microorganism hydrolysis, they can exert different biological functions (Meisel, 1997; Korhonen & Philanto, 2006; Erdmann et al., 2008; Guilloteau et al., 2009). Bioactive peptides have been reported to exert several different beneficial effects to health, which include antihypertensive, antioxidative, antithrombotic, opioid agonist and antagonist, immunomodulatory, hypocholesteloremic and antimicrobial effects (Meisel, 1997; Korhonen & Philanto, 2006; Hartmann & Meisel, 2007; Erdmann et al., 2008; Guilloteau et al., 2009).

The interactions between proteins or peptides and the cell membrane are frequently linked to the biological functions exerted by these proteins or peptides (Mozsolits et al., 1999; Matos et al., 2008; Hohlweg et al., 2012). Therefore, knowledge about how these interactions take place can contribute important information to understand the mechanisms involved in the biological functions of some proteins and peptides. These interactions between proteins or peptides and cell membranes can include the mechanism of how peptides attach to the cell membrane, as well as the insertions or transport of peptides across the cell membrane.

The interaction between protein and peptides and the cell membrane has been studied by several authors (Ramaswami et al., 1992; Colotto et al., 1993; Hianik et al., 1998; Hianik et al., 1999; Mozsolits, et al., 1999; Krivanek et al., 2000; Romanowski et al., 2002; Koenig & Gawrisch,

2005; Rybar et al., 2007; Uhríková et al., 2007; Matos et al., 2008). Frequently studies of interactions between proteins and cell membranes have been performed using model membranes because of the complexity of the cell membrane. Cell membranes are composed of several components that increase the number of factors involved in the interaction with other molecules like proteins or peptides and this complicates the interpretation of results (Baginski et al., 2006; Peetla et al., 2009). Therefore many studies are conducted using lipid vesicles or planar bilayers that usually are made from no more than two types of lipids that facilitate the interpretation of the results from experiments of how the membrane interacts with proteins or peptides (Baginski et al., 2006; Peetla et al., 2009). In this study, two types of liposomes where chosen to conduct the experiments, unilamellar vesicles (UV) and multilamellar vesicles (MLV). Refined lecithin was used to prepare both liposomes, with the multilamellar vesicles prepared by dispersion of refined lecithin in ultrapure water. Because of the self association properties of phospholipids in aqueous solutions, the dispersion allows the creation of multilamellar vesicles (Hianik, 2006; Luckey, 2008). The unilamellar vesicles were prepared by an extrusion technique (Hope et al., 1985; MacDonald et al., 1991), passing a multilamellar vesicles suspension through an extruder device containing a polycarbonate filter with pore sizes of 0.2 µm, 20 times.

Five bioactive peptides were used, three of which were dairy bioactive peptides received from Glanbia Nutritionals Inc. (Fitchburg, WI, USA), which were obtained from whey protein. Dairy proteins are sources of an important amount of bioactive peptides which have been reported to exert several effects (Hartmann & Meisel, 2007; Korhonen, 2009). The two main protein components from milk, casein and whey proteins, are reported to have several bioactive peptides within their structure that can be released during human digestion or food processes and can be found in fresh milk or products derived from milk (Gill et al., 1996; Chabance et al., 1998; Meissel, 1998; Korhonen & Pihlanto, 2006; Hartmann & Meisel, 2007;

Erdmann et al., 2008; López-Expósito & Recio, 2008; Guilloteau et al., 2009, Jäkälä et al., 2009; Korhonen, 2009). Two bioactive peptides from hemp seeds were also analysed, and these peptides were received from Mr.Girgih, a Ph.D candidate at the University of Manitoba, who obtained the peptides by enzyme digestion and separated them by ultrafiltration as explained in Girgh et al. (2011). The amount of bioactive peptides isolated from plant sources remains small compared with other sources such as dairy or egg proteins, although bioactive peptides have been isolated from proteins of plants such as pea, wheat, soy and hemp seed and amaranth Silva-Sanchez et al., 2008; Yoshie-Stark et al., 2008; Guang & Phillips, 2009; Tang et al., 2009; Tiengo et al., 2009; Orlovskaya et al., 2010; Roy et al., 2010; Lico et al., 2012).

Volumetric properties such as volume and compressibility of biomolecules in aqueous solutions, determined from ultrasonic velocity and density measurements of the solutions, have been used to analyse the structure and dynamics of biomolecules in solutions (Chalikian & Breslauer, 1998; Gekko, 2002; Taulier & Chalikian, 2002; Chalikian, 2003; Pfeiffer et al., 2008). Information obtained from determination of volumetric properties provides a valuable tool for understanding conformational transitions, structural dynamics and interactions of biomolecules as they accomplish their biological functions.

In addition, the volumetric properties, such as specific volume and compressibility, have frequently been used to analyse the structure and conformational transitions of proteins (Kharakoz, 1997; Chalikian et al., 1995; Gekko, 2002; Taulier & Chalikian 2001; Valdez et al., 2001; Taulier & Chalikian, 2002; Chalikian & Filfil, 2003; Lee et al., 2008; Pfeiffer et al., 2008; Chalikian & Macgregor, 2009; Lee et al., 2010). However, these parameters are usually applied to highly purified proteins or peptides, and have not been used yet to characterize the structure and behavior in aqueous solutions of peptides isolated from food sources, which have not been

purified, containing trace of other components, but that have a composition that meets a commercial application.

The first objective of this study was to characterize the volumetric properties in aqueous solution of the three dairy bioactive peptides and the two hemp seed bioactive peptides, analyzing the apparent specific volume and apparent specific compressibility at low concentrations and comparing their partial specific volume and partial specific adiabatic compressibility in order to obtain information about their thermodynamic properties.

The second objective was to analyse differences in particle size, and size distribution between the two different liposomes, UV and MLV, prepared with and without the extrusion technique. The volumetric properties of the two types of liposomes, UV and MLV, in aqueous solutions were subsequently characterized. The characterization was based on the ultrasonic velocity and density of UV and MLV in aqueous solutions, in order to calculate the apparent specific volume and apparent specific adiabatic compressibility, and then compare the values of these parameters for the different types of liposomes. The volumetric properties have been used to analyse the physical properties of model membranes as well as the structural dynamics within the model membranes (Hianik et al., 1988; Uhríková et al., 2007).

The final objective was to analyse the interaction between each of the three dairy peptides and the two types of liposomes, and to determine if these interactions change over time. In this study, the ultrasonic velocity and density was measured over time in mixes of each dairy peptide and the two types of liposome. Based on the additive properties of the volumetric properties (Hianik et al., 2011), the experimental values of the mixes were compared with the theoretical additive values in order to determine if potential interactions between the peptides

and liposomes would affect the volumetric parameters, thereby giving guidance on membrane-peptide interactions.

2. Literature Review

2.1. Volumetric Properties

Thermodynamic properties, such as volume and compressibility volumetric properties of proteins, peptides and amino acids are a valuable tool for understanding the structure and conformational transitions of these biomolecules and their interactions with the solvent and other solutes; this is very important for the way these molecules exert a biological function (Gekko, 2002; Taulier & Chalikian, 2002; Chalikian, 2003; Pfeiffer et al., 2008).

The interactions between solvent and proteins are especially important for protein dynamics which seem to be essential for the biological activity of proteins, but these interactions are also important for peptides. As Murphy et al. (1998) pointed out, the measurement of specific volume of proteins and peptides can help us to understand solvent-solute interactions and the features of protein hydration.

Several structural techniques, such as X-ray crystallography, NMR spectroscopy and differential scanning calorimetry, have been used to understand hydration of biological molecules. These techniques are very useful to determine some volumetric properties in very localised molecular areas. However uses of high precision density measurements and ultrasonic velocity measurements have proved to be quite useful to measure the effect of the hydration in the entire solution instead of as a localized effect (Chalikian & Breslauer, 1998).

2.1.1. Specific Volume

Volume is a physical property of any material that can be described as the three-dimensional space occupied by the material. The volume of a solution, with ideal interactions between the solvent and the solute, would be described as the sum of the volume of all its components and calculated by the addition of the original volume of the solvent and the volume of solute added (Murphy et al., 1998).

The volume of a solution is a function of the volume of its components at a specific temperature and pressure. Density (ρ), defined as the sum of the masses of the components divided by the volume of the solution, is the inverse of the specific volume ($1 / \rho$) which is the volume occupied by a solution per unit of mass (Kupke, 1973). Density measurements are generally the way to measure the volume of a solution and the apparent volume of its components (Durchschlag & Jaenicke, 1982; Zamyatnin, 1984; Bánó & Marek, 2006; Lee et al., 2008; Marcus, 2011).

2.1.1.1. Apparent Specific Volume

The study of volumetric properties of solutions of biological components like proteins, and especially the analysis of solute-solvent interactions is made convenient by using the apparent specific volume and apparent specific compressibility of the solute which includes the changes of the volumetric properties as a function of the amount of solute that is added to the solution (Chalikian et al., 1994). The apparent specific volume, $\overline{V}$, can be defined as the change in the volume of the solution as a unit mass of the solute is added and dissolved at finite concentration (Kupke, 1973). The apparent specific volume can be calculated based on density

measurements of the solvent and the solutions at finite concentrations (Kupke, 1973; Durchschlag & Jaenicke, 1983; Chalikian et al., 1998).

$$\overline{\mathrm{V}} = \left(\frac{1}{\rho_0}\right)\left(\frac{1-(\rho-\rho_0)}{c}\right) \quad [1]$$

where ρ and ρ_0 are the densities of the solution and the solvent, and *c* is the concentration of the solute. The apparent specific volume of proteins, peptides and amino acids in aqueous solutions, which do not interact with the solvent in an ideal manner, is influenced by solvent-solute interactions (Murphy et al., 1998; Sirotkin et al., 2012).

2.1.1.2. Partial Specific Volume

The partial specific volume ($\overline{\mathrm{V}}^0$) is generally defined as the change in volume of the solution when a known amount of solute is added at constant temperature and pressure at infinite dilution (Kupke, 1973; Chalikian et al., 1998; Murphy et al., 1998; Bánó & Marek, 2006; Lee et al., 2008; Marcus, 2011). The partial specific volume is calculated by the extrapolation of the apparent specific volume to zero concentration as:

$$\bar{V}^0 = \lim_{c\to 0} \bar{V} \quad [2]$$

The apparent specific volume, $\overline{\mathrm{V}}$, includes the contribution arising from solute-solvent interactions and solute-solute interactions. In order to determine the contribution corresponding only to the solute-solvent interaction, the value of the apparent specific volume, $\overline{\mathrm{V}}$, is extrapolated to zero concentration to obtain the partial specific volume, $\overline{\mathrm{V}}^0$, where the solute-solute interactions diminish (Chalikian et al., 1994; Marcus, 2011).

The partial specific volume has been described as the result of two contributions; the intrinsic volume or molecular volume (V_M), the volume assigned to the solute molecule, and the hydration contribution (ΔV_h), which is the change of the solvent volume resulting from the perturbation of the water molecules of the hydration shell by the solute (Chalikian and Breslauer, 1998; Murphy et al., 1998).

$$\bar{V}^0 = V_M + \Delta V_h \quad [3]$$

The interaction between the solvent and the surface of the protein, peptide or amino acid causes a change in some thermodynamic properties of the solvent. The layer of solvent molecules which interacts with different chemical groups present on the exposed surface of the protein or peptide shows a different behavior from other solvent molecules which are not in contact with the solute. Differences in specific volume between water molecules in the hydration layer compared to the water molecules in bulk water can be measured; these changes can help us to understand the amount of water molecules that interact with the protein or peptide.

However, according to several authors (Chalikian, 2003; Chalikian & Filfil, 2003; Bánó & Marek, 2006) the partial specific volume also can be described as consisting of four contributions as:

$$\bar{V}^0 = V_M + V_T + V_I + \beta_{T0} RT \quad [4]$$

where V_M is the intrinsic volume which is defined by the molecular size; V_T is the thermal volume, which results from the molecular vibrations of the solute and the solvent; V_I is the interaction volume which results from the changes in volume due to the interactions between the solute and water molecules in the hydration layer; β_{T0} is the coefficient of isothermal

compressibility of the solvent; R is the universal gas constant and T is the temperature (Chalikian, 2003; Bánó and Marek, 2006). Bánó & Marek (2006) pointed out that for large molecules, such as proteins in water, the value of $\beta_{T0}RT$ (1.83 $Å^3$) can be neglected.

Chalikian (2003) pointed out two definitions of intrinsic volume which can be used. One definition is the Voronoi volume where the volume is calculated according to a geometric theorem developed by Voronoi in which the atoms are surrounded by a polyhedron. The other definition is the molecular volume which is directly related to the molecular weight. Bánó & Marek (2006) pointed out that the molecular volume generally is described by the Van der Waals shell model or as the volume enclosed by the molecular surface of a protein.

The thermal volume V_T is related with the thermal motion of the solvent surrounding the surface of the solute (Chalikian, 2003; Bánó & Marek, 2006). The interaction volume V_I is related with the perturbation of the water molecules which surround the protein (Chalikian, 2003; Bánó & Marek, 2006); according to Bánó & Marek (2006) these water molecules can be classified into three groups: water molecules bonded to the polar protein surface by hydrogen bonds, water molecules that only interact with the non-polar protein surface and water molecules that are located in the next hydration shell. These three groups of water molecules show different behavior than water molecular in bulk water, with the water molecules interacting with the polar protein surface the most important for defining the interaction volume (Bánó & Marek, 2006).

The partial specific volume of biomolecules also has been calculated using additive schemes; where it is assumed that the individual volume of the units of a protein, as amino acids or small peptides, are additive, having the same hydration and contributing to the protein

volume with the same volume as the individual units have in solution (Kharakoz & Sarvazyan, 1993; Chalikian et al., 1994; Kharakoz, 1997).

2.1.1.3. Volume Determination Experimental Techniques

Experimental techniques for the determination of the volume of a solution can be divided into two groups: First, density measurements; the apparent specific volume of a solution can be calculated based on density of the solution and the solvent. There are several methods to measure density, like pycnometry, falling drop method and vibrating tube densitometry; this last method has been used to determine volumetric properties of proteins due to the high accuracy of the measurements and its compatibility with protein solutions (Schwitzer & Hedwig, 2005; Bánó & Marek, 2006, Lee et al., 2008). The second experimental approach to determine the specific volume is dilatometry which measures directly changes of volume, usually using capillary detectors (Zamyatnin, 1984).

Among the several options to measure densities in liquids, the use of devices based on the vibrating, oscillating tube principle have proved to be of high accuracy in density measurements and have advantages like the use of small amounts of liquid and the ability to use a large range of operating temperature (Picker et al., 1974; Zamyatnin, 1984; Retsina et al., 1986).

Vibrating tube density meters are based on the principle that the vibration of a solid which is surrounded by a liquid is mediated by the properties of the liquid, and it is specially related to the density of the liquid. Therefore, in these equipments the density of the solution is calculated based on the vibration frequency, determined by highly precise electronic

techniques, of a solid tube filled with the solution (Zamyatnin, 1984; Retsina et al., 1986). Generally, the configuration of vibrating tube density meters consists in a hollow tube with a "U" shape that is fixed as both ends (Retsina et al., 1986).

2.1.2. Compressibility

Elastic and hydration properties of proteins and peptides can give information about their structure, dynamics in solution and their functionality. The compressibility of a solution and the apparent compressibility of proteins and peptides in solution are parameters used to characterize the elastic and hydration properties of proteins and peptides (Heremans & Smeller, 1998; Pfeiffer et al., 2008).

The compressibility (*K*) of a solution can be defined as the change in volume as the pressure changes (Chalikian & Breslauer, 1998; Pfeiffer et al., 2008) and can be isothermal or adiabatic depending on the conditions, with isothermal compressibility taken at constant temperature and adiabatic compressibility taken at constant entropy. Therefore, according to Sarvazyan (1991) and Taulier & Chalikian (2002), the isothermal compressibility of a solution, K_T, can be defined as the change of its volume V as the pressure P changes [equation 5] at constant temperature, which is usually associated with a negative value due to the pressure increment causing a reduction of the volume of the solution.

$$K_T = \beta_T V = -(\partial V/\partial P)_T \quad [5]$$

where T is the absolute temperature and β_T is the isothermal compressibility coefficient of the solution $\beta_T = -V^{-1}(\partial V/\partial P)_T$.

The adiabatic compressibility of a solution, K_S, is the change of the volume V of the solution as the pressure P changes [equation 6] at constant entropy, which also is associated with a negative value due to a pressure increment causing a reduction of solution volume (Sarvazyan, 1991; Taulier & Chalikian, 2002).

$$K_S = \beta_S V = -(\partial V/\partial P)_S \quad [6]$$

where S is the entropy and β_S the adiabatic compressibility coefficient of the solution $\beta_S = -V^{-1}(\partial V/\partial P)_S$.

In aqueous dilute solutions, the isothermal and adiabatic compressibility are similar because of the large heat capacity of the water and its low compressibility (Sarvazyan, 1991).

The isothermal and adiabatic compressibility coefficients can be related by the equation (Taulier & Chalikian, 2002):

$$\beta_T = \beta_S + \alpha^2 T/\rho C_P \quad [7]$$

where α is the coefficient of thermal expansion, and C_p the specific heat capacity at constant pressure.

Ultrasonic velocity and density measurements can be used to determine the adiabatic compressibility coefficient based on the Newton-Laplace equation,

$$\beta_S = \frac{1}{\rho U^2} \quad [8]$$

where β_S is the coefficient of adiabatic compressibility of the solution and ρ and U are the density and sound velocity of the solution (Sarvazyan, 1991; Pavlovskaya et al., 1992; Kharakoz

& Sarvazyan, 1993; Nölting, 1995; Heremans & Smeller, 1998; Taulier & Chalikian, 2002; Chalikian, 2003; Pfeiffer et al., 2008).

In order to analyse the elastic properties of proteins or peptides in aqueous solution the apparent specific adiabatic compressibility and partial specific adiabatic compressibility values are generally used (Chalikian et al., 1994; Taulier & Chalikian, 2002). Therefore, the apparent specific adiabatic compressibility, $\overline{K}_S$, is the change of the compressibility of the solution as a specific amount of the solute is added and the concentration of the solute varied [equation 9] (Sarvazyan 1991; Chalikian & Breslauer, 1998). The apparent specific adiabatic compressibility of a solution is normally associated with the apparent volume that a solute occupied within a solution at a given concentration.

$$\overline{K}_S = \frac{(K_S - K_{S0})}{cV} \qquad [9]$$

where K_S is the adiabatic compressibility of the solution, K_{S0} is the adiabatic compressibility of the solvent, V is the volume of the solution and c is the concentration of the solute.

The partial specific adiabatic compressibility, $\overline{K}_S^0$, is the change of the compressibility of the solution as a specific amount of the solute is added, but at infinite dilution [equation 10] (Sarvazyan 1991; Chalikian & Breslauer, 1998). At infinite dilution the solute-solute interactions vanish and the partial specific adiabatic compressibility is only influenced by the solute-solvent interactions (Chalikian et al., 1994).

$$\overline{K}_S^0 = \lim_{c \to 0} \overline{K}_S \qquad [10]$$

In the study of solute-solvent interactions, the apparent compressibility will be affected by the solute-solvent interactions as well as the solute-solute interactions. The interactions of

solute-solute molecules diminish as the concentration is reduced, therefore the partial compressibility calculated by extrapolation to zero concentration of the apparent compressibility describes more accurately solvent-solute interactions (Chalikian et al., 1994). However, as happens with the partial specific volume, accurate experimental values of density and ultrasonic velocity are needed, especially at low concentrations (Marcus, 2011).

The partial adiabatic compressibility can be analysed as the sum of different contributions; intrinsic compressibility of the solute, K_M, and the changes in compressibility due to hydration ΔK_h.

$$\bar{K}_S^0 = K_M + \Delta K_h \qquad [11]$$

The partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^0$, which describes the relation between compressibility and specific volume, can be determined based on ultrasonic velocity and density measurements as explained previously and is calculated as (Gekko et al., 2004):

$$\bar{\beta}_S^0 = \frac{\bar{K}_S^0}{\bar{V}^0} = -\frac{1}{\bar{V}^0}\left(\frac{\partial \bar{V}^0}{\partial P}\right) \qquad [12]$$

Then $\bar{\beta}_S^0$ can be calculated based on concentration of the solution, the density of the solution (ρ) and the solvent (ρ_0), and the adiabatic compressibility coefficient of the solution (β_s) and the solvent (β_{s0}) calculated by equation [8] with density and ultrasonic velocity as:

$$\bar{\beta}_S^0 = \frac{\beta_{s0}}{\bar{V}^0} \times \lim_{c\to 0} \frac{\beta_s/\beta_{s0} - (\rho - c)/\rho_0}{c} \qquad [13]$$

Experimentally, adiabatic conditions are generally chosen because the isothermal conditions normally provide less accurate results (Chalikian et al., 1994). However, some authors (Sarvazyan, 1991; Kharakoz & Sarvazyan, 1993; Nölting, 1995) have pointed out that the

adiabatic condition of the ultrasonic measurements is normally achieved for the solvent but not for the solute. Normally the short time of the ultrasonic measurements allows us to consider there is not heat exchange within the solvent molecules in aqueous solutions, but that assumption is not necessarily true for solute molecules. These authors consider it is only possible to achieve pseudoadiabatic conditions, and therefore to calculate pseudoadiabatic compressibility which is between the adiabatic and the isothermal compressibility. Nevertheless these previous studies, conducted in order to analyse the possibility of achieving adiabatic conditions during ultrasonic velocity measurements, were conducted only with large globular proteins solutions.

2.1.2.1 Apparent Specific Adiabatic Compressibility

Compressibility of solutions of biological components like proteins as well as specific volume, is influenced by the solute-solvent interactions and it is convenient to use the apparent specific adiabatic compressibility and partial specific adiabatic compressibility (Chalikian et al., 1994).

The apparent specific adiabatic compressibility can be calculated based on the density and ultrasonic velocity measurements, and can be defined as the change in apparent specific volume of the solute as the pressure changes as (Chalikian et al., 1994; Gekko et al., 2004):

$$\overline{K}_s = \beta_{s0} \times \frac{\beta_s/\beta_{s0} - (\rho - c)/\rho_0}{c} \qquad [14]$$

where β_s and β_{s0} are the adiabatic compressibility coefficient of the solution and the solvent; ρ and ρ_0 are the density of the solution and the solvent, and c is the concentration of the solution.

2.1.2.2. Partial Specific Adiabatic Compressibility

Finally, the partial specific adiabatic compressibility, which describes the change of the apparent specific volume of the solute as the pressure changes at infinite dilution, where the solute–solute interactions are diminished, can be calculated as:

$$\overline{K}_s^0 = \beta_{s0} \times \lim_{c\to 0} \frac{\beta_s/\beta_{s0} - (\rho - c)/\rho_0}{c} \quad [15]$$

2.1.2.3. Ultrasound Waves to Measure Elastic Properties

In equation [8] the coefficient of adiabatic compressibility was related to the ultrasonic velocity. The ultrasonic velocity can be a useful tool in order to measure molecular transitions of proteins and peptides that occur in aqueous solutions and during the interactions of proteins or peptides with other molecules. The great sensitivity of the ultrasound measurements can give information on the changes in the solute-solvent interactions occurring during protein solvation and its dynamics in solution (Sarvazyan, 1991; Chalikian, 2003). Additionally, and regarding the dependence of chemical reactions on temperature and pressure, ultrasonic techniques have the advantage that they can be performed at a specific temperature and pressure, as well as being independent of the need for chemical probes, which like other chemical components, can alter the nature of the original system (Sarvazyan, 1991; Chalikian, 2003; Kaatze et al., 2008).

Kentish & Ashokkumar (2011) describe sound waves as pressure waves passing through a medium. These are longitudinal waves that differ according to their frequency. Ultrasound is the sound that has a frequency above 20 kHz (Mason & Saéz Bernal, 2012).

Ultrasonic techniques are based on the propagation of sound, and also its attenuation, reflection, absorption or refraction when passing through a liquid sample (Sarvazyan, 1991). The ultrasonic techniques include ultrasonic spectroscopy and ultrasonic velocimetry (Sarvazyan, 1991; Kaatze et al., 2008). According to McClements (1991), the ultrasound can propagate through a material by different ways. Ultrasonic velocity and attenuation measurements are used to assess the propagation of compressional waves which are transmitted by successive compressions and expansions within the elastic limit of the material. Therefore the physical properties of the material, such as density, elasticity and composition can be related to the velocity of propagation of ultrasonic waves passing through a material (McClements, 1991).

The ultrasound velocity of a solution is the result of the relation shown in equation [8]. Therefore depending on the molecular characteristics of different compounds in solution the compressibility values affect the ultrasonic velocity of the solution (Sarvazyan, 1991, Kaatze et al., 2008).

Measurements of the ultrasonic velocity can be used to determine the elastic properties of a sample, the conformational transitions of a solute and the solute-solvent interactions; and it has become a very important method in biophysical studies (Sarvazyan, 1991). Ultrasonic velocity measurements are similar to those from ultrasonic spectroscopy, which measures the absorption of ultrasonic pulses at different frequencies and is normally used to analyse the aggregation or relaxation processes of biomolecules (Sarvazyan, 1991; Povey et al., 2011).

There are several methods to measure ultrasonic velocity. In the case of solutions of biological compounds the method generally involves specific requirements, like a small size of the sample and high precision of the measurement. For example, the size of the sample should of 1 mL or less, and the precision around 10^{-4}%. The need to use a small sample, around 1 mL, or

less, is because with this size it is possible to achieve good temperature control to avoid the variations and error in the measurement due to the temperature dependence on the ultrasound velocity (Sarvazyan, 1991, Kaatze et al., 2008). The precision of around 10^{-4}% is needed to allow working with low concentrations, where the solute-solute interactions are reduced and the changes in ultrasonic velocity are very small (Sarvazyan, 1991, Kaatze et al., 2008).

Two kinds of methods are usually used to determine ultrasonic velocities in biological compounds. First, the sing-around or pulse method which consists in an ultrasonic pulse which passes through the solution and then is retransmitted after it is received. This method can achieve the precision of 10^{-4}% needed for accurate measurements but with the disadvantage that it requires a sample that is large, around 10mL (Sarvazyan, 1991, Kaatze et al., 2008).

Secondly, the continuous wave or resonator method can be used, where the resonance frequency of an ultrasonic pulse is measured by two parallel transducers located at different side of a resonator cavity, where one is the transmitter and the other the receiver (Eggers & Funck, 1973; Sarvazyan, 1991, Kaatze et al., 2008). Then the ultrasonic velocity is determined by the difference between the resonance frequency of the solvent and the solution. This method can achieve a precision around 10^{-4}% and also have the advantage that it only needs a small volume sample, around 0.1 mL. A temperature stability around 10^{-3}K is required to have accurate measurements, because of the temperature dependence of the ultrasonic velocity (Sarvazyan, 1991). The control of sample temperature becomes an important requirement for any method, and because it is easier to control the temperature for a small sample, the use of the resonator method (0.1 mL sample) is also advantageous (Sarvazyan, 1991, Kaatze et al., 2008).

2.1.3. Interpretation of Specific Volume, Compressibility and Their Changes

Volumetric properties have been used in several studies (Durchschlag & Jaenicke, 1982; Gekko & Hasegawa, 1986; Kharakoz & Sarvazyan, 1993; Chalikian et al., 1994; Harpaz et al., 1994; Chalikian et al., 1995; Chalikian et al., 1996; Kharakoz, 1997; Heremans & Smeller, 1998; Murphy et al., 1998; Calandrini et al., 2000; Taulier & Chalikian, 2001; Valdez et al., 2001; Taulier & Chalikian, 2002; Chalikian, 2003; Chalikian & Filfil, 2003; Gekko et al., 2004; Bánó & Marek, 2006; Lee et al., 2008; Pfeiffer et al., 2008) in order to understand protein hydration and its changes during protein denaturation. These same measurements can help us to characterize the conformational state of different proteins and peptides.

2.1.3.1. Specific Volume Changes in Proteins and Peptides

Studies of proteins in the unfolded state can give a lot of information about protein dynamics. The unfolded state of a protein usually is described as an extended chain; this conformation has the amino acid chains in random coil conformation where most of the atomic groups are exposed (Murphy et al., 1998).

The specific volume changes which happen in the conformational changes of protein reflect alterations in protein hydration and intrinsic packing (Chalikian & Filfil, 2003). This difference in molecular volume can give a hint about the conformational state of the proteins. However the specific volume changes which occur during the transition of a protein from a folded to an unfolded state are very small, generally around 1% or 2% of the partial specific volume value of the protein, and moreover, these changes can be positive or negative

(Chalikian, 2003). However, according to Chalikian (2003), the changes in specific volume resulting from pressure induced denaturation at elevated pressure are positive.

Generally the transition of a protein from folded to an unfolded conformation is mediated by different states of a protein that is more or less unfolded, which can be classified in three main classes: compact intermediate states, unfolded states, and fully unfolded state. The compact intermediate state has a compact structure, retaining part of the secondary structure, and almost no tertiary structure, and also it has a large core of amino acids inaccessible to the solvent; the folded state has a partially extended structure with all secondary structure lost, but a small core of amino acids remains inaccessible to the solvent; finally, the fully unfolded state has a completely extended chain of amino acids with almost no amino acids inaccessible to the solvent (Chalikian, 2003).

The conformational changes in a protein from a folded to an unfolded state are supposed to cause important changes in the specific volume due to the changes in the amount of surface atomic groups exposed to the solvent which influences the interaction between protein and the water molecules in the hydration layer. The hydration layer, whose dimensions are no larger than two layers of water molecules, are the water molecules whose physicochemical properties are altered by the interaction with the solute and have properties that are different from those of the water molecules of the bulk water (Chalikian et al., 1994). A possible interpretation of the change of specific volume is that during these conformational transitions of proteins, a decrease of intramolecular voids within the polypeptides chains occurs due to the unfolded process, which causes a decrease of intrinsic volume, and an increase in protein surface exposed to the water and therefore the protein hydration (Chalikian, 2003). Another important aspect is the change in the thermal volume; as pointed out by Chalikian

(2003), the thermal volume would be related with the amount of surface exposed to the solvent, hence when a protein unfolds an increase of surface exposed causes an increase of the thermal volume which seems to compensate for the decrease of volume caused by the elimination of intramolecular voids between the polypeptide chains of the protein structure.

Harpaz et al. (1994) found differences in volume between amino acids and small peptides in solution, and their contribution to the total volume of a protein molecule when these same amino acids or small peptides were within a protein structure. Additionally, Harpaz et al. (1994) reported differences in volume between non-polar and polar groups of amino acids and between non-polar and polar groups present within the structure of peptides and protein structure. These differences were due to the effect on the volume of the charges of the non-polar and polar groups when they are exposed to the water and when they are within the protein structure.

Changes in the specific volume of proteins, peptides and amino acids are also influenced by the ionization/neutralization of their chemical groups in contact with the solvent. This effect was studied by Rasper & Kauzmann (1962) who found that neutralization of carboxylic groups in proteins results in a volume increase of globular proteins (11 cm^3mol^{-1}). A similar effect was found for the neutralization of the amino groups, resulting in a volume increase of around 16 to 18 cm^3mol^{-1}. It is important to mention that the ionization/neutralization of carboxylic and amino groups in small molecules, such as amino acids or small peptides, produces changes in the specific volume different than the changes occurring in the proteins containing carboxyl or amino groups (Rasper & Kauzmann, 1962). Kharakoz (1997) found that ionization contributes about 3% to the partial specific volume of unfolded proteins. However, this contribution of 3%, was calculated based on the partial specific volume of each amino acid of the protein structure

and It was done assuming that the contribution of each amino acid to the partial specific volume of a protein is additive.

The small changes in volume during conformational transitions of proteins have been documented in several studies (Harpaz et al., 1994; Hackel et al., 1999; Chalikian, 2003; Chalikian & Filfil, 2003). Valdez et al. (2011) found small change in volume (0.006 ± 0.003 mL g^{-1}) between two forms of the myelin basic protein. Additionally, it is important to mention that the method used to induce denaturation could affect the results of the change in specific volume during a conformational transition in the protein. The nature of the interaction between the polypeptide chains and denaturating agents is not well understood yet (Hackel et al., 1999).

The changes in specific volume as proteins change their conformation or denature are caused by the effect of the protein surface on the hydration layer and therefore by the changes in the amount of protein structure that is exposed to solvent and the amount of protein buried in the interior (Murphy et al., 1998; Chalikian & Filfil, 2003). These differences are mainly caused by the electrostriction effect that occurs in charged groups of the amino acids exposed to the aqueous solvent (Harpaz et al., 1994). The electrostriction effect has been reported in biological compounds such as proteins to be related to the amount of charged groups in the protein structure that are exposed to the solvent (Pfeiffer et al., 2008; Marcus, 2011).

Electrostriction can be defined as the contraction of a material exposed to an electric field. In solutions, electrostriction is seen as the apparent diminution of the volume of a solute when it is dissolved in a solvent, and can be described as:

$$\Delta V = V_M - \bar{V}^0 \qquad [16]$$

where V_M is the intrinsic volume, which is the solute volume assigned to a solute molecule and $\bar{V}^0$ is the partial specific volume. The electrostriction effect can occur in any solvent, although it is especially important in aqueous solution (Desnoyers, 1965; Marcus, 2011).

The electrostriction effect in aqueous solution is caused by the electric field of a dissolved solute which affects the water molecules located in the water shell around the solute. This electric field causes a compression effect on the water molecules, breaking the hydrogen bonds that interact with the solute, and then reducing the space between the water molecules and the solute causing a compression of the solvent. There are small or negligible effects on the ion molecule (Marcus, 2011).

Generally the electrostriction effect is analysed at infinite dilution with the partial specific volume because at this condition the solute-solute interaction diminishes. For electrolyte solutions these conditions consider a total dissociation of ions. However, for the analysis of the electrostriction at finite dilution, besides the solute-solvent interactions, it is necessary to also consider solute-solute interactions and the dynamics of the solvation effect as the solvent molecules enter and leave a close association with the solute molecule at different rates depending on the nature of the solute (Marcus, 2005; Marcus, 2011).

Chalikian & Filfil (2003) have reported changes in specific volume caused by denaturation of proteins with different molecular weight, and the dependence of these changes on the degree of denauration of the proteins. These results confirmed the small magnitude of changes in specific volume during protein denaturation which suggests that there is a the compensation between the different contributions to the specific volume: intrinsic volume, thermal volume and interaction volume, which due to microscopic changes occurring during the

denaturation process cause an increase of some contributions while the other decreases depending on the conformation state (Chalikian, 2003).

Electrostriction has an effect on the hydration properties of the solute and hence on the compressibility, and as pointed out by Pfeiffer et al. (2008), the negative effect on the compressibility caused by the hydration of solutes is generally associated with electrostriction.

2.1.3.2. Compressibility Changes of Peptides and Proteins

The compressibility will vary depending on the size of the molecule, but between globular proteins and peptides with different sizes, the changes in compressibility could result from different factors. In globular proteins, due to the imperfect packing of the folded conformation, the interior voids result in an important contribution to the intrinsic compressibility which could be substantially lowered in unfolded proteins or peptides with low molecular weight. Unfolded proteins or peptides don't have the same amount of voids as globular proteins. Also it is important to account the effect on the compressibility caused by changes in the pH of the solution. This effect is usually low for globular proteins, but it can be an important effect for peptides due to pH changes which affect the protonation of the carboxylic and amino groups exposed to the solvent (Sarvazyan et al., 1979; Sarvazyan, 1991; Chalikian et al., 1994; Chalikian, 2003).

Compressibility is usually calculated assuming a linear relation between pressure and density. However according to Sarvazyan (1991) the non-linearity between these two parameters can also be used to analyse solute-solvent interactions. Measurement of the dependence of ultrasonic velocity on pressure and temperature is thought to be the easiest

method to calculate the non-linearity based on the relation between the adiabatic compressibility coefficient and density [equation 8] (Sarvazyan, 1991).

Compressibility values for proteins, peptides and amino acids in aqueous solutions can be either positive or negative. Positive compressibility values indicate a highly compressible interior, while negative compressibility values indicate a non-compressible interior like in fibrous proteins and amino acids, where the compressibility values are driven primarly by the negative compressibility contribution of the hydration of the solute caused by solute-solvent interactions (Gekko & Hasegawa, 1986; Kharakoz & Sarvazyan, 1993; Apenten et al., 2000). Generally the compressibility of amino acids in aqueous solutions is negative, the compressibility of proteins in the native state is positive and the compressibility of proteins in the unfolded state is usually negative. This behavior can be caused due to the negative compressibility of the water in the hydration shell compared to its behavior as bulk water. For amino acids and unfolded proteins the hydration contribution to the compressibility is very important because the intrinsic compressibility tends to be small. In contrast, for the native state of proteins the contribution of the intrinsic compressibility is usually large due to the internal voids in its structure (Heremans et al., 1998). Apenten et al. (2000) confirmed this behavior, concluding that protein compressibility is the result of two antagonistic factors, the intrinsic compressibility and the compressibility due to the hydration effect. Therefore the changes in compressibility can be used as an indicator of the folded to unfolded protein conformation process.

Protein transitions from a folded to an unfolded conformation are generally classified by three different states representing species of protein that are more or less unfolded: compact intermediate states, partially unfolded states, and fully unfolded states (Chalikian, 1993). These conformation transitions result in changes in the partial molar adiabatic compressibility (K_s^0),

according to Taulier & Chalikian (2002). The transition of a globular protein from a folded state to a fully unfolded state is mediated by different conformation states with different compressibility values. The transition from a native protein to a compact state has a small increase of K_S^0 between 1 to 4 x10^{-6} cm^3 mol^{-1} bar^{-1}. For the transition from a native protein to a partially unfolded state, K_S^0 shows a small decrease between 3 to 7 x$10^{-6}$$cm^3$ $mol^{-1}$$bar^{-1}$; finally a full transition from the native to the fully unfolded state shows a large decrease of K_S^0 between 18 to 20x10^{-6} cm^3 mol^{-1} bar^{-1} (Taulier & Chalikian, 2002). In the partially unfolded state the tertiary structure is completely lost and a partial secondary structure remains; for the fully unfolded state, theoretically it is accepted that the structure that remains is a random coil (Chalikian, 2003; Chalikian & Filfil, 2003; Lee et al., 2008). These changes in compressibility are common for all the proteins, in other words these changes are present in all globular proteins during the conformational changes. Variation in compressibility, as a result of an unfolding of proteins, happens because the unfolding process causes changes in the packed interior of the protein, which affects the intrinsic compressibility, and additionally increase the solvent accessible surface, which also affects the hydration contribution to the compressibility (Taulier & Chalikian, 2001; Taulier & Chalikian, 2002; Lee at al., 2008).

Chalikian & Breslauer (1996), found a similar result in a study about the changes in compressibility in different conformations of the protein cytochrome C subjected to acid induced denaturation. In this study a decrease of the protein compressibility as the denaturation process occurred was found and was associated with the increase of solvent accessible surface and decrease of hydrophobic character of the overall structure.

During the conformation transition of proteins and peptides, another effect that influences compressibility is the kind of secondary structure that proteins or peptides adopt.

According to Gekko & Hasegawa (1986), it seems that the helix structure has large compressibility. Therefore, proteins with a larger proportion of the helix secondary structure have large compressibility value compared with other proteins. Additionally, the presence of disulphide bonds or non-protein attached groups as metal, carbohydrate, lipid or coenzymes can also influences the compressibility of globular, concluding that structural factors seem to be important due to their influence on the intrinsic compressibility of protein and peptides.

Beside the influential factors of intrinsic compressibility, it is also important to account for factors that influence the hydration compressibility of proteins. The properties of water molecules in the hydration shell around biological compounds in solution are an important factor that influence the compressibility of a solution. As it was mentioned previously in this section and in section 2.1.3.1., the properties of the water molecules surrounding the solute are different from the rest of water molecules in bulk water, with a different compressibility. The amount of water molecules in the hydration shell is directly related to the hydration contribution of the compressibility. Therefore, the amount of exposed surface of the solute will affect the amount of water molecules and the size of the hydration shell and the hydration contribution to compressibility. The number of water molecules in the hydration shell of biomolecules is known as the hydration number (Sarvazyan, 1991; Chalikian et al., 1994). The hydration of biomolecules in solution is a result of the surface area of this solute exposed to the solvent and the characteristics of the groups on the surface area, which can affect the thickness of the hydration shell (Sarvazyan, 1991; Chalikian et al., 1994).

The difference in compressibility of the water molecules in the hydration shell of a solute, such as a protein or a peptide, is related to the electrostriction effect. The electrostriction effect, explained in the previous section 2.1.3.1., causes a contraction in the

volume of the water molecules in the water shell that surround a solute. This contraction, which is caused by the electric field of the exposed surface of the solute, has a negative effect on the compressibility (Pfeiffer et al., 2008; Marcus, 2011).

The compressibility of small peptides is generally affected by the hydration contribution. Small peptide structure, similar to that of unfolded proteins or amino acids, have a mainly extended structure which causes the intrinsic compressibility to be small, and the compressibility is influenced mainly by the hydration contribution that is negative (Chalikian et al., 1998; Pfeiffer et al., 2008).

Finally the compressibility can be influenced by the stability of the proteins which is directly affected by the physicochemical properties of the solvent. Calandrini et al. (2000) pointed out the complexity of the changes when a co-solvent, such as alcohol, is added inducing changes in the protein stability. At low concentrations of alcohol the structure seems to be more tightly packed at the folded conformation, but at higher concentrations the alcohol induced a destabilization of the folded structure causing changes in compressibility.

2.1.3.3. Other Biological Compounds

The compressibility of other compounds, such as lipid bilayers (model membranes), which are used in studies to increase understanding of the complexity of the dynamics of the cell membrane, can be analysed. Lipid bilayers can be formed by a mixture of different phospholipids and other components like cholesterol. Volumetric properties can help to understand the changes in conformation and phase transitions caused by composition or change in temperature. The conformation of phospholipids bilayer can vary depending on the

size of the phospholipids in the structure, the amount of double bonds, and the presence of other components like cholesterol (Hianik et al., 1998; Uhríková et al., 2007). Analysis of the changes of the volumetric properties can help to understand how the physical properties of the model membranes are affected by the composition, transition phase temperature and interactions with other molecules as proteins or peptides (Hianik et al., 1998; Krivanek et al., 2001; Rybar et al., 2007; Uhríková et al., 2007).

2.2. Proteins, Peptides and Amino Acids

2.2.1. Chemical Structure and Physical Properties

Proteins and peptides are polymers of amino acids which are joined by peptide bonds. This bond is planar but allows an amount of torsional flexibility which leads to several possible energetically favorable conformations for a given peptide or protein. There are 20 different amino acids, each of which has a basal structure with an amide and carboxylic group and a distinctive group named as a side chain (Creighton, 1993; Sewald & Jakubke, 2002; Langel et al., 2010).

Protein conformation can be classified into four types, based on the complexity of the structural conformation: primary, secondary, tertiary and quaternary (Creighton, 1993; Sewald & Jakubke, 2002; Langel et al., 2010).

The primary structure consists of the sequence of amino acids, which will later define the following more complex structures (secondary, tertiary). The amino acid composition and sequence defines the intramolecular interactions, and these interactions permit the secondary

structure and the folded arrangement of the tertiary structure (Creighton, 1993; Sewald & Jakubke, 2002).

The secondary structure of a protein is the most energetically favorable conformation adopted by a polypeptide chain allowed by the torsion of the peptide bond at different angles, and it is generally classified into two main conformations, namely α-helix, and β-sheets (Creighton, 1993; Vila et al., 1998; Sewald & Jakubke, 2002).

The tertiary structure is a global and precisely defined three dimensional organization of the polypeptide chain. The tertiary structure is largely maintained by the interaction between amino acid side chains. Two features determine the tertiary structure: the primary structure (amino acid sequence) and the environment (solvent, pH, electrolytes concentration) (Creighton, 1993; Sewald & Jakubke, 2002).

The quaternary structure occurs in some proteins that exist as aggregates of two or more polypeptide chains (monomers). The center of the interfaces between monomers are usually similar to the interiors of the individual monomers in that they are closely packed and involve primarily hydrophobic interactions between non-polar side chains (Creighton, 1993).

The physicochemical properties of proteins in solution depend on the specific conformation of the protein and the amino acid residues present on their surface which are exposed to the solvent (Harpaz et al., 1994; Avbelj, 2000; Trevino et al., 2007; Chen et al., 2008).

Many important biomolecules like globular proteins have to be in aqueous solution in order to perform their biological function (Raschke, 2006; König & Boresch, 2009). Each protein in solution has a unique tertiary structure while peptides also have a complex structure, usually adopting a specific secondary structure (Creighton, 1993). Because the conformation of proteins

and peptides is not completely rigid important dynamic changes occur which are related with the protein or peptide's biological function (Creighton, 1993; Sewald & Jakubke, 2002).

The solubility of the protein will be mainly defined by both the hydrophobicities and the hydrophilicities of the side chains of the amino acids at the surface (Raschke, 2006; Trevino et al., 2006). The polar side chains at the surface promote better solubility of the protein molecule, because the exposed charges of the polar side chains will tend to interact with water. The most favorable interactions with aqueous solvent are provided by charged and polar groups of the hydrophilic side chains (Raschke, 2006). König & Boresch (2009) pointed out that the amino acids in the interior of the protein structure contribute very little to the solvent affinity of the protein, because the solvent interacts in a limited way with the internal structure of the protein.

Generally, the solubility of proteins in aqueous solution varies very much, since it is related with the amino acid sequence of its surface, as well as with pH and other solutes in the aqueous environment. Some structural proteins that have polar side chains at the surface could need a pH far from the isoelectric point to raise the electrostatic repulsion needed to keep molecules in solution (Trevino et al., 2006).

The pH of the solution can affect the stability of the folded structure and cause the protein structure to unfold, letting many non-polar amino acid residues be exposed to the aqueous solution, and thus increasing the non-polar surface area of the protein molecules. Because the interactions between water molecules (polar) and the non-polar surface of proteins are thermodynamically unfavorable, protein solubility is reduced (Creighton, 1993; Kyte, 1995; Trevino et al., 2006; Langel et al., 2010).

Protein structure is related to the behavior of water as a solvent. Agarwal et al. (2010) pointed out that water has a large dipole moment and polarizability which results in a very high dielectric constant, making water an excellent solvent of ionic or polar solutes. Water molecules in the hydration layer around the surface of a protein or peptide have different chemical arrangements compared to bulk water molecules.

The physicochemical properties of proteins originate from their three dimensional structure. Calandrini et al. (2000) suggest that the free energy of stabilisation of the three dimensional structure of the proteins partly arises from hydrophobic interactions, which refers to the interaction induced by the solvent which prefers to interact with other solvent molecules instead of the solute molecules; this effect induces greater interaction between molecules of the solute.

Taulier & Chalikian (2002) pointed out that protein folding occurs spontaneously, and they suggest the hypothesis that the lowest free energy state is achieved in the native and folded conformation of the protein. The transitions of folding and unfolding of proteins seem to involve cooperativity reactions, and are affected by pH, temperature, pressure and any denaturating agent (Taulier & Chalikian, 2002).

2.2.2. Peptides Bioactivity

Proteins are very important structural and functional components in the cell and have well known importance as part of nutrition. Besides the nutritional benefit, proteins, and peptides within the protein´s structure, have been found to have functional properties for the food industry like foaming, emulsifying, and film forming capacity due to their physicochemical

properties (Kylara & Panyam, 2003). Moreover, the physiologically active (bioactive) properties of many proteins and peptides from different sources, vegetal and animal, have been receiving an increasing interest (Korhonen & Pihlanto, 2006; Hartmann & Meisel, 2007).

The bioactivity of peptides can be described as a physiological effect in addition to the nutritional benefit; however this description is very broad. According to Owusu-Apenten (2010), the bioactivity of any peptide, of animal or vegetal source, is an effect which exerts a benefit in health not related with its nutritional function.

According to Korhonen & Pihlanto (2006), bioactive peptides can be defined as specific parts of proteins which provide a positive effect on body functions and results in a health benefit. Some bioactive peptides are produced naturally by the human body to exert a specific physiological action in different sites such as the gastrointestinal tract and the brain (Sewald & Jakubke, 2002; Owusu-Apenten, 2010).

The bioactive peptides from food sources are not active within the protein structure, but as the peptides are released, they can exert their functionality. The peptides in general can be released by enzymatic hydrolysis during digestion, proteolysis by microorganisms during fermentation or the ripening process; and proteolysis by enzymes from microorganisms or plants (Meissel, 1998; Korhonen & Pihlanto, 2006; Erdmann et al., 2008; Tidona et al., 2009).

The bioactive peptide sizes can vary from di-peptides to quite large and complex structures that can include glycol, propryl and acyl groups, whole bioactive peptides can also exert one or multiple effects (Gill et al., 1996; Korhonen & Pihlanto, 2006). Depending on the amino acid sequence, these peptides can exert different physiological actions which comprise multiple effects (Guilloteau et al., 2009).

2.2.2.1. Dairy Bioactive Peptides

The interest about the physiological benefits of dairy proteins, beyond their nutritional value, and especially about bioactive peptides within dairy protein structures, has been rising in the past number of years. Several studies have been performed in order to test their different physiological functionalities (Gill et al., 1996; Chabance et al., 1998; Meissel, 1998; Korhonen & Pihlanto, 2006; Hartmann & Meisel, 2007; Erdmann et al., 2008; Guilloteau et al., 2009, Jäkälä et al., 2009; Korhonen, 2009; López-Expósito & Recio, 2009; Kamau et al., 2010; Phelan et al., 2009).

Bioactive peptides have been isolated from different fractions of dairy proteins, such as caseins and whey protein and several different effects have been reported (Hartmann & Meisel, 2007; Korhonen, 2009).

A dairy bioactive peptide of a given sequence can exert several functions depending on different segments of that sequence. This is contrary to the endogenous bioactive peptides which are usually very specific and have only one function. Casein bioactive peptides, that exert several effects, have been reported. These effects can be associated with different segments within the amino acid sequence of the peptides, and these segments can be overlapped within the whole structure of the bioactive peptide (Meisel, 1997; Hartman & Meisel, 2007).

Because the bioactive peptides need to be released from their protein source to exert their effects and these releases have different pathways, like human digestion, food fermentation or ripening and microbial proteolysis, it is important to know if these peptides can resist the process and finally be absorbed and transported to the blood stream. Several studies have been made in order to understand the resistance of bioactive peptides to human digestion. The results of these studies have been varied, but have shown that some peptides actually can

reach the blood stream (Chabance et al., 1998; Quirós et al., 2009; Phelan et al., 2009; Picarriello et al., 2010). However, according to Möller et al. (2008), it is also known that not all bioactive peptides are absorbed during digestion, and some of them can act directly to produce a physiological effect via receptors in the gut.

2.2.2.1.1. Bioactivity Reported

The bioactivity reported from dairy peptides is quite large. In Fig 2.1. is shown some of the main effects reported from bioactive dairy peptides.

Opioid peptides were the first bioactive peptides isolated, and they are also called "exorphins" due to them having certain similarity with hormones like endorphins and enkephalins. Casomorphins are an important group of opioid peptides; they are isolated from caseins and have different agonist effects like gut regulation of electrolyte absorption and the effects derived from this absorption (Hartmann & Meisel, 2007).

Dairy bioactive peptides with inhibitory effects over the Angiotensin I-converting enzyme (ACE) have been isolated. Peptides with different amino acid sequence have been reported with this inhibitory effect that in general helps in regulation of blood pressure. Several of these dairy bioactive peptides that have been found are short, between 2 or 3 amino acids residues, which make them more resistant to digestive enzymes, and easily absorbed to exert their effect. Also it has been found that these peptides frequently contain polar amino acids (Meisel, 1997; Meisel, 1998; Hartmann & Meisel, 2007; Saito, 2008; Madureira et al., 2010).

Fig 2.1. Main effect associated with dairy bioactive peptides (Korhonen & Pihlanto, 2006).

Antihypertensive	Cardiovascular System
Antioxidative	
Antithrombotic	
Hypocholesterolemic	
Opioid agonist	Nervous System
Opioid antagonist	
Mineral binding	Gastrointestinal System
Anti-appetizing	
Antimicrobial	
Immunomodulatory	Immune System
Cytomodulatory	

The immunomodultory effects of dairy peptides varied from lymphocyte proliferation, natural killer cell activity, antibody synthesis and cytokine regulation. Casokins are one example of bioactive peptides which have some effects like enhancement of the immune response, and regulation of the cytokine or antibody synthesis (Meisel 1997; Hartmann & Miesel, 2007).

The absorption of the bioactive peptides in order that they exert a physiological effect is very important and there is still not too much understanding yet. Several pathways are involved in absorption of these peptides; these pathways vary depending on the size, the amino acid structure, charge and hydrophobicity (Lipka et al., 1996; Walter et al., 1996; Daniel, 2004; Poliwoda et al., 2007, Guang & Phillips, 2009; Phelan et al., 2009). According to Phelan et al. (2009), small di- and tri- bioactive peptides can be transported via a specific transporter named PepT1, and larger oligopeptides can be transported via a paracellular route which allows the

peptides to cross within the intercellular space between the cells. Furthermore, bioactive peptides can also be transported by a vesicle-mediated transporter in a process named transcytosis.

The stability and resistance of bioactive peptides during digestion is critical in order to exert their effects in the intestinal tract or to be absorbed. The resistance of bioactive peptides to the digestion process varies a lot, so it is necessary to determine individually the resistance of any bioactive in order to understand its ability to exert an effect. Bioactive peptides that have been isolated and shown to have a proven physiological effect in studies in vitro, have been found with further analysis to not resist gastrointestinal conditions (Chabance et al., 1998; Madureira et al., 2010).

2.2.2.2. Plant Origin Bioactive Peptides

Plant proteins are a source of bioactive peptides. Although milk and egg are two of the most important sources of bioactive peptides, plant proteins from pea, wheat, soy and hemp seed and amaranth have been found to exert physiological effects (Silva-Sanchez et al., 2008; Yoshie-Stark et al., 2008; Guang & Phillips, 2009; Tang et al., 2009; Tiengo et al., 2009; Orlovskaya et al., 2010; Roy et al., 2010; Lico et al., 2012).

2.2.2.2.1. Bioactivity Reported

The bioactivity reported in peptides from plant sources is not as abundant as it is for dairy bioactive peptides, however effects like opioid agonist, immudolatory, anti-hypertensive and antioxidant effects have been reported for peptides from different plant sources like wheat,

rice, peas, hemp proteins and other (Gill et al., 1996; Hartmann & Meisel 2007; Erdmann et al., 2008, Tang et al., 2009; Orlovskaya et al., 2010).

ACE inhibitor peptides are some of the peptides that can be isolated from plants. These bioactive peptides have been found in several different plant sources such as soybean, wheat, sunflower, rice, corn, broccoli and garlic (Guang & Phillips, 2009) and also ACE inhibitory properties have been found in bioactive peptides from pulse crops (Roy et al., 2010). Bioactive peptides isolated from amaranth have shown ACE inhibitory, antithrombotic, antioxidant immunostimulatory properties as well as effects against cancer and hypertension (Silva-Sanchez et al., 2008; Tiengo et al., 2009). Nevertheless more in vivo studies are necessary to confirm bioactivity in amaranth and pulse crop peptides (Silva-Sanchez et al., 2008; Roy et al., 2010). Studies that have been made already suggest that ACE inhibitor peptides from plants are small, with 2 to 5 amino acids, that also help them to be more resistant and more easily absorbed, and they typically contain proline, lysine and arginine amino residues (Erdman et al., 2008, Roy et al., 2010). Some ACE inhibitor peptides from plants have also shown additional antioxidant effects (Roy et al., 2010).

Hemp seed protein hydrolysates, obtained by different enzymes, have shown antioxidant effects. This suggests that it is important to conduct more studies on peptides isolated from hemp seed proteins in order to find if they can produce other effects (Tang et al., 2009). Girgih et al. (2011) also found antioxidant effects in vitro of hemp seed protein hydrolysates.

2.3. Model Membranes

The basic unit of the cell membrane is a bilayer formed by phospholipids and sphingolipids organized in two layers with their polar head groups along the two surfaces and their acyl chains forming the non-polar domain in between (Luckey, 2008). Other important components in the cell membrane are proteins which are in the lipid bilayer and these proteins cannot be removed without disrupting the membrane. These proteins can be integral proteins or peripheral proteins. The integral proteins have two segments, one is a transmembrane segment which is hydrophobic and interacts with the lipids in the interior of the membrane and the other segment, which is hydrophilic, is in contact with the aqueous exterior or interior of the lipid bilayer. The peripheral proteins are connected with the membrane surface by short hydrophobic segments and electrostatic forces (Hianik, 2006).

Cellular membranes are very complex heterogeneous systems modulated by many components; therefore, studies of molecular interaction of different compounds with the cell membrane are very difficult (Baginski et al., 2006; Peetla et al., 2009). Results coming from experiments with cells are usually susceptible to several interpretations. Hence other simpler systems such as model membranes can be used. Model membranes such as lipid vesicles, lipid monolayer and bilayers are good representatives of cellular membranes (Baginski et al., 2006; Peetla et al., 2009). These systems usually contain only one or two types of lipids being much simpler than regular cellular membranes (Baginski et al., 2006).

2.3.1. Lipids in the Cell Membrane

The cell membranes are constituted by lipids, proteins and carbohydrates. The lipid component includes phospholipids, sphingolipids and sterols. Membranes are responsible for the selective permeability that regulates the transport of many nutrients and exclusion of harmful agents (Hianik, 2006; Luckey, 2008).

Lipids are a group of heterogeneous organic compounds which are defined as biological substances that are generally hydrophobic in nature and in many cases soluble in organic solvents (Baum, 1987).

Phospholipids are the most abundant lipids in the cell membrane. Due to their amphipathic nature, they play an important role in the structure of the cell membrane; as a result of the hydrophobic effect phospholipids are able to self assemble into a bilayer, having an arrangement where the non polar section is in the middle of the cell membrane and at both side ends are the polar sections in contact with the aqueous solvent (Hianik, 2006; Luckey 2008).

Sphingolipids are another lipid component of the cell membrane; these compounds are not built on a glycerol backbone but on sphingosine, a long chain amino alcohol, to which a fatty acyl chain is attached in an amide linkage. The most common sphingolipids are the sphingomyelins, which are sphingophospholipids with phosphocholine or phosphoethanolamine head groups (Baum, 1987; Luckey, 2008).

The third main lipid components are the linear isoprenoids and the sterols, of which the most abundant compound in animal cell membranes is cholesterol. The linear isoprenoids include compounds derived from five-carbon units called isoprene (2-methyl-1, 3-butadiene) (Baum, 1987; Luckey, 2008).

2.3.2. Phospholipids in the Cell Membrane

Phospholipids are lipid compounds found in all living organisms; they are particularly abundant in the biological membranes that surround cells. Phospholipids are any lipid that contains a phosphate group. Phospholipids play vital roles within the organism, from taking part in fat metabolism to being involved in the transport of certain molecules across the cell membranes (Hianki, 2006; Luckey 2008).

There are different classifications in the literature for phospholipids; one common way is to classify phospholipids in two groups: phosphoglycerides and sphingolipids (Baum, 1987; Hianik, 2006). Fahy et al. (2005) proposed that phospholipids also can be classified as a different category that only covers the molecules of glycerides with a phosphate group, classifying them in a different group from compounds such as sphingolipids which are commonly found associated within the phospholipids category.

Phospholipids are the most polar of lipids, and they contain both hydrophilic and hydrophobic groups. Therefore phospholipids are amphipathic molecules in whose structure the polar region (hydrophilic) and the non-polar region (hydrophobic) can be readily distinguished. The glycerolphosphocholine moiety and the two carboxyl ester groups constitute the polar region, while the acyl chains, that extend away from the plane of the head group, constitute the non-polar region (Jain, 1988). In many phospholipids in biological membranes, the acyl chain C1 is saturated and is 16 or 18 carbons long while the C2 is frequently unsaturated and usually longer (Luckey, 2008).

In the phospholipids that form the cell membrane the phosphate group is usually associated with choline in phosphatidyl choline (PC), ethanolamine in phosphatidyl ethanolamine (PE), serine in phosphatidyl serine (PS), glycerol in phosphatidyl glycerol (PG),

glycerol phosphate, threonine and inositol in phosphatidyl inositol (PI, Jain, 1988; Hianik, 2006). In animal cell membranes phosphatidylcholine (PC) is the most abundant class of lipid. Phospholipids pack preferentially in bilayer form in aqueous dispersions (Jain, 1988).

The spontaneous self association of some lipids such as phospholipids is a very important effect which is present in biologically important components like the cell membrane (Jain, 1988; Hianik, 2006). The hydrophobic effect is the preference of the non-polar molecules to interact with each other instead of interacting with water molecules. It is the driving force for the self-association of amphipaths, that include the phospholipids, but as Luckey (2008) pointed out, the spontaneous formation of each type of lipidic aggregate is also driven by the structure of water. Hydrophobic compounds cannot interact favorably with water via ionic, polar, or hydrogen bonding interactions. Amphipathic molecules aggregate in water to maximize favorable interactions, for example, the interactions that maximize orientational and configurational entropy. The most important factors involved are:

I. The change of free energy when a methylene group is removed from water to a non-polar environment. Even though changes depend also on the nature of the non-polar environment, this change is always negative. The gain in free energy arising from the hydrophobic effect increases linearly with the chain length (Jain, 1988).
II. The change of electrostatic energy for the removal of a charged group from water to a non-polar environment. Then, the overall free energy needed is about 6.28 kJ/mol (Jain, 1988), which means that energy within the system is needed to drive this change.

The overall gain in free energy when transferring a phospholipid molecule from water or from a hydrocarbon medium to a polar-non-polar interface is over –6.28 kJ/mol (Jain, 1988).

The insertion of a non-polar molecule such as a lipid, with a long acyl chain, into the water creates a reordering of the water molecules around the hydrocarbon chain to form a hydrogen-bonded cage. When the water molecules rearrange to form the cage around the non-polar chain, their mobility is drastically reduced, resulting in a large loss of entropy. The best way to lower this entropic cost is to rearrange the non-polar section into large aggregates, which reduces the total surface area of the non-polar material exposed to the water and hence reduces the amount of immobilized water molecules (Jain, 1988).

The bilayer is only one of the possible lipid aggregates that spontaneously form when amphipathic lipids are mixed with water (Chatterjee & Agarwal, 1988, Luckey, 2008). Generally, amphipathic molecules such as lipids aggregate in aqueous solution and can form a variety of phases, including monolayers, micelles, hexagonal, cubic and lamellar phases (Fig. 2.2.), among which the predominant phase in a given system depends on the structure and conformation of the amphipathic molecules, and the water content and environmental factors, such as ionic strength, pH, and the temperature of the system.

Most amphipaths with a long polymethylene chain form a thin film at the air-water interface (Jain, 1988; Hianik, 2006). Such an arrangement avoids energetically unfavorable interactions between acyl chains and polar solvents like water, and also avoids unbalanced cohesive forces between the water molecules at the interface. Due to the difference of polarity, amphipathic molecules localize the polar groups to the water and the acyl chains extend into the air (Jain, 1988; Hianik, 2006). An interface of water with another non-polar solvent also offers an ideal environment for the formation of a monolayer; here the acyl chains are in the non-polar medium. If the hydrophobic effect is dominant, as is the case with most of the

phospholipids, amphipathic molecules tend to segregate at the air-water interface to form insoluble monolayers (Jain, 1988).

Amphipaths with relatively large head groups form aggregated structures called micelles, in which polar groups are on the surface and the chains are segregated from the aqueous phase. In organic phases containing small amounts of water, the energetically favorable organization is inverted; where the polar groups are oriented toward the center and enclose a small drop of water (Jain, 1988).

A hexagonal phase (Fig. 2.2.) occurs when lipid molecules are organized into long tubes (5-12 nm). These tubes are arranged with hexagonal symmetry. Compared with other phases formed in a lipid-water system, this polymorph is characterized by a hydrophobic surface and low water content. Some lipids, such as phosphatidylethanolamine, can form hexagonal phases when they are dispersed alone in water. The transition from hexagonal phase to a bilayer is thermotropic, because it depends on temperature. This transition is rapid, cooperative, and occurs without significant change in hydration or a major reorganization or exposure of hydrocarbon chains (Jain, 1988; Luckey, 2008).

The lamellar phase (Fig. 2.2.) is the most naturally occurring phase for phospholipids when they are dispersed in excess water, and this is observed in biological systems as a lipid bilayer (Jain, 1988; Lewis et al., 1989; Hianik, 2006).

Phospholipid bilayers can either exist in a liquid form phase or in a gel solid phase. Phospholipids are in an ordered gel phase at low temperature. Above a melting temperature (T_m) that is a characteristic for each lipid, the bilayer is present in a phase, termed liquid-crystalline (l_q) or liquid disordered (l_o), in which the lipid acyl chains are liquid and disordered

(Baginski et al., 2006). The phase behavior of the lipid bilayer is largely determined by the strength of the van der Waals interactions between the adjacent lipid molecules (Jain, 1988).

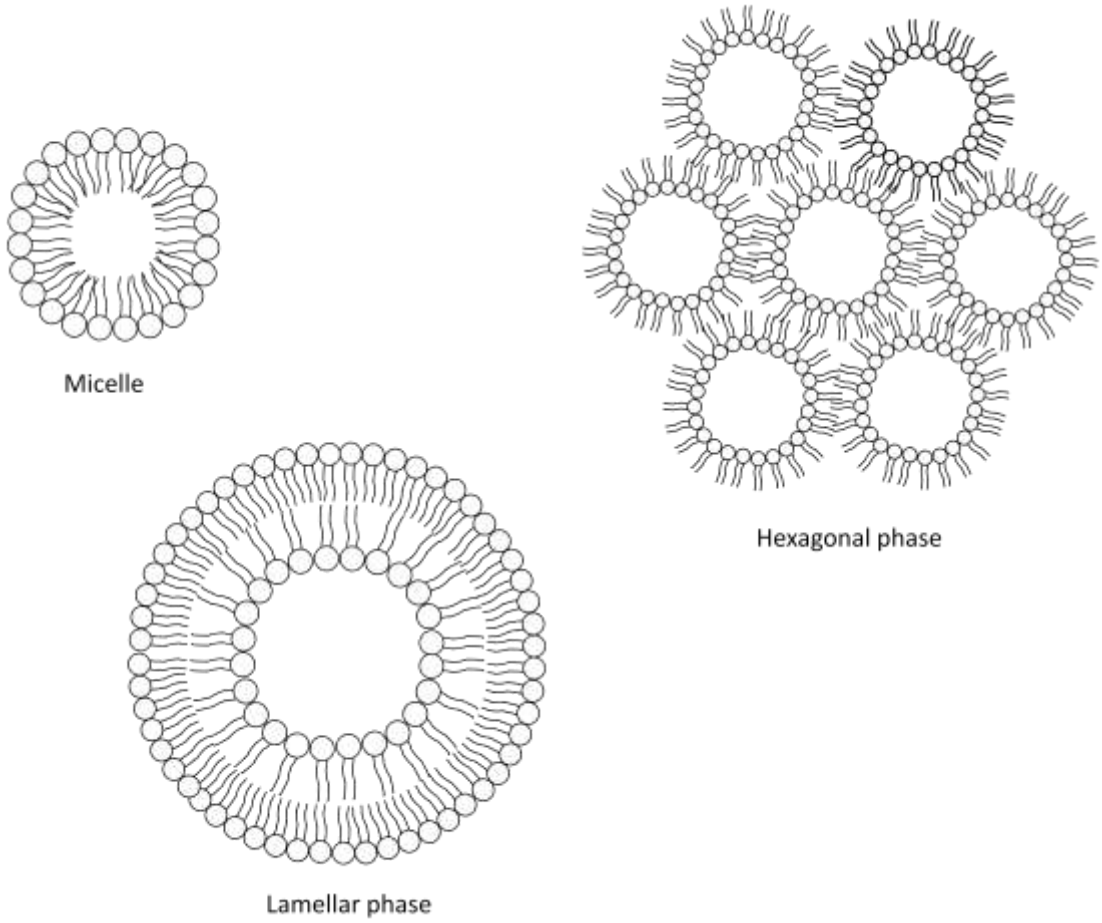

Fig. 2.2. Schematic representation of different lipid aggregates.

The gel-to-fluid phase transition temperature (T_m) of a variety of phospholipids is determined by different factors such as the chain length, degree of unsaturation, and structure of the head group. Generally T_m changes not only with the chain length but with the state of ionization and the extent of hydrogen bonding. The hydrogen bonding and ion binding effects are much more pronounced in liposome structures that contain phosphatidic acid. Thus, an isothermal chain melting can be induced in bilayers by changing pH (Jain, 1988).

In the gel phase of the bilayer, acyl chains are tilted relative to the plane of the membrane, and in the fluid phase, chains lie perpendicular to the surface. A fluid phase bilayer is about 15% thinner than in the gel phase. An increase in the acyl chain length is accompanied by an increase in the thickness of the fluid bilayer (Jain, 1988).

In the gel phase, lipid chains are rigid, but NMR (Nuclear Magnetic Spectroscopy) has shown that in a fluid membrane the acyl chains are not rigid. Lipid molecules move within the bilayer in three different modes: rotational, lateral, and transverse (Luckey, 2008).

2.3.3. Bilayers and Liposomes

Different types of model membranes can be used in order to study different phenomena related with the cell membrane (Baginski et al., 2006; Hianik, 2006; Peetla et al., 2009).

Planar bilayer models which separate two aqueous compartments are used to study electrical properties. Pure lipid bilayers are not permeable to ions, so introduction of molecules that form ion channels can be closely monitored (Luckey, 2008; Peetla et al., 2009). Peptides, small proteins, and other lipids will diffuse into the bilayer when added to one of the aqueous compartments (Luckey, 2008).

Supported bilayer models, which are planar lipid bilayers that sit on a glass, quartz or gold support, allow direct observation of their surface using atomic force microscopy and fluorescence. Supported bilayers can be a fusion of lipid vesicles of the desired composition on the surface of the support in an aqueous environment or by sequential deposition of monolayers, which allows asymmetric bilayers to be formed (Luckey, 2008).

Liposomes result when bilayers forming lipids are mechanically dispersed in aqueous suspensions due to the tendency for bilayer edges to seal so the acyl chains are not exposed to the water. Depending on the method used it is possible to have unilamellar or multilamelar liposomes. Liposomes also can be classified by size (Fig. 2.3.). (Ostro, 1983; Chatterjee & Agarwal, 1988; Luckey, 2008; Peetla et al., 2009).

- The multilamellar liposomes (MLV) contain concentric spheres of lipid bilayers, and may be made by simple shaking of a thoroughly dried lipid film into an aqueous solution. Multilamellar liposomes are usually polydisperse, having different diameters that can be from 0.2 to 50 µm, and could have different amounts of concentric layers, usually as many as 20. These model membranes usually are used in studies of lipid phase transitions and in studies of binding of proteins or peptides (Luckey, 2008; Peetla et al., 2009).
- The unilamellar liposomes (UV) could be classified as:
    - Small unilamellar (SUV) vesicles that are usually with diameters between 20 and 50 nm. Due to the small size of the SUVs they have an extreme curvature and they are very asymmetric. For example a SUV of phosphatidylcoholine of 22 nm diameter has 1900 and 1100 molecules in the outer and inner leaflet (phospholipids layer) of the lipid bilayer, respectively (Luckey, 2008; Peetla et al., 2009).
    - Large unilamellar Vesicles (LUV) usually have diameters from 100 nm to 5 µm. LUVs have the advantage of large encapsulated volumes, up to 50 L/mol of lipid, but also have disadvantages that include heterogeneous size distributions and fragility of larger vesicles (Luckey, 2008; Peetla et al., 2009).

- The giant unilamellar vesicles (GUV) are usually from 5 to 300 µm in diameter. These GUV are the size of a cell and they are large enough to insert microelectrodes or visualize the surface in optical microscopy (Luckey, 2008; Peetla et al., 2009).

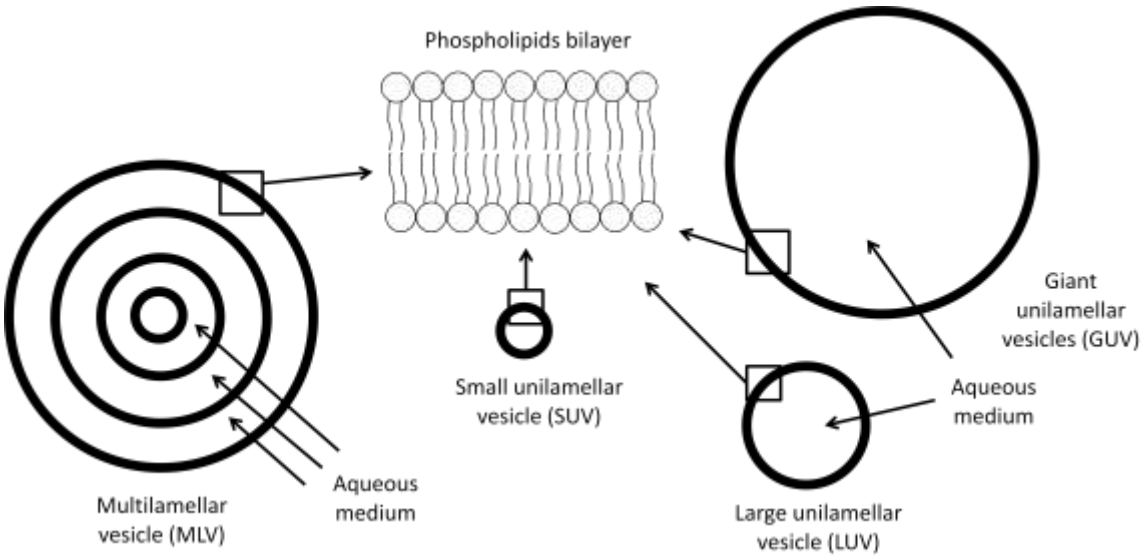

Fig. 2.3. Schematic representation of different types of liposomes.

2.3.3.1. Preparation of Model Membranes

SUVs can be obtained by exposing MLV to extensive sonication, or they can be made also by extrusion of MLV through polycarbonate filters of defined pore size, after which they can be sized by gel filtration or gradient centrifugation. SUVs are also formed by injection of lipids in organic solvent into aqueous media, followed by removal of the organic solvent (Ostro, 1983; Luckey, 2008; Peetla et al., 2009).

According to Peetla et al. (2009), SUVs and LUVs are typically prepared by dissolving lipids in organic solvents (a 2:1 mixture of chloroform: ethanol) and then the solvents are evaporated under vacuum conditions to form thin films of lipids at the bottom of a round-

bottom flask. The dried lipids are rehydrated in a desired buffer to form liposomes. The liposome suspension is subjected to vortexing until complete dissolution of the lipid film, followed by ultrasonication. This procedure usually results in multilamellar liposomes which normally are subjected to cycles of freeze/thawing in order to improve the uniformity of liposome size.

Large unilamellar vesicles (LUV) can be obtained using extruders with polycarbonate filters; nowadays extruders have been used under nitrogen pressures that help attain more uniformly sized LUVs, especially during repeated extrusions (Hope et al., 1985, MacDonald et al., 1991).

GUVs can be obtained by slow hydration of lipids, at low ionic strength and high lipid concentration, followed by sedimentation through sucrose to eliminate multilamellar vesicles and amorphous material. According to Peetla et al. (2009), GUVs can be prepared by electroformation. In this method the lipid film is dried under an oscillating electric field. Typically a standard wave generator is used to apply 1 V at 10 Hz between electrodes onto which a thin film of lipids has been dried in the presence of water to form GUVs.

2.3.4. Studies with Liposomes and Peptides

The interactions between peptides and a lipid membrane is an area of research where there are still many questions to be resolved. The complexity of these interactions arises first due to the mixed nature of the lipids in the membrane and second due to the variety of peptides that can be involved in these interactions. Khandelia et al. (2008) pointed out that the association of peptides or proteins with the membrane affected the structural and dynamical

properties of the lipid bilayer on different length and time scales. Many peptides exert a biological effect (antimicrobial, antifungal, antiviral, etc.) when they interact with the membrane. These effects are, in many cases, mediated by structural changes in the lipid bilayer.

The associations of peptides with the lipid bilayer have different mechanisms. Most often, the peptides have an amphiphilic character, and the hydrophobic effect acts to locate the peptide in the hydrophilic-hydrophobic interfacial layer of the membrane. Electrostatic effects may also be involved. Upon association with the membrane, the peptides often change their conformation (Khandelia et al., 2008).

Electrostatic interactions between peptides and lipid membranes are very important in order to define which kind of interaction mechanism occurs with different charged peptides. Tang & Deber (2004) pointed out that peptides with charged amino acids in both termini can affect the peptide-membrane interactions. The positive charged residues can bind strongly to anionic lipid surfaces via electrostatic interactions, which may prevent the transbilayer insertion of the peptides and/or prevent their transport across a cellular membrane if binding is strong enough. In a study conducted by Tang & Deber (2004), Lys was replaced in a peptide in order to reduce the charges on the peptide; the resulting peptide molecule was then more hydrophobic while retaining acceptable water solubility and was able to readily penetrate the membrane even with a charged group at both termini.

An important interaction of peptides with model membranes is related to passive transport across a membrane. The concept of membrane as a barrier implies that at least some substances can pass across it (Jain, 1988). The permeability properties of the cell membrane are determined by the lipid and protein components, and in general the lipid bilayer is readily penetrated by non-polar substances while proteins in the membrane make channels and

transporters for ions and hydrophilic substances. Romanowski et al. (2002) pointed out that the total flow across the membrane is related to the product of the water-membrane partition coefficient, and the diffusion coefficient of the permeant molecules.

Due to the forces related to the interaction and permeability of molecules across the membrane, the composition of peptides can affect this permeability, as pointed out by Ramaswami et al. (1992) in a study of water soluble peptides that have limited permeability from the bloodstream into the brain. According to Ramaswami et al. (1992), lipophilicity did not play an important role in the ability of a particular peptide to cross the blood-brain barrier.

The driving force for the net movement of solutes by diffusion across the membrane is the concentration gradient. Permeation across the bilayers may also be viewed as a process that tends to equalize the electrostatic interactions of an amphipathic molecule between the two phases (Jain, 1988).

According to Romanowski et al. (2002) the permeability coefficient is proportional to the diffusion coefficient of the permeant, D, and the water-membrane partition coefficient as:

$$P = \frac{DK}{X} \qquad [17]$$

where X is the membrane thickness, and the water-membrane partition is defined as $K = [C_{mem}]/[C_{\infty}]$ where C_{mem} and C_{∞} are the concentration of the solute in the membrane and in the bulk solvent, respectively.

This permeability of a specific solute can be enhanced by: increasing water-membrane partitioning, increasing the diffusion coefficient constant within a membrane interior, and/or by controlling the interfacial resistance of the membrane.

According to the results of a study related to the permeability of a cyclic bioactive peptide, conducted by Romanowski et al. (2002), the ionic character of peptides and the hydrophobicity of peptides, which plays an important role in the diffusion within the model membrane, influences the permeability coefficient. That suggests the peptide permeability is mediated by both attraction and repulsion forces.

In another study conducted also with cyclic bioactive peptides (enkephalines), Ramaswami et al. (1992) found that cyclic enkephalines had only weak interactions with neutral lipid model bilayers, and that the conversion of cyclic peptides to acyclic peptides increased the number of low energy conformations available for the peptides, increasing the extent of interactions with the lipid bilayer.

Measures of the volumetric properties of lipid bilayers have been used in order to analyse the mechanical properties and structural dynamics of lipid bilayers, as well as examine the effect of the composition and phase transition temperature on the physical properties of the lipid bilayer. Additionally the volumetric properties also have been used to analyse the effect of the interactions of lipid bilayers with protein and peptides on the physical properties of lipid bilayers (Strom-Jensen et al., 1984; Colotto et al., 1993; Hianik et al., 1997; Hianik et al., 1998, Hianik et al., 1999; Krivanek et al., 2001; Krivanek et al., 2002; Rybar et al., 2007; Uhríková et al., 2007; Hianik, 2011; Hianik et al., 2011).

Determination of the volumetric properties of lipid bilayers has shown that changes in the chain length and degree of saturation of the lipids cause changes in their mechanical properties, due to a change in the packing of the lipid structure (Hianik et al., 1998; Uhríková et al., 2007). Hianik (2011) pointed out that measurements of the mechanical properties of the lipid bilayers give information about the stability and shape of the lipid bilayers used as model

membranes and can provide better understanding about the mechanics of membrane interactions with proteins.

Determination of the changes in the volumetric properties of model membranes (lipid bilayers) as they interact with proteins or peptides can help us to understand the disturbance of the lipid structure caused by their interaction with proteins, particularly how this disturbance affects the physical properties of the model membranes (Hianik et al., 1997; Hianik et al., 1999; Krivanek et al., 2001; Krivanek et al., 2002; Rybar et al., 2007; Uhríková et al., 2007). Interactions between proteins or peptides with cell membranes play an important role in the stability and functioning of a cell membrane. Therefore, understanding the mechanical properties of the model membranes as they interact with proteins or peptides can help us to increase knowledge about the mechanism involved in the dynamics of cell membranes (Hianik, 2011).

3. Materials and Methods

3.1. Materials

3.1.1. Bioactive peptides

Hemp seed peptides with antioxidant activity were received from Abraham Girgih, Ph.D candidate at University of Manitoba, who obtained the peptides by enzyme digestion and separated by filtration as explained in Girgh et al. (2011). Two fractions were selected, the fraction which passed through a 1 kDa filter (1 kDa), and the peptides that passed through a 3 kDa filter (3 kDa), but from which the fraction that had passed through the 1 kDa filter had been filtered out.

Dairy peptides were produced by Glanbia Nutritionals Inc. (Fitchburg, WI, USA) from whey milk protein and identified by the letters A, C, and D. The main composition of these peptides is shown in Table 3.1.

Table 3.1. Dairy peptides main composition.

	Amount
Protein, dry basis	>90%
Moisture	<5.0%
Fat	<0.7%
Minerals	<3.5%
Lactose	<1.0%

3.1.2. Chemicals and Reagents

The chemicals and reagents used in this study are listed in Table 3.2.

Table 3.2. Chemicals and Reagents

Chemicals	**Manufacturer**
Ethanol 95%	Commercial Alcohol, Brampton, ON, Canada.
Phosphatidylcholine	Alfa Aesar, Ward Hill, MA, USA.
Acetone 95%	Sigma-Aldrich, St. Louis, MO, USA.
Potassium chloride	Sigma-Aldrich, St. Louis, MO, USA.
Mucasol	Merz Hygiene GmbH, Germany.

3.1.3. Instruments and Supplies

The chemicals and reagents used in this study are listed in Table 3.3.

Table 3.3. Instruments and Supplies

Instruments and Supplies	**Manufacturer**
0.1 mL, 1 mL mechanical pipettes	Fisher Scientific Inc., Nepean, ON, Canada.
250 µL syringe	Hamilton Company, Switzerland.
3.0 mL PP/PE syringe without needle,luer tip	Sigma-Aldrich, St. Louis, MO, USA.
DMA 5000 Density meter	Anton Paar, Austria.
ResoScan System	TF Instruments Inc., Germany.
Vacuum pump RV8	BOC Edwards, Germany.
Analytic Balance d=0.1 mg	Denver Instruments, USA.
Direct Q UV3 Ultrafiltration system	EDM Millipore, USA.
Water Bath	Thermo Fisher Scientific, USA.
Mini-Extruder	Avanti Polar Lipids Inc., USA
Rotary Shaker	Fermentation Design Inc., Allentown, PA, USA
Rotary Shaker	New Brunswick Scientific Co., Edison, NJ, USA
Mastersizer 2000	Malvern Instruments Ltd., UK

3.2. Methods

3.2.1. Preliminary Analysis

Preliminary analyses using potassium chloride (KCl) serial dilutions were made to determine the variation of the ultrasonic measurements and the error inherent to the preparation of serial dilutions. Ultrapure degassed water (UDWater) and KCl, a highly soluble material, were used to prepare serial dilutions according to the procedure shown in Figure 3.1. The ultrapure water was obtained from a Millipure system "Direct-Q 3" and degassed using a vacuum pump at a pressure between 25 and 30 mmHg for 1 h-25min.

The solutions were made up gravimetrically and the molal concentration of each sample was calculated from the corresponding weight of the KCl and UDWater used (KCl g UDWater g^{-1}). Two stock solutions (of different initial concentration) were made up to prepare the serial dilutions with 3 replicates for each stock solution. The ultrasound velocity and attenuation measurements were done using the procedure of section 3.2.3.

3.2.2. Serial Dilution Preparation of Hemp Seed and Dairy Peptides

Hemp seed peptide fractions 1 kDa and 3 kDa as well as dairy peptides A, C and D (whey protein peptides) were used to prepare serial dilutions. Two different stock solutions concentrations, 20 mg UDWater g^{-1} and 15 mg UDWater g^{-1}, were used for each peptide to prepare the serial dilutions in triplicate.

Serial dilutions were made with ultrapure and degassed water (UDWater). The serial dilutions were made gravimetrically.

The stock solution was prepared using a 50 mL clean beaker which was set on an analytical balance; the peptide was weighed and, using a mechanical pipette set at 1mL, UDWater was added carefully over the walls of the beaker to avoid air incorporation. The stock solution beaker was immediately covered with parafilm to avoid water evaporation which can affect solution concentration. The solution was gently shaken for a specific period of time for each peptide. A rotary shaker (New Brunswick Scientific Company, Edison, NJ, USA) set at velocity 5 was used in all preparations. The mixing time was decided based on the solubility of each peptide. Solubility trials were done previously with the dairy peptides A, C and D, defining the time for peptide dissolution, which was the time where no suspended particles were observed or no change happened in more than 2 hrs. The procedure used to prepare the serial dilutions is shown in Figures 3.2. and 3.3. and detailed as follows:

1. Weigh corresponding peptide sample in a 50 mL clean beaker.
2. Add:
    a. 5 g of UDWater for the hemp seed peptides, using a mechanical pipette.
    b. 20 g of UDWater for the dairy peptides, using a mechanical pipette.
3. Gently shake the sample for:
    a. 5 hr for the 1kDa hemp seed peptide.
    b. 1 hr for the 3kDa hemp seed peptide.
    c. 1 hr for dairy peptides A and C.
    d. 2 hr for dairy peptide D.
4. Extract half of the stock solution (around 2.5 g for hemp seed peptides and 10 g for dairy peptides) using a mechanical pipette, and deposit it in another clean beaker set on the analytical balance. The stock solution beaker is sealed again with parafilm.
5. Add the same weight of UDWater as the weight extracted from the stock solution.

6. Cover and seal the beaker with parafilm.
7. The solution is gently shaken for 10 min (for both hemp seed peptides and dairy peptides).
8. Extract half of the first dilution using a mechanical pipette, and deposit in another clean beaker set on the analytical balance. The first dilution solution beaker is sealed again with parafilm.
9. Add the same weight of UDWater as the weight extracted from the first dilution solution.
10. Cover and seal the beaker with parafilm.
11. The solution is gently shaken for 10 min.
12. The process is repeated from step 4 until one gets to the fifth dilution.

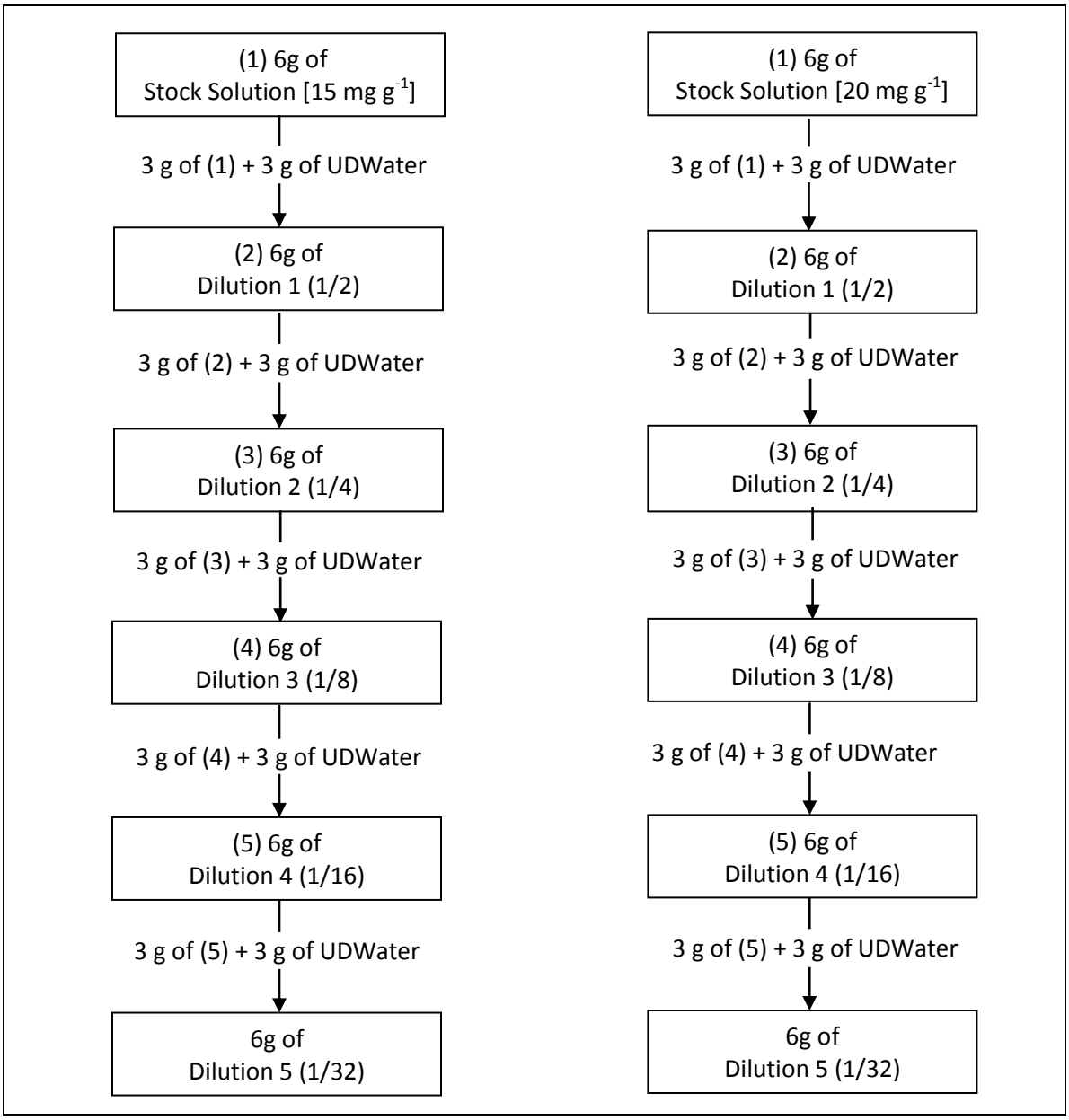

Fig. 3.1. The procedure to make KCl serial dilutions for preliminary analysis.

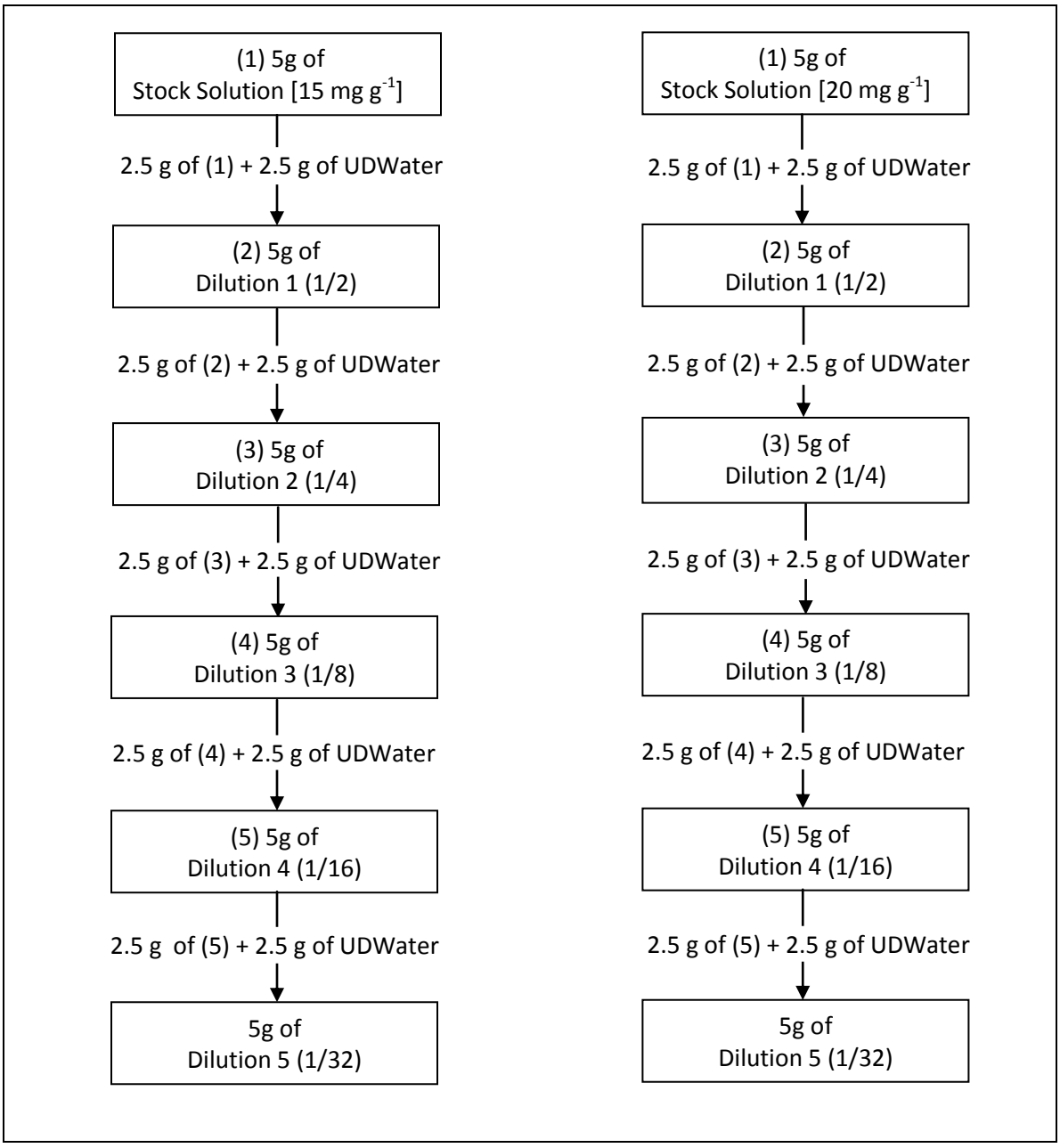

Fig. 3.2. The procedure used to make hemp seed peptide (1 kDa, 3 kDa) serial dilutions.

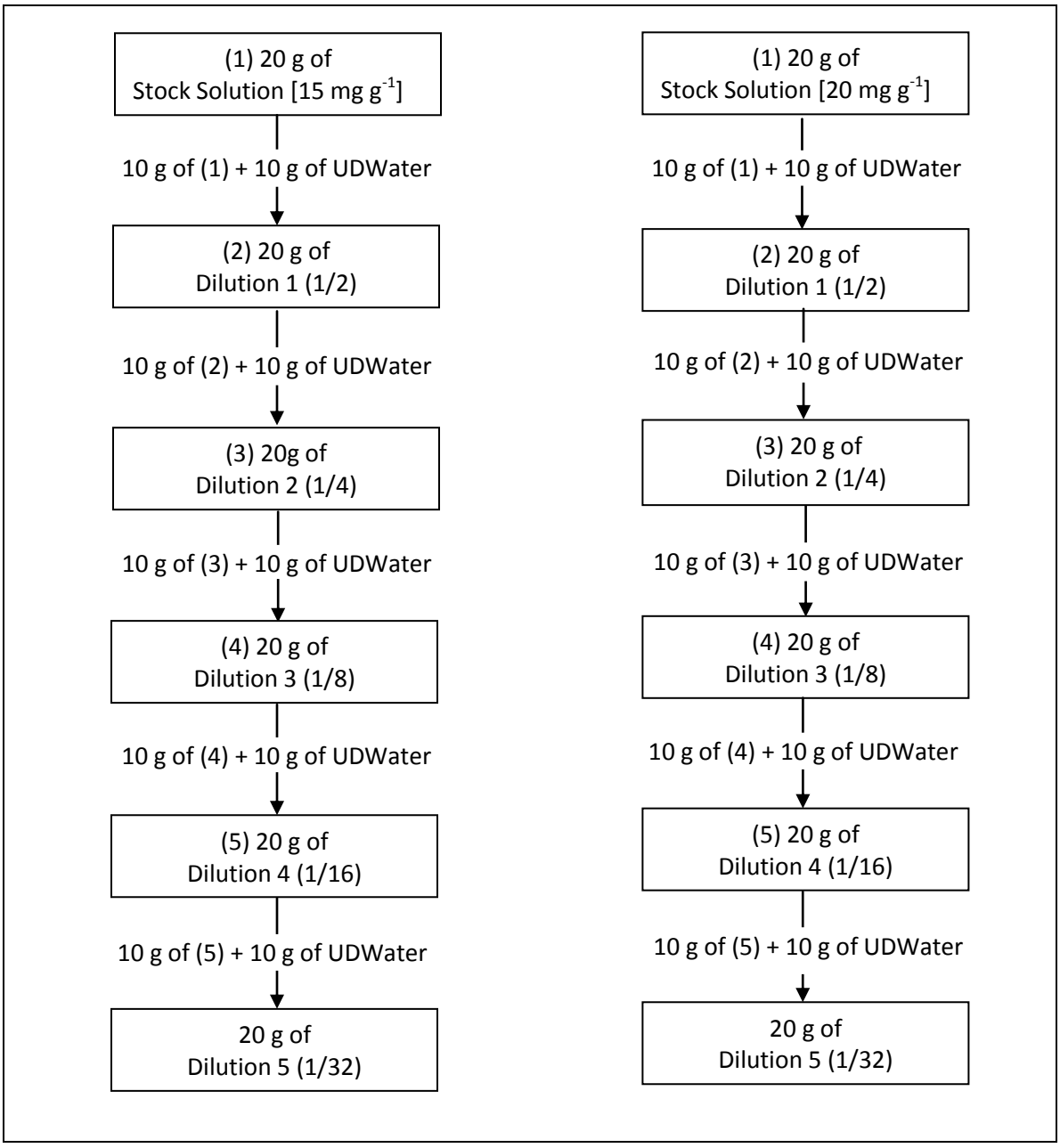

Fig. 3.3. The procedure used to make dairy peptides (A, C and D) serial dilutions.

3.2.3. Ultrasound Velocity and Attenuation Measurements of KCl, Hemp Seed Peptide and Dairy Peptide Solutions

Ultrasound velocity and attenuation measurements were performed on all serial dilutions (KCl, hemp seed peptides and dairy peptides). These measurements consist in measuring the velocity and the attenuation of ultrasonic waves passing through a liquid sample. In order to measure this, a ResoScan System (TF Instruments Inc, Germany) was used. The system includes a resonator unit with two cells, with a maximum volume of 250 µL for each cell, to measure the velocity of the ultrasonic waves passing through liquid samples and the attenuation of the ultrasonic waves. The resonator unit works at a frequency range between 7 and 8.5 MHz. The resonator unit is in a metal block thermostat that provides temperature control with a precision of ±0.005°C.

3.2.3.1. Initial Cleaning

The equipment was cleaned at the beginning of all measurements with UDWater. Both cell cavities were rinsed thoroughly with UDWater several times. After the initial cleaning procedure UDWater was added in both cell cavities and a "Resoscan Check" was performed to assure that the cells were properly cleaned from the previous use and that the ultrasonic velocity difference between the two cells was less than 0.025 m s^{-1}. If the difference was higher, a cleaning procedure was applied to both cavities and a "Resoscan Check" was then performed, repeating this cleaning procedure until the ultrasonic velocity difference requirement between the two cells (less than 0.025 m s^{-1}) was fulfilled.

3.2.3.2 Measurement

The measurements were performed from the lowest to highest concentration solution of the corresponding serial dilution, starting with the fifth serial dilution (1/32). Three replicates were measured for each serial dilution and three subsamples were measured for each replicate. The ultrasound velocity and attenuation measurements were carried out at 24.985°C and a glass Hamilton syringe (250 µL) was used to fill the cells according to the procedure described below:

1. Fill cell 1 with 200 µL of UDWater.
2. Extract 200 µL from the fifth dilution beaker to wash cell 2; repeat this step two more times.
3. Extract another 200 µL from the fifth dilution beaker, reseal the beaker with parafilm.
4. Fill cell 2. It was important to add the solution slowly to assure that there were no air bubbles in the syringe before filling the cell. Place the metal cap back on to seal cell 2.
5. Wait until the screen shows the temperature is stable.
6. Wait another 2.5 min or until set temperature (24.985°C) is reached.
7. Start measurement.
8. Stop measurement after 10 readings were obtained.
9. Drain cell 2 with the Resopump and repeat steps 3,4,5,6 for the second and third subsample.
10. Drain cell 2 with the Resopump and repeat steps 2 to 9 with the next sample.

3.2.4. Density Measurements of Hemp Seed Peptide and Dairy Peptide Solutions

Density measurements were performed on all the serial dilutions of hemp seed peptides (1 kDa, 3 kDa) and dairy peptides (A, C and D) serial dilutions. A density meter DMA 5000 (Anton Paar, Austria) was used for all density measurements. This equipment uses the principle based on the dependence of the oscillation rate of a borosilicate glass U-tube on the density of the liquid present within the tube, giving highly accurate density measurements. Two platinum thermometers were used to control the temperature with a precision of 0.001°C.

3.2.4.1. Initial and Final Check

At the beginning and at the end of the day it was necessary to perform an “Air Check” and a “Water Check”.

The “Air Check” was done to prove the equipment was properly cleaned after previous use. The cell must be cleaned and dry. If the “Check Air” didn’t pass, a cleaning procedure was performed as follows:

1. Using a syringe (3 mL), rinse the cell thoroughly with 20-30 mL of UDWater.
    a. If necessary, using a 3 mL syringe, wash the cell with 5-10 mL of 1% Mucasol solution, and repeat step 1.
2. Using another syringe, rinse the cell with around 9 mL of ethanol (95%) or acetone (95%).
3. Connect the rubber pipe and turn on the fan for about 5 min, and perform a “Check Air“.

After the “Air Check” is passed, it is necessary to perform a “Water Check”, in order to prove the equipment is properly calibrated. Using a syringe used just for UDWater, fill the cell with UDWater and press OK. If the water Check did not pass, a cleaning procedure (as above) was performed.

3.2.4.2. Measurements

The measurements were performed for all samples from the lowest to highest concentration solution of the corresponding serial dilution, starting with the fifth serial dilution (1/32). Three replicates were done for each serial dilution. Two subsamples were measured for each hemp seed peptide solution and 3 subsamples for each dairy peptide solution. The density measurements were done using a new polypropylene/polyethylene (PP/PE) syringe (3 mL) for each sample. The density measurements were conducted at 24.985°C with the procedure as follows:

1. Extract between 2 and 3 mL of sample (for the hemp peptide solution, just between 1 and 2 mL was used).
2. Fill the cell with the sample, leaving the syringe attached. The cell needs around 1 mL of sample to be filled; the other 1 or 2 mL were used to wash the cell with the sample.
3. Check on the screen for the absence of air bubbles in the cell.
4. Start the analysis.
5. After the analysis, using the same syringe, repeat steps 1 to 4 for the subsamples (2 for hemp seed peptides, and 3 for dairy peptides).

6. After finishing analyses of the subsamples, wash the cell thoroughly with UDWater, using between 20 and 30 mL.
7. Wash the cell with ethanol (95%) or acetone (95%) and dry it by connecting the rubber pipe and turning on the fan for about 5 min.
8. Repeat steps 1 to 7 for the rest of the serial dilutions.

3.2.5. Unilamellar and Multilamellar Vesicles (Model Membranes) Preparation

Phosphatidylcholine (PC) was used to create multilamellar and unilamellar vesicles as model membranes in order to analyse the interaction of bioactive peptides with model membranes.

3.2.5.1. Multilamellar Vesicles (MLV)

Phosphatidylcholine (PC) was used. PC was weighed in a test tube, dispersed in UDWater, and vortexed for about 20 s, using a molal concentration of 20 mg UDWater g^{-1}. The PC suspension in UDWater was left for 30 min in order to hydrate the PC, then vortexed again for about 20 s, and transferred to a beaker.

3.2.5.2. Unilamellar Vesicles (UV)

Phosphatidylcholine (PC), was used. PC was weighed in a test tube, dispersed in UDWater, and vortexed for about 20 s, using a molal concentration of 20 mg UDWater g^{-1}. The PC suspension was hydrated for about 30 min, and then it was placed in a water bath at 42 °C

for another 30 min to ensure temperature homogenization, and then vortexed again for about 20 s.

An extrusion device from Avanti Polar was used in order to create the unilamellar vesicles. The extrusion device consists of a mini-extruder, a heating block and two 1 mL Hamilton gas-tight glass syringes. The PC suspension was extruded at 42°C to obtain unilamellar vesicles using the following procedure:

1. Place the heating block on a hot plate at 45°C.
2. The extrusion device was assembled with a polycarbonate membrane with 0.2 µm pores and placed on the heating block.
3. One syringe is filled with UDWater and placed on the extrusion device at one of its ends; on the other end, an empty syringe is placed.
4. First extrusion was made just with UDWater to pre-wet the extrusion device parts, including the membrane, and increase the efficiency of the extrusion.
5. Extract 1 mL of PC suspension from the test tube with one syringe and place it at one end of the extrusion device; the empty syringe is placed at the other end.
6. Wait 3 min for temperature stabilization.
7. Extrude the sample by using the syringe filled with PC suspension to push the liquid through the extrusion device, so that the extruded sample fills the other syringe. Repeat this process 20 times.
8. Transfer the extruded sample to a beaker and repeat the procedure until the amount of sample required is obtained.
9. At the end, remove and dispose of the polycarbonate membrane and clean the syringes and all the parts of the extrusion device with a dishwasher detergent and UDWater.
10. Finally, rinse the syringes and all the parts of the extrusion device with isopropyl alcohol.

3.2.6. Particle Size Measurements of Unilamellar (UV) and Multilamellar Vesicles (MLV)

Particle size measurements of the UV and MLV liposomes were conducted in the Mastersizer 2000 (Malvern Instruments Ltd., UK), equipment that measures the size of a particle based on the way the sample particles scattered light of a laser beam directed at them. The measurements were done using the following procedure:

1. Enter a specific standard operation procedure (SOP) for your sample, including the general description of the kind of sample and its refractive index.
2. Conduct a standard cleaning procedure twice in order to ensure the correct cleanliness of the dispersion unit.
3. Conduct a cleaning check; if it is not passed, conduct a new standard cleaning procedure and repeat the cleaning check until it is passed.
4. Select the specific standard operation procedure (SOP) for your sample that was entered previously.
5. Extract the liposome suspension from the test tube with a plastic Pasteur pipette.
6. Add the sample into the sample dispersion unit by drop until the obscuration rate, which is the parameter used by the equipment to determine the requisite sample amount, is between 10 and 15.
7. Start the measurement.
8. Save the result.
9. Conduct a standard cleaning procedure twice.
10. Conduct a cleaning check; if it is not passed, conduct a new cleaning procedure and repeat the cleaning check until it is passed.
11. Continue with the next sample.

3.2.7. Unilamellar (UV) and Multilamellar (MLV) Serial Dilutions Preparation

The UV and MLV stock solutions were prepared at 20 mg UDWater g^{-1} molal concentration in triplicate, following the procedure in section 3.2.5. Rotary shaker equipment set at velocity 4 was used in all serial dilutions. The procedure used to prepare the serial dilutions is shown in Figure 3.4 and detailed as follows:

1. Transfer, using a mechanical pipette set at 1000 µL, between 15-20 g of the corresponding UV or MLV suspension, to a 50 mL clean beaker. Seal the beaker with parafilm.
2. Extract half (about 7.5-10 g) of the stock solution using a mechanical pipette and deposit it in another clean beaker set on the analytical balance. The stock solution beaker is sealed again with parafilm.
3. Add the same weight of UDWater as the weight extracted from the stock solution. Cover and seal the beaker with parafilm.
4. The solution is gently shaken for 10 min to homogenise it.
5. Extract half of the first dilution using a mechanical pipette, and deposit in another clean beaker set on the analytical balance. The first dilution solution beaker is sealed again with parafilm.
6. Add the same weight of UDWater as the weight extracted from the first dilution solution. Cover and seal the beaker with parafilm.
7. The solution is gently shaken for 10 min.
8. The process is repeated from step 2 until one gets to the fifth dilution.

3.2.8. Ultrasound Velocity and Attenuation Measurements of Unilamellar (UV) and Multilamellar (MLV) Serial Dilutions

Ultrasound velocity and attenuation measurements were performed on UV and MLV serial dilutions following the procedure of section 3.2.3.

3.2.9. Density Measurements of Unilamellar (UV) and Multilamellar (MLV) Serial Dilutions

Density measurements were performed on UV and MLV serial dilutions following the procedure of section 3.2.4. Three replicates were measured for each serial dilution and three subsamples were measured for each solution.

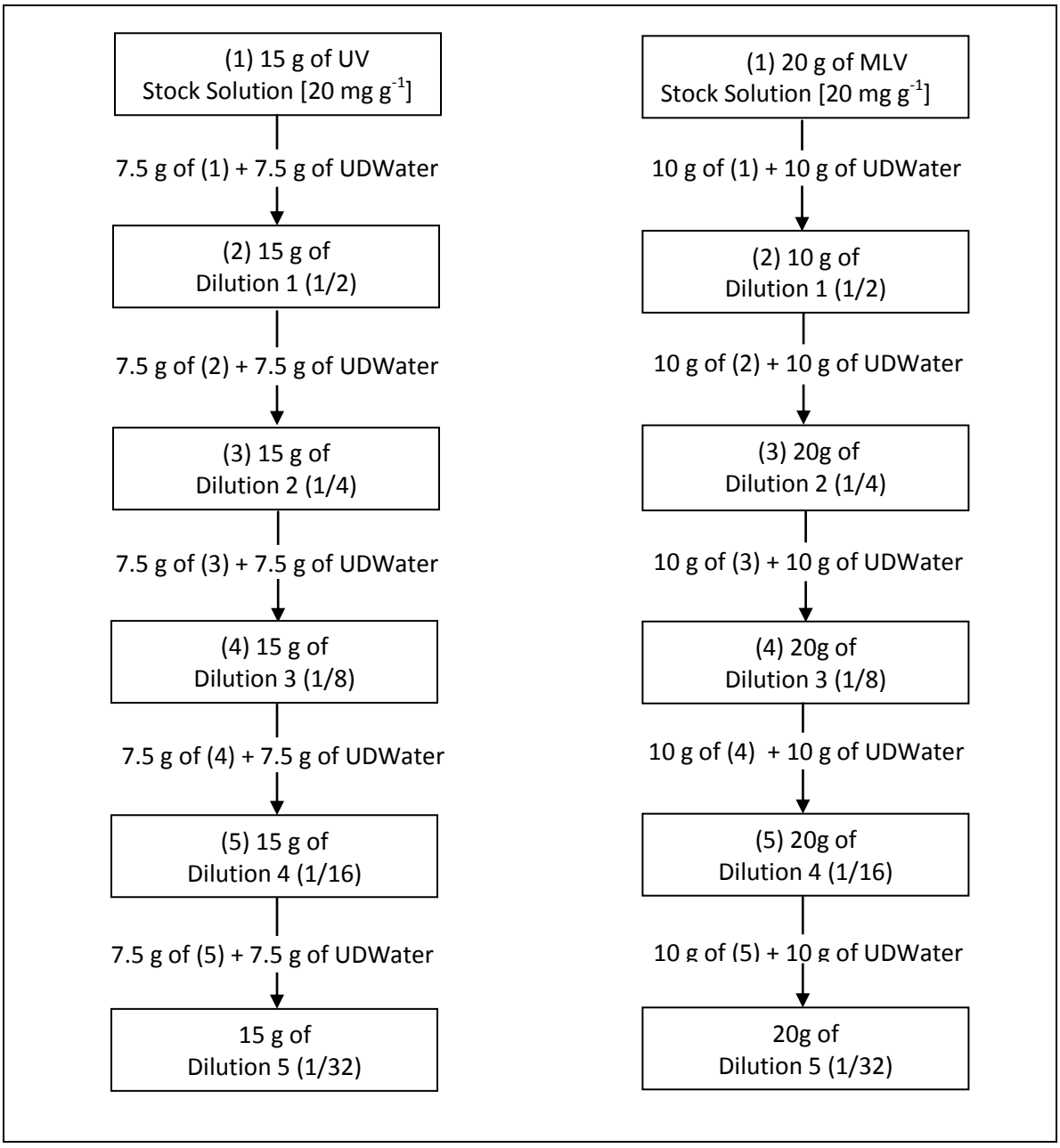

Fig. 3.4. The procedure used to make UV and MLV serial dilutions.

3.2.10. Sample Preparation and Measurements of Liposome Vesicles and Dairy Peptide Mixes

3.2.10.1. Preparation of Unilamellar and Multilamellar Vesicles

1. Prepare multilamellar vesicles (MLV) by mixing PC and UDWater with molal concentration of 20 mg UDWater g^{-1} following the procedure in section 3.2.5.1.
2. Prepare unillamelar vesicles (UV) by mixing PC and UDWater with molal concentration of 20 mg UDWater g^{-1} following the procedure in section 3.2.5.2.

3.2.10.2. Dairy Peptide Solution Preparation

1. Prepare solutions of dairy peptides A, C & D with UDWater at two final concentrations for each peptide, as follows:
    a. Weigh 300 mg of the dairy peptide (A, C or D) in a beaker and add 40 g UDWater, getting 15 mg g^{-1} concentration. Seal the beaker with parafilm.
    b. Weigh 400 mg of the dairy peptide (A, C or D) in a beaker and add 40 g UDWater, getting 20 mg g^{-1} concentration. Seal the beaker with parafilm.
2. Place each beaker on a rotary shaker set at velocity 4 for 1 hr for dairy peptides A and C, and 2 hrs for dairy peptide D. Mixing times were the same as those used for the stock solutions prepared for the dairy peptide serial dilutions as shown in section 3.2.2.

3.2.10.3. Preparation of the Samples to Analyse the Interaction between Liposome Vesicles and Dairy Peptides

In order to analyse the interaction between vesicles (both UV and MLV) with the dairy peptides using ultrasonic velocity and densitometry techniques, the UV and MLV were prepared

at a molal concentration of 20mg UDWater g^{-1} and mixed with the 3 dairy peptides (A, C and D). These mixes were prepared at 2 molal concentrations (i.e., 20 mg UDWater g^{-1} and 15 mg UDWater g^{-1}) by using the following procedure:

1. Weigh in a beaker 7.5g of liposome vesicle (UV, MLV) suspension using a mechanical pipette set at 1000 µL.
2. Add 2.5g of dairy peptide solution using a mechanical pipette set at 1 mL.
3. Seal the beaker with parafilm.
4. Place the beaker on the rotary shaker set at velocity 4. The moment when the beaker was placed on the shaker was defined as "time 0".
5. Measure ultrasound velocity and attenuation, and density of all samples. The measurements of ultrasound velocity and density were conducted as a function of time.

Each mix was prepared adding 2.5 g of the corresponding dairy peptide solution to 7.5 g of liposome suspension. The final concentrations of all liposome vesicle-dairy peptide mixes are shown in Table 3.4.

Table 3.4. Final concentration of UV, MLV and dairy peptide components in each liposome vesicle-dairy peptide mix.

	UV-dairy peptide (A,C or D)	UV-dairy peptide (A,C or D)	MLV-dairy peptide (A,C or D)	MLV-dairy peptide (A,C or D)
MLV			15 mg g^{-1}	15 mg g^{-1}
UV	15 mg g^{-1}	15 mg g^{-1}		
Dairy peptide (A,C or D)	3.5 mg g^{-1}	5 mg g^{-1}	3.5 mg g^{-1}	5 mg g^{-1}

3.2.11. Measurement of Ultrasound Velocity and Attenuation, and Density of Mixes

The measurements of ultrasound velocity and density were conducted over time as the interaction between peptide and vesicles happened.

3.2.11.1. Ultrasound Velocity and Attenuation Measurements for the Liposome Vesicle-Dairy Peptide Mixes

The measurements were done over time at 5, 15, 30, 45 and 60 min from the moment the dairy peptide solution was added to the beaker with the liposome suspension and placed on the rotary shaker (Fermentation Design Inc., Allentown PA, USA). Three replicates were done and measured for each mix. The ultrasound velocity and attenuation measurements were carried out at 24.985°C and a glass Hamilton syringe (250 µL) was used to fill the cells using the following procedure. An initial cleaning of the ResoScan was performed before the measurements as described in section 3.2.3.

1. Fill cell 1 with 200 µL of UDWater.
2. Take the beaker with the liposome vesicle-dairy peptide mix 5 min after it was placed on the rotary shaker.
3. Extract 200 µL from the beaker with the liposome vesicle-dairy peptide mix to wash cell 2; repeat this step one more time.
4. Extract another 200 µL from the beaker, reseal the beaker with parafilm.
5. Fill cell 2. It was important to add the solution slowly and to assure the absence of air bubbles in the syringe before filling the cell. Place the metal cap back to cover cell 2.
6. Place the beaker back on the shaker.

7. Wait until the screen shows the temperature is stable.
8. Start measurement. Record the time at the first reading.
9. Stop measurement after 10 readings are obtained.
10. Take the beaker 15 min after it was initially placed on the shaker.
11. Drain cell 2 with the Resopump and repeat steps 3 to 9.
12. Take the beaker 30, 45 and 60 min after it was initially placed on the shaker, drain cell 2 with the Resopump and repeat steps 3 to 9.

3.2.11.2. Density Measurements of the Liposome Vesicle-Dairy Peptide Mixes

The measurements of density were done over time as the interaction between peptide and vesicles happened. The measurements were done after 5, 15, 30, 45 and 60 min from when the beaker was placed on the shaker. Three replicates were done and measured for each mix. The density measurements were done using a new polypropylene/polyethylene syringe (3 mL) for each sample, and done at 24.985°C with the following procedure. An initial and final check was performed as described in section 3.2.4.

1. Take the beaker with the liposome vesicle-dairy peptide mix 5 min after it was placed on the rotary shaker.
2. Extract between 1 and 1.5 mL of sample from the beaker; reseal the beaker with parafilm.
3. Fill the cell with the sample, leaving the syringe attached. The cell needs around 1 mL of sample to be filled; the residual amount of sample was used to wash the cell.
4. Check on the screen for the absence of air bubbles in the cell.
5. Start the analysis.

6. Record the time when the measurement was done.
7. Use the same syringe to push out the sample from the cell.
8. Take the beaker 15 min after it was initially placed on the shaker.
9. Repeat steps 2 to 7.
10. Repeat steps 1 to 7 for reading at 30, 45, and 60 min
11. After finishing analyses, wash the cell thoroughly with UDWater, using between 20 and 30 mL.
12. Wash the cell with ethanol (95%) or acetone (95%) and dry it by connecting rubber pipe and turning on the fan for about 5 min.

4. Preliminary Analyses Results and Discussion

The preliminary analyses involve ultrasonic velocity measurements of potassium chloride (KCl) solutions and further determination of the partial specific volume and apparent specific adiabatic compressibility. The ultrasonic measurements of potassium chloride (KCl) serial dilutions were performed in order to know the range of precision of the ResoScan System (TF Instruments Inc., Germany). Potassium chloride was chosen due to its highly hydrophilic properties. The determination of the partial specific volume and partial specific compressibility was done in order to evaluate these two parameters, which are highly sensitive to very small changes in the volumetric properties of the molecules caused by solute hydration, with a highly soluble molecule such as KCl in aqueous solution. Potassium chloride serial dilutions were carefully prepared as explained in 3.2.1.

4.1. Ultrasonic Velocity Results

Ultrasonic velocity measurements of potassium chloride serial dilutions were performed as explained in 3.2.3. The ultrasonic velocity was measured as a function of KCl concentration. A linear relationship between ultrasonic velocity and concentration was found for KCl serial dilutions (Fig. 4.1.). The serial dilutions were made using ultra pure water at two initial stock solutions with different concentration, 20 mg g^{-1} and 15 mg g^{-1}. Three replicates and three subsamples were used for the analysis. Due to the preparation procedure, dilutions were done gravimetrically and the exact weight of the solute and water added was recorded, and hence the concentration of the replicates was slightly different.

In order to assure the precision in both cells of the resonator, measurements were performed in both cells, first filling cell 1 of the resonator with water and cell 2 with KCl solution, and second filling cell 1 with KCl solution and cell 2 with water. The ultrasonic velocity results showed very small variation between readings obtained from the different cells (1 or 2), showing the resonator provides high precision readings of ultrasonic velocity in both cells.

The linear regressions calculated for the ultrasonic velocity had very good R^2 values for measurements of KCl solution in both cells, with equal values of 0.9999. The variation between the slope and the intercept of both linear regressions was < 0.09 % and < 0.003%, respectively, confirming the similarity of the results obtained in both cells and similar precision achieved in the measurements in both cells too. In order to confirm the linear relation including the whole concentration range of the serial dilutions, linear regressions were calculated for the ultrasonic velocity within the lower concentration range (0.45-2.5 mg g^{-1}, Fig. 4.2.) and the higher concentration range (3.75-20 mg g^{-1}, Fig. 4.3.). Although the ultrasonic velocity values at the lower concentrations range had R^2 values, 0.9998 and 0.9991, for cell 1 and cell 2, respectively; which is a slightly lower than from the R^2 values at the higher concentration range, all fits of velocity against concentration were very good and confirm the good linear fit for all the concentrations used.

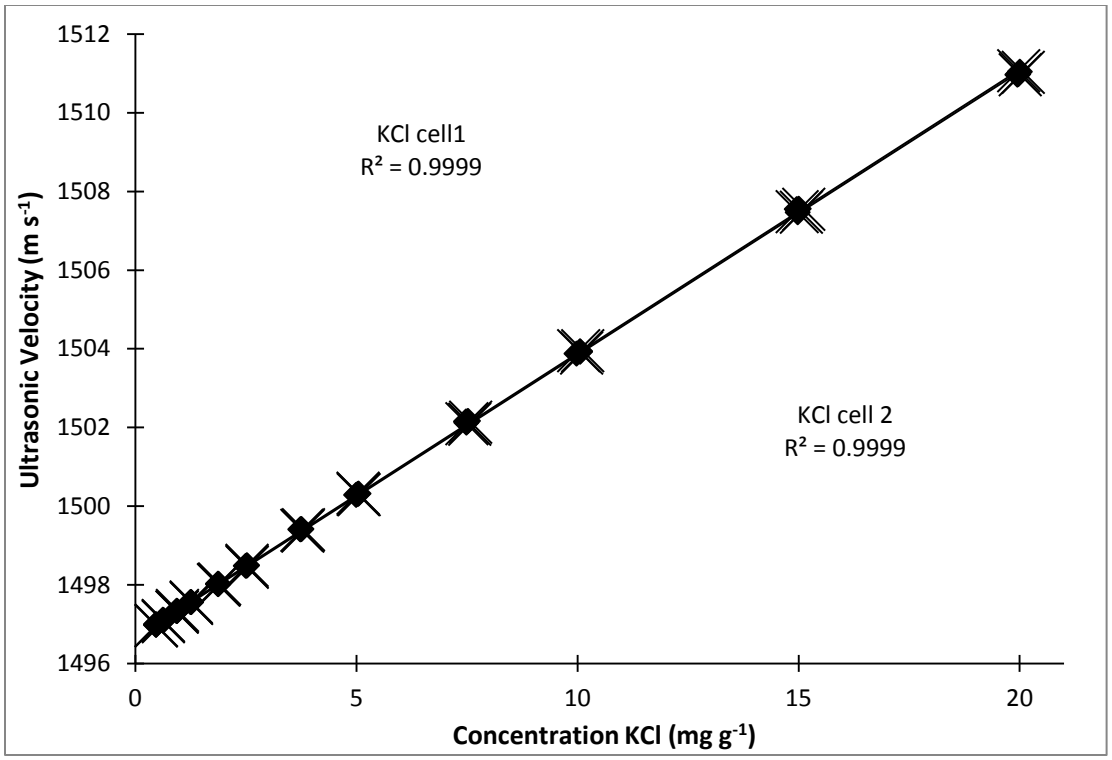

Fig. 4.1. Ultrasonic velocity of serial dilutions of KCl measured in both cells (Cell 1 & Cell 2) of the Resoscan System, KCl cell 1 (◆) and KCl cell 2 (×).

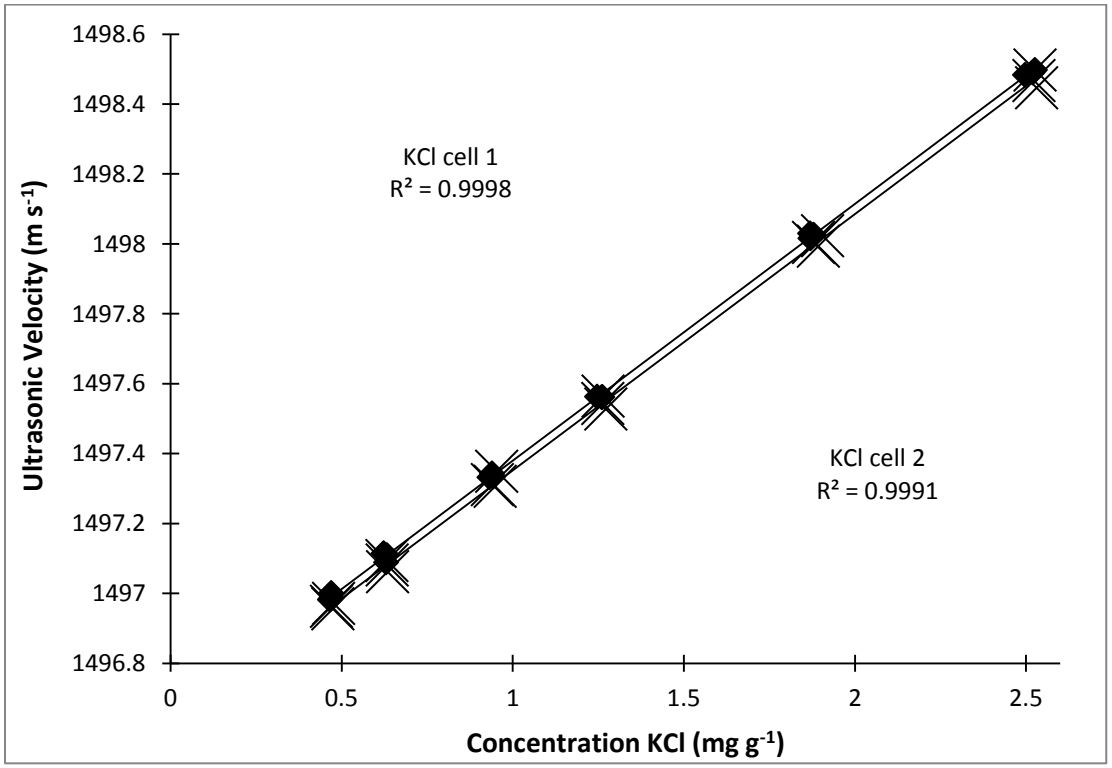

Fig. 4.2. Ultrasonic velocity of serial dilutions of KCl, at low concentrations, measured in both cells (Cell 1 & Cell 2) of the Resoscan System, KCl cell 1 (◆) and KCl cell 2 (×).

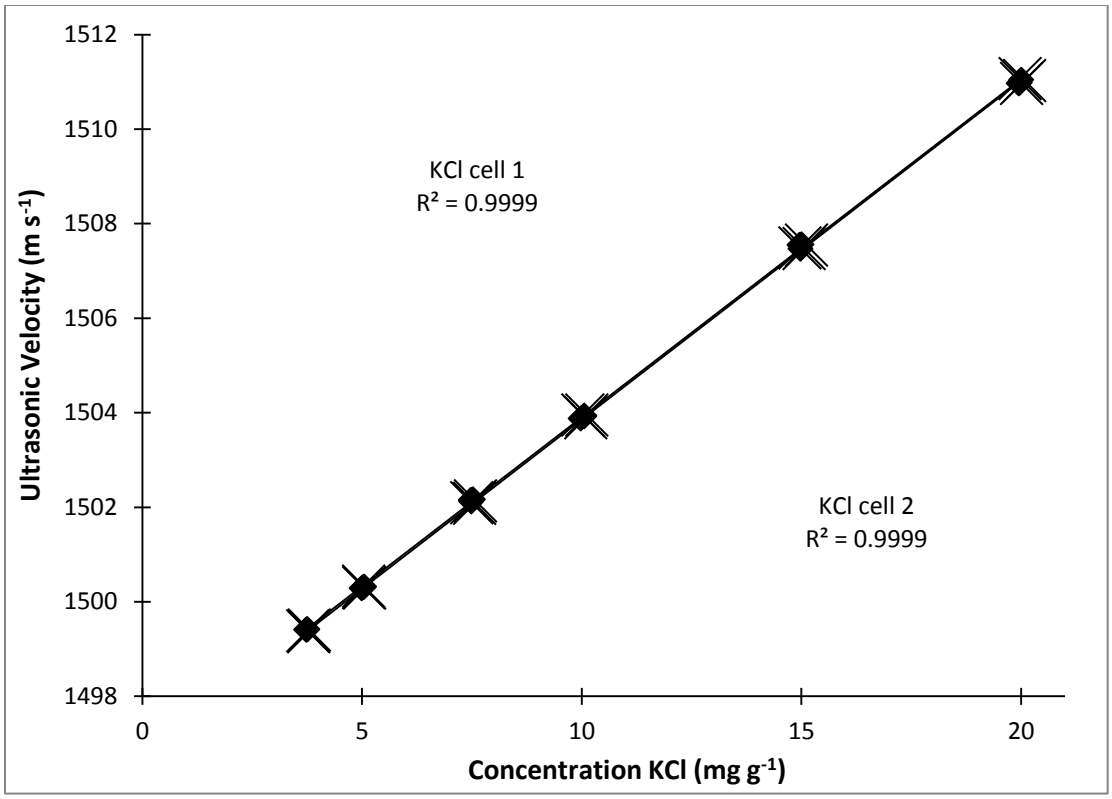

Fig. 4.3. Ultrasonic velocity of serial dilutions of KCl, at high concentrations, measured in both cells (Cell 1 & Cell 2) of the Resoscan System, KCl cell 1 (◆) and KCl cell 2 (×).

The coefficient of variation (CV) of the three replicates and three subsamples made for each serial dilution was calculated for the ultrasonic velocity results. The CV values between subsamples describe the variation mainly caused by the equipment, and the CV between replicates describes the variation mainly caused by errors in the preparation of the solutions. CV values between subsamples of KCl serial dilutions (Fig. 4.4.) were very small, with the highest value $< 0.005\%$, and with most of the values $< 0.002\%$. However, there is a difference between the variability of the subsample measurements done in cell 1 and in cell 2. In the cell 1 subsamples, the CV was $< 0.0005\%$, showing outstanding precision between subsamples, while subsample CV values in cell 2 were still excellent, but varied from 0.0005% to 0.005%.

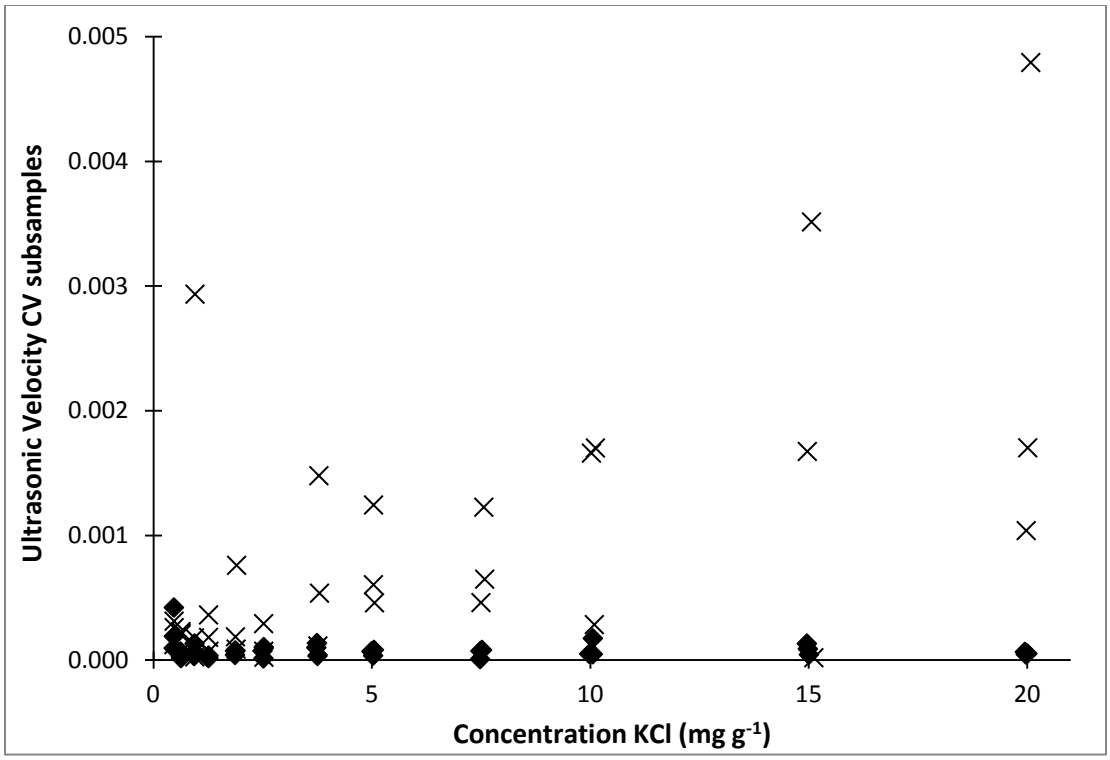

Fig. 4.4. Ultrasonic velocity CV between subsamples of the serial dilutions of KCl measured in both cells of the Resoscan System, KCl cell 1 (◆) and KCl cell 2 (×).

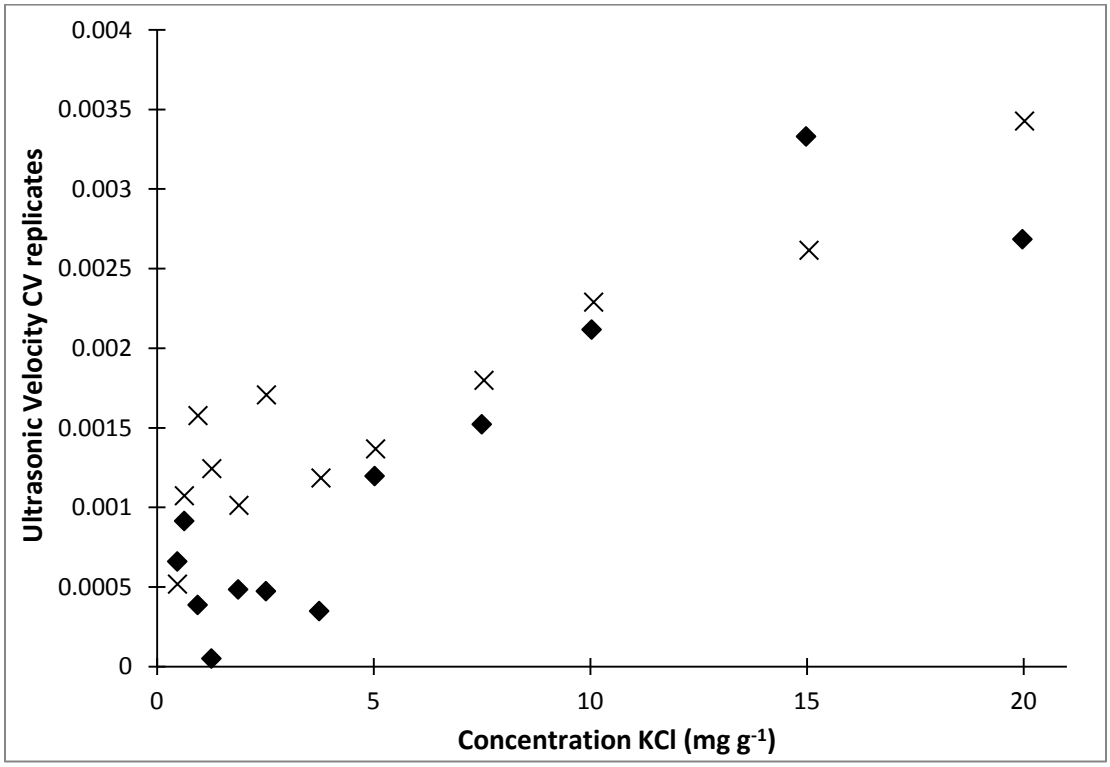

Fig. 4.5. Ultrasonic velocity CV between replicates of the serial dilutions of KCl measured in both cells of the Resoscan System, KCl cell 1 (◆) and KCl cell 2 (×).

CV values between the replicates of the KCl serial dilutions (Fig. 4.5) were very small in both cells, with the highest values $<$ 0.0035%, confirming the high precision of the measurements in both cells. One point that should be emphasized about the replicate CV is its manner of calculation. According to the procedure used to prepare the serial dilutions gravimetrically, the exact concentration of each solution was recorded, so that there are slight differences between the concentrations of the three replicates at any given dilution; therefore the CV measured for the "replicates" was done using the average concentration of each dilution. As a result, CV values reported in Fig. 4.5. are an overestimation of the variability between replicates.

4.2. Density Results

The density data of KCl solutions were obtained from Perven (2012). The experimental density measurements were performed on a DMA 5000 (Anton Paar, Austria) density meter at 25°C. Three different concentration KCl solutions with three subsamples were used to derive a linear regression which was used to interpolate values for the corresponding density values to the same concentrations used in the serial dilution made for the ultrasonic velocity measurements. The linear regression had a very good R^2 with a value of 1.0000, providing great confidence in the density values interpolated from the linear regression equation.

4.3. Specific Volume

The apparent specific volume, $\bar{V}$, was calculated from the corresponding density interpolated from the linear regression equation of the density values to the concentration used

in the serial dilution for the ultrasonic velocity measurements. In order to maintain confidence in the data, only the concentration values within the range used by Perven (2012) were calculated (3.7276 – 14.9103 mg mL^{-1}). The apparent specific volume was calculated as $\bar{V} = \frac{1-(\rho-c)/\rho_0}{c}$ and is shown in Fig. 4.6. A rapid decrease in the apparent specific volume occurred starting at about a concentration of 10 mg mL^{-1}. The reduction in the apparent specific volume can be caused by electrostriction of the water molecules around the KCl molecules (Marcus, 2011).

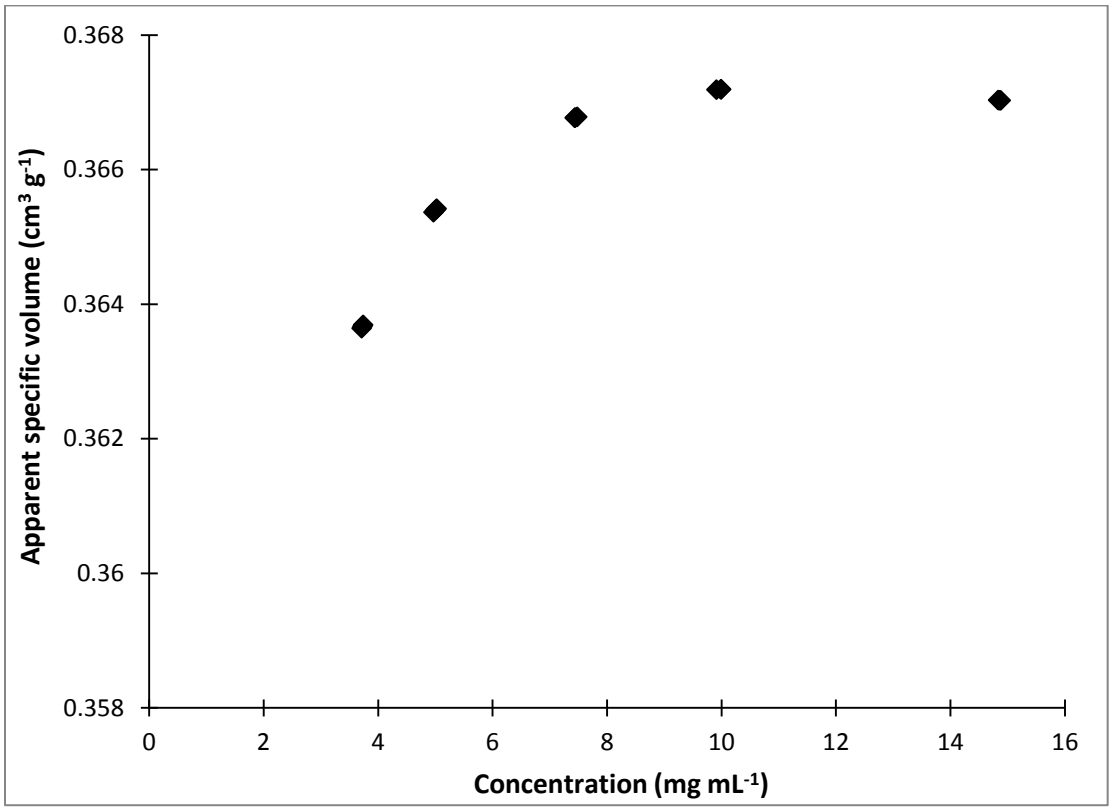

Fig. 4.6. Apparent specific volume of serial dilutions of KCl.

The partial specific volume $\bar{V}^0$ was calculated by the extrapolation of the apparent specific volume to zero concentration as $\bar{V}^0 = \lim_{c\to 0} \bar{V}$. However, because the apparent specific volume rapidly decreased from concentration values below 7.5 mg mL^{-1}, a proper

equation to describe the behavior of the experimental data was not found. In order to approach as much as possible to the correct value, the extrapolation to zero was done using just the two smallest concentration values (Fig. 4.7.). It is recognized that even this value is an overestimate of the partial specific volume of KCl. The partial specific volume obtained (Table 4.1.) is comparable with results obtained by Zen (1957), 0.354 cm^3 g^{-1}, and with other cited results reported in the same paper, 0.354- 0.360 cm^3 g^{-1}.

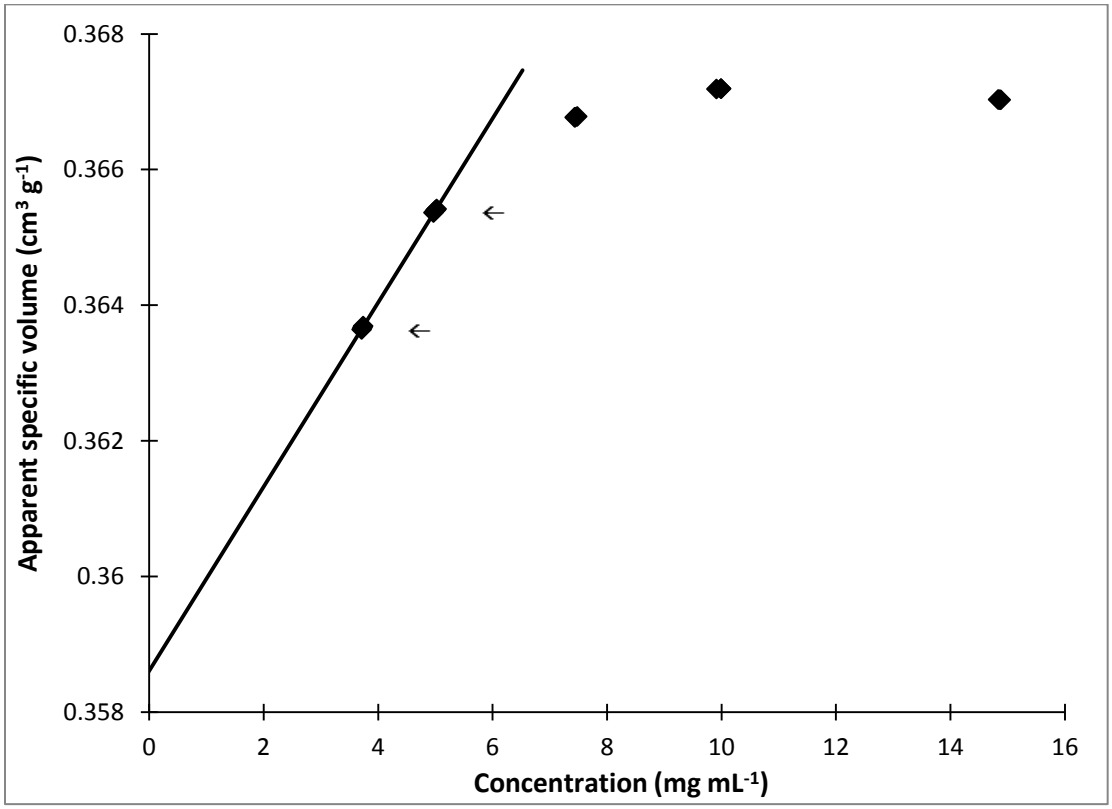

Fig. 4.7. Linear regression of the two lowest concentration values of the apparent specific volume of serial dilutions of KCl.

Table 4.1. Partial specific volume of KCl.

	$\overline{V^0}$ (cm^3 g^{-1})
KCl	0.359

4.4. Compressibility

The adiabatic compressibility coefficient, β_s, was calculated according to the Newton-Laplace equation $\beta_s = \frac{1}{\rho u^2}$, where β_s is a function of the density (ρ) and ultrasonic velocity (u). The relative adiabatic compressibility coefficient, β_s/β_{s0} which expresses the relation between the adiabatic compressibility coefficient of the solution, β_s, against the adiabatic compressibility coefficient of the solvent β_{so}, had a linear relation.

The apparent adiabatic specific compressibility, $\overline{K}_s$, which expresses the change in the apparent specific volume as a function of a change of pressure, was calculated as $\overline{K}_s = \beta_{so} \frac{\beta_s/\beta_{so} - (\rho - c)/\rho_0}{c}$ (Fig. 4.8.).

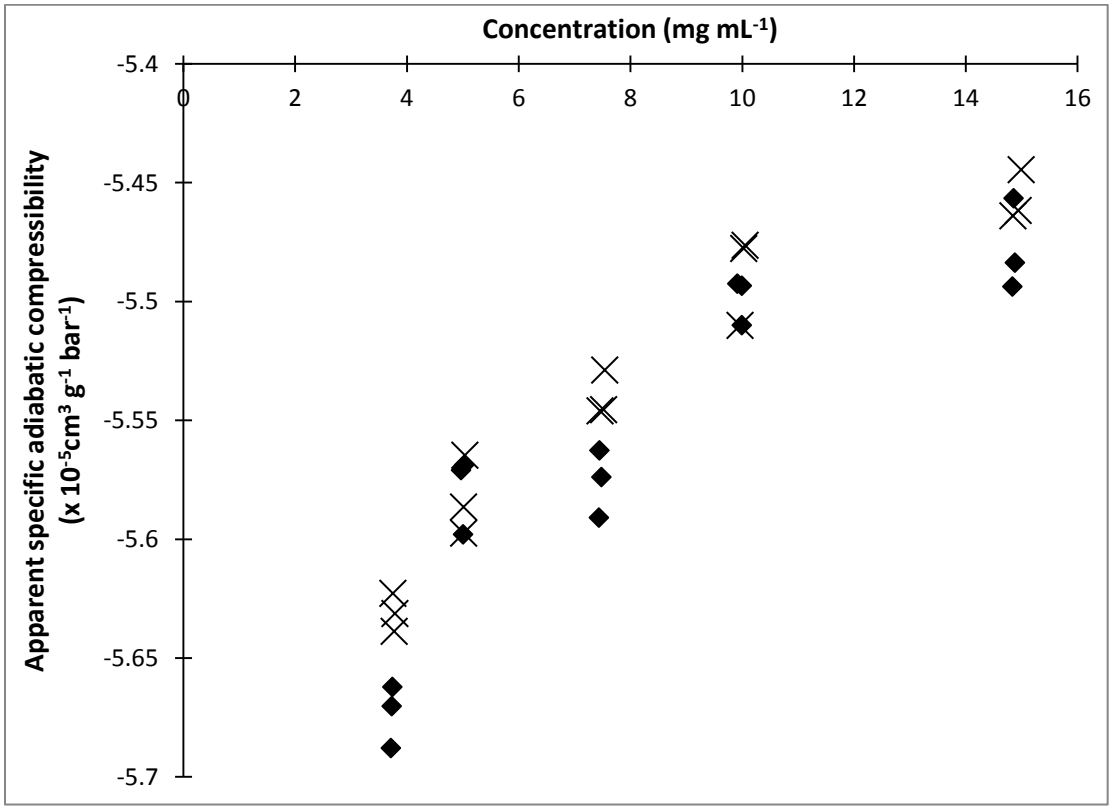

Fig. 4.8. Apparent specific adiabatic compressibility of KCl serial dilutions of KCl measured in both cells of the Resoscan System, KCl cell 1 (◆) and KCl cell 2 (×).

The partial specific adiabatic compressibility coefficient, $\bar{\beta}_s^0$, which describes the relation between the partial specific adiabatic compressibility, $\bar{K}_s^0$, and partial specific volume, $\bar{V}^0$, was calculated as $\bar{\beta}_s^0 = \frac{\bar{K}_s^0}{\bar{V}^0} = \frac{\beta_{so}}{\bar{V}^0} \lim_{c\to 0} \frac{\beta_s/\beta_{so} - (\rho - c)/\rho_0}{c}$, where the term $\frac{\beta_s/\beta_{so} - (\rho - c)/\rho_0}{c}$ is extrapolated to zero while β_{s0} and $\bar{V}^0$ are constant values. However, as with the apparent specific volume, because the term $\frac{\beta_s/\beta_{so} - (\rho - c)/\rho_0}{c}$ had a rapid decrease from concentrations below 5 mg mL^{-1}, a proper equation to describe this behavior of the experimental data was not found, and in order to approach as much as possible to the correct value, the extrapolation to zero was done using just the two smallest concentration values (Fig. 4.9), although it is likely that even this value is overestimated. The partial specific adiabatic compressibility coefficient $\bar{\beta}_s^0$ values are shown in Table 4.2.

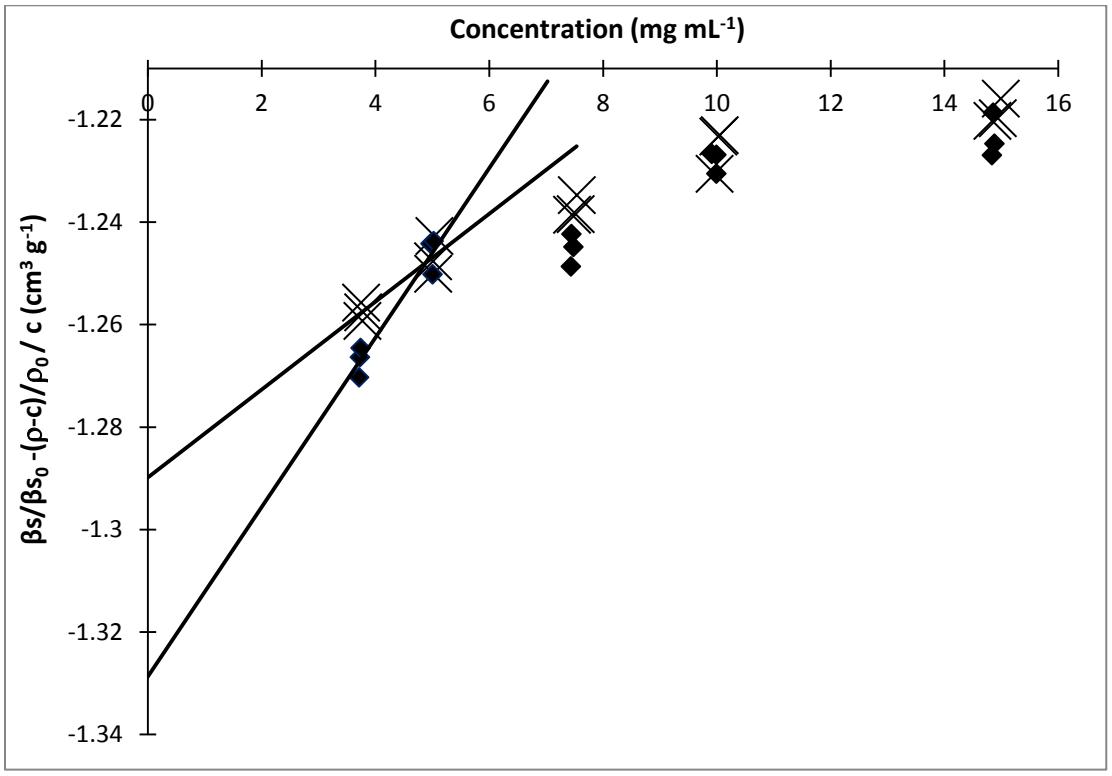

Fig. 4.9. Linear regression of the two lowest concentration values of $\frac{\beta_s/\beta_{so} - (\rho - c)/\rho_0}{c}$ of KCl serial dilutions measured in both cells (Cell 1 & Cell 2) of the Resoscan System, KCl cell 1 (◆) and KCl cell 2 (×).

Table 4.2. Partial specific adiabatic compressibility coefficient of KCl.

	$\overline{\beta}_S^0$ (x10^{-6} bar^{-1})
KCl cell 1	-165.91
KCl cell 2	-161.0

4.5. Discussion

KCl serial dilutions showed a linear relationship between ultrasonic velocity (Fig. 4.1.) and concentration and according to Pavlovskaya et al. (1992) we can treat them as ideal solutions. The propagation of the ultrasonic waves is related with the cycles of compression and expansion of the molecules in the solution, and so the velocity of the propagation is related to the elasticity, density and composition of the molecules of the solvent (ultrapure water) and the solute (KCl) (McClements, 1991). Therefore, the linear relationship between the ultrasonic velocity and concentration showed that the addition of a solute, which has a different density and elasticity to the solvent, affects the ultrasonic velocity of the solution, increasing the ultrasonic velocity of the solution linearly as the solute is added.

The ultrasonic velocity of the pure solvent (ultrapure water) and the solution are different due to three main reasons: 1) the water molecules in the pure solvent have a hydrogen bonded network which is more less compressible during the propagation of ultrasound waves by the cycles of compression and expansion, 2) in the solution, the solute added occupies space and has a less compressible structure than the water molecules, causing a different velocity for the propagation of the ultrasonic waves, 3) additionally, the water molecules that interact with the solute are affected by the electric field of the ions, and so their structure becomes dipolar and this also changes the way that the ultrasound waves propagate.

The apparent specific volume of the KCl serial dilution (Fig. 4.6.) was seen to continuously decrease starting at about 7.5 mg mL^{-1}. The effect was seen to become more pronounced as the concentration diminished. The rapid change in the specific volume as the concentration diminished did not allow us to have certainty about the behavior at lower concentrations.

The change in the apparent specific volume for this concentration range, which was about 0.057 cm^3 g^{-1} if the mean value at the lowest concentration is subtracted from the mean value at the highest concentration, can be caused by the electrostriction effect. According to Desnoyers et al. (1965), electrostriction is defined as an apparent reduction in the volume of an ion when it is dissolved. Electrostriction is an effect caused by ion-solvent interactions and it is the compression of the solvent caused by its exposure to an electric field. The charge of an electrolyte in aqueous solution causes water molecules around the electrolyte to be affected by the electric field and the hydrogen bond network breaks, reducing the space between the water molecules and the ion, causing a compression of the solvent volume. The electrostriction effect affects the water molecules around the ion reducing its volume, but seems to not affect the volume of the ion itself (Marcus, 2011). The solvation of ions in aqueous solution is dynamic and the ion interactions with water molecules and with other ions is influenced by the nature of the ion but also by the concentration, so that at higher concentrations ion pairing is a common occurrence (Marcus, 2005). The interactions between ions in aqueous solutions include the pairing of two ions (association) or the disassociation of them; the pairing of two ions reduces the surface exposed to the water, and hence causes a reduction of the number of water molecules on the water shell that are affected by the electrostriction effect (Marcus 2005). Therefore, ion dissociation has the opposite effect, the disassociated ions have a larger surface exposed to the water than the paired ions, causing an increase in the number of water

molecules in the hydration shell exposed to the electrostriction effect. The dilution of the KCl solutions reduces the number of ion-ion interactions, because a greater number of ions are disassociated. The reorganization of water increases the number of water molecules in the water shell around the ions, which are affected by the electrostriction effect, and produces a lower apparent specific volume than that of the water molecule in the bulk water. This increase of water shell molecules affected by electrostriction as the solution is diluted decreases the apparent specific volume (Fig. 4.6.).

The partial specific volume (Table 4.1.), calculated as the value of the apparent specific volume at infinite dilution, was 0.359 $cm^3 g^{-1}$, which is comparable with the result obtained by Zen (1957), of 0.354 $cm^3 g^{-1}$, and it is in the range of other values referred to in the same paper, 0.354 $cm^3 g^{-1}$ - 0.360 $cm^3 g^{-1}$. However as was remarked previously, density measurements at lower concentrations are needed to confirm this value, because as remarked by Marcus (2011), highly accurate density values, at least at a concentration as low as 0.01 mol dm^{-3} (0.7455 g dm^{-3} for KCl), are needed to avoid inaccurate determination of partial specific volume values.

The apparent specific adiabatic compressibility of the serial dilution (Fig. 4.8.) was seen, as was the apparent specific volume, to continuously decrease starting at the highest concentration, 15 mg mL^{-1}; the total decrease, between the highest and the lowest concentration was about 1.7-1.9 x 10^{-6} $cm^3 g^{-1}$ bar^{-1}. The apparent compressibility of electrolytes solutions is also affected by the electrostriction effect of the water molecules in the hydration shell around the electrolytes (Afanas'ev & Tyunina, 2001). Therefore, the decrease of the apparent specific adiabatic compressibility of the KCl solutions might be caused by an increase in the number of water molecules affected by electrostriction as the concentration decreases. As explained with the apparent specific volume, the reduction in the concentration of the KCl

solutions leads to a decrease of the ion-ion interactions causing a higher amount of ions to disassociate, and these dissociated ions allow more water molecules in the hydration shell than the paired ion (Marcus, 2005; Marcus, 2006; Marcus, 2011). The concentration dependence of the compressibility of KCl solutions caused by the electrostriction effect has already been reported by Afanas'ev & Tyunina (2001).

The partial specific adiabatic compressibility coefficient (Table 4.2.) calculated at infinite dilution allows analysis of the apparent compressibility influenced only by the ion-solvent interaction and not the ion-ion interactions (Marcus, 2011). However, as explained previously, the value obtained (-164 x 10^{-6} bar^{-1}) could be overestimated. Afanas'ev & Tyunina (2001), Afanas'ev & Ustinov (2009) and Afanas'ev (2011) have already studied the adiabatic compressibility of KCl solutions but at higher concentrations than the ones used in this study. In these papers, a concentration dependence of the compressibility of electrolyte solutions was found, as well as a concentration dependence of the hydration number which influenced the solvent-solute interaction of electrolytes in aqueous solution. Afanas'ev & Tyunina (2001) reported values for the apparent specific compressibility of potassium chloride in the range between -3.1 cm^3 g^{-1} bar^{-1} to -6.7 x10^{-6} cm^3 g^{-1} bar^{-1}. However, these values are apparent values and not the partial specific compressibility coefficient, and were obtained at a much higher concentration range than analysed in this study between (45 mg g^{-1} and 300 mg g^{-1}).

5. Hemp Seeds Peptides Results and Discussion

Characterization of the volumetric properties of hemp seed bioactive peptides in aqueous solutions involves ultrasonic and density measurements and determination of the apparent specific volume and partial specific volume as well as the apparent specific adiabatic compressibility and partial specific adiabatic compressibility. Hemp seed peptides, 1 kDa and 3 kDa fractions, used to prepare serial dilutions made up gravimetrically (section 3.2.2.) were obtained from Girgih et al. (2011). Both peptides were analysed in the same study investigating their biological activity as antioxidants. These peptides were obtained by enzymatic hydrolysis and separated by filtration. In this study, the 1 kDa fraction, size $<$ 1 kDa, and the 3 kDa fraction, $<$ 3kDa and $>$ 1kDa, were used. Because the fractions used were just ultrafiltered but not dialyzed, the fractions are not pure protein and also contain other components, such as salts and sugars, which were not determined. According to Girgih et al. (2011) the 3 kDa fraction was 85% protein and the 1 kDa fraction was 64% protein. Density and ultrasonic velocity measurements were measured as a function of peptide concentration at 24.985°C; this temperature was set because it was the most stable temperature reached close to 25°C in the ResoScan system, and the same temperature was used with the density meter DMA 5000 which has accurate temperature control. Temperature stability is very important for the precision of ultrasonic measurements (Sarvazyan, 1991).

5.1. Ultrasonic Velocity Results

Ultrasonic velocity (u) of hemp seed peptide serial dilutions was found to have a linear relationship between ultrasonic velocity and concentration (Fig. 5.1.).

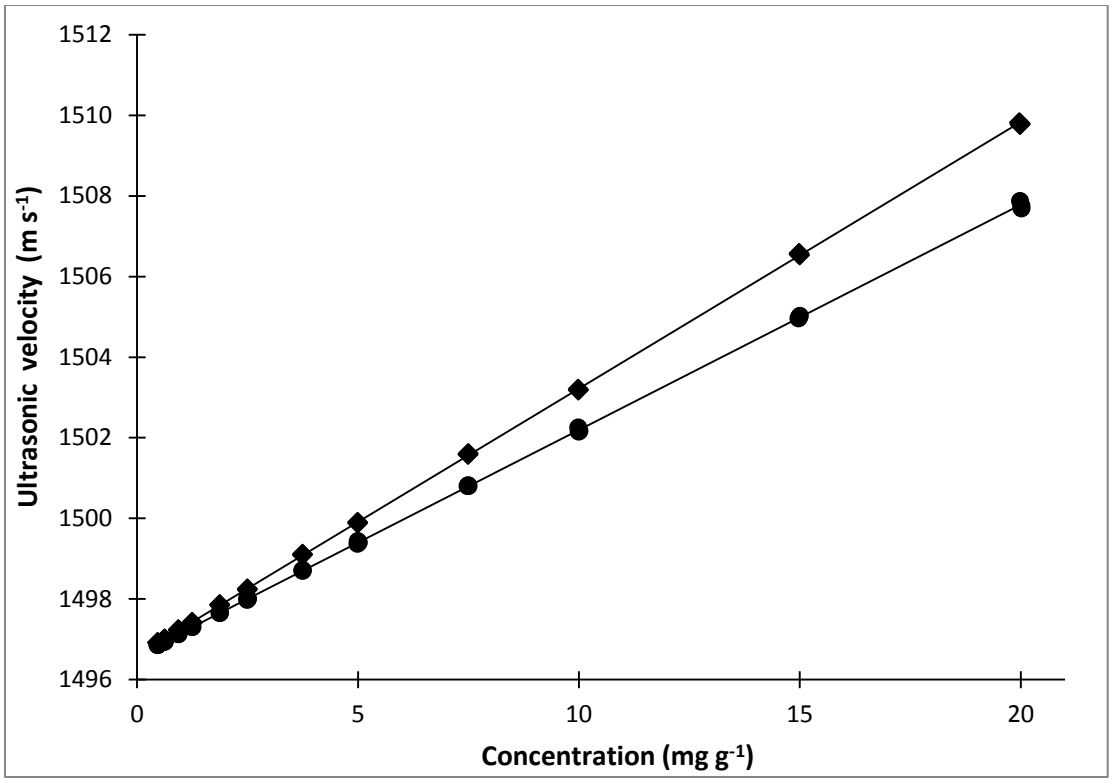

Fig. 5.1. Ultrasonic velocity of hemp seed peptide serial dilutions, 1 kDa (◆) and 3 kDa (●).

The R^2 for the linear regressions calculated for the ultrasonic velocity of both hemp seed peptides serial dilutions, 1 kDa and 3 kDa, were very good, with values of 1.0000 and 0.9999 respectively. In order to confirm a linear relationship, including all of the concentration range of the serial dilutions, linear regressions were calculated for the ultrasonic velocity in the lower concentration range (0.45-2.5 mg g^{-1}, Fig. 5.2.) and in the higher concentration range (3.75-20 mg g^{-1}, Fig. 5.3.). The R^2 values for 1 kDa and 3 kDa peptides in the lower concentration range, with values of 0.9990 and 0.9993, respectively, were slightly lower than R^2 values for 1 kDa and 3 kDa peptides in the higher concentration range, with values of 0.9999 and 0.9998 respectively. The very good fit over both concentrations confirms the good linear fit over all the concentrations range used.

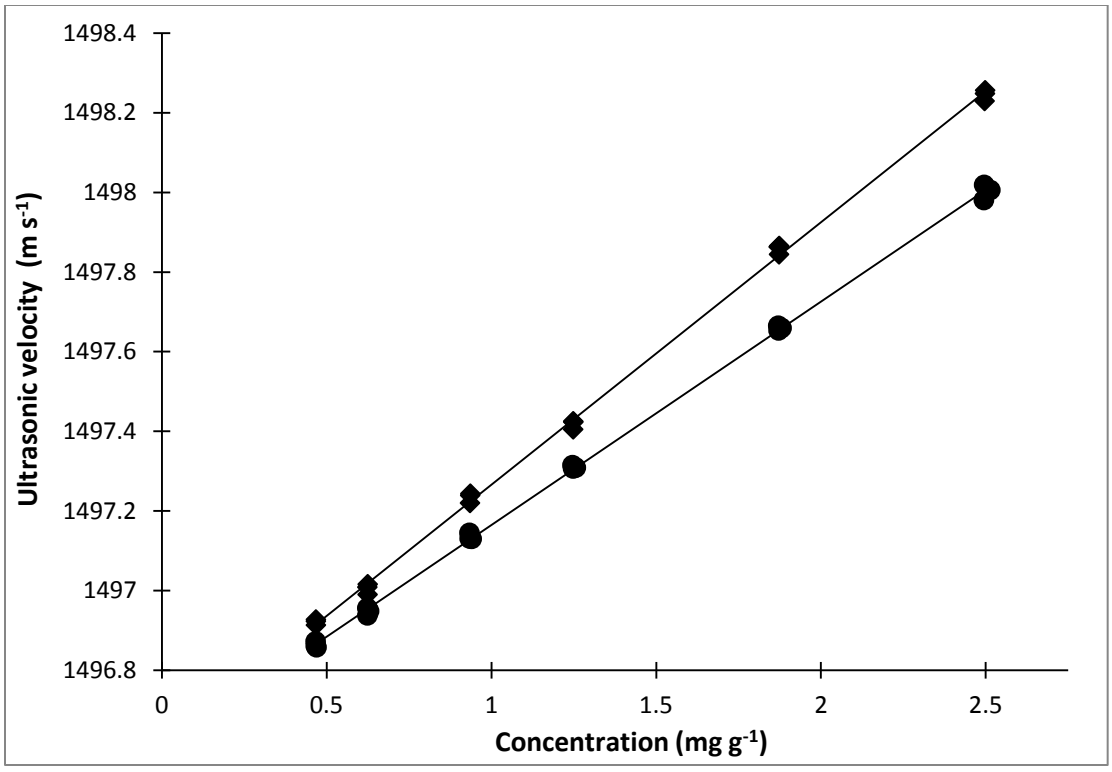

Fig. 5.2. Ultrasonic velocity of hemp seed peptide serial dilutions in the low concentration range, 1 kDa (◆) and 3 kDa (●).

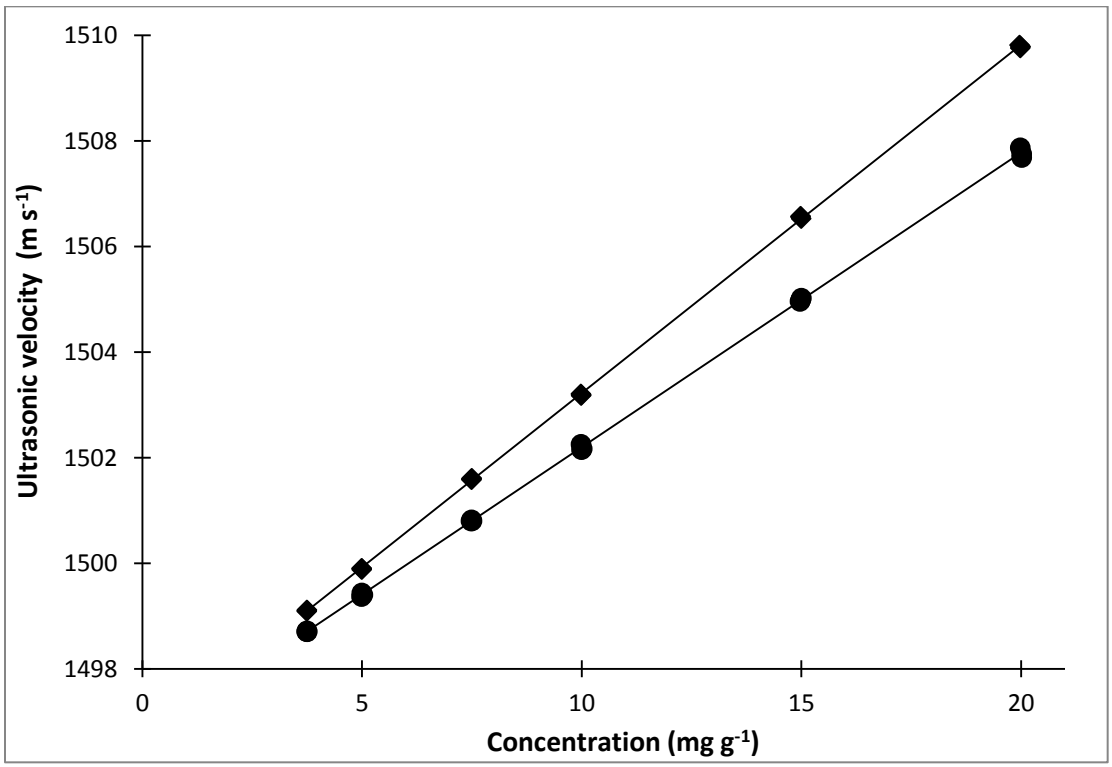

Fig. 5.3. Ultrasonic velocity of hemp seed peptide serial dilutions in the high concentration range, 1 kDa (◆) and 3 kDa (●).

Serial dilutions were made using ultrapure water and two initial stock solutions with different concentration, 20 mg g^{-1} and 15 mg g^{-1}. Three replicates and two subsamples were used for the analysis, but due to the preparation being done gravimetrically, the exact weight of the solute and the water added was recorded, and so the concentration of the replicates at a given dilution was slightly different.

The coefficient of variation (CV) of the ultrasonic velocity from serial dilutions of both hemp seed peptides, 1 kDa and 3 kDa, was calculated between the three replicates and the two subsamples. Subsample CV (Fig 5.4.) values were low in both peptides, 1 kDa and 3 kDa, with values $<$ 0.0045% and $<$ 0.0035%, respectively, with most of the values $<$ 0.0035% for both peptides.

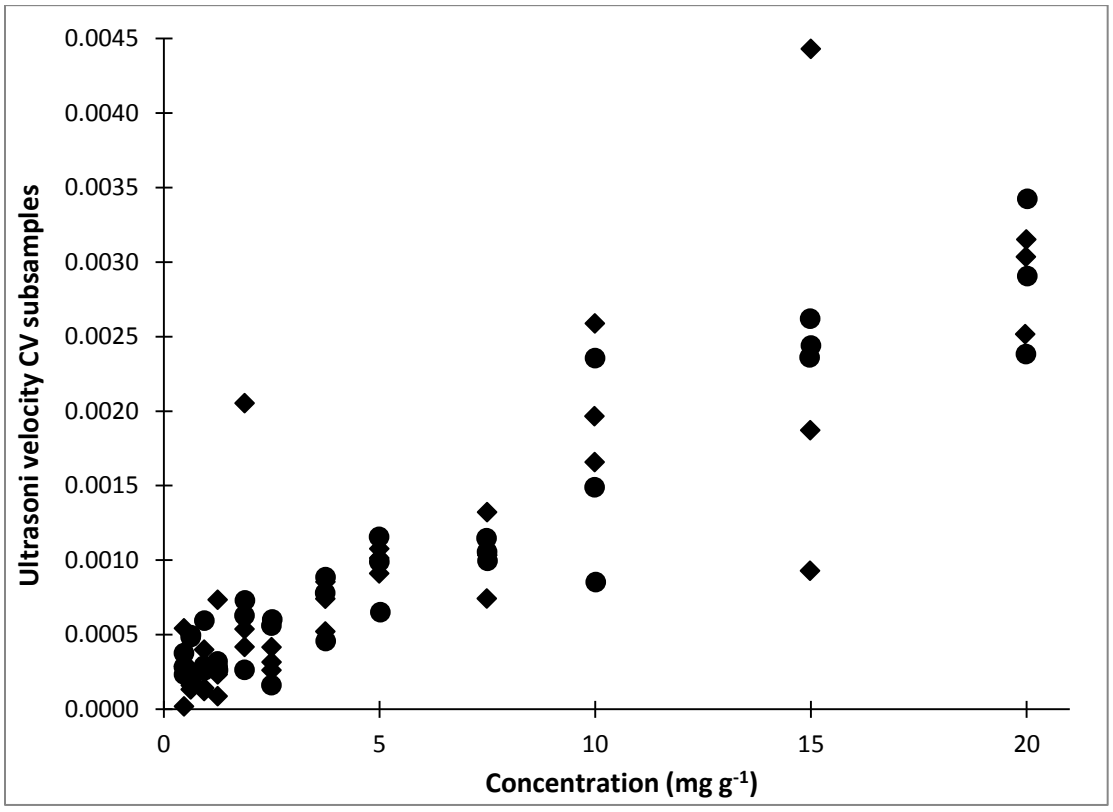

Fig. 5.4. CV of ultrasonic velocity for subsamples of hemp seed peptide at different serial dilutions, 1 kDa (◆) and 3 kDa (●).

CV values for replicates were also low for both peptide solutions, with values < 0.002% and < 0.0065%, respectively. Although 3 kDa peptide solutions had a higher CV than those of 1 kDa, most of the values were < 0.004% except for one value at the highest concentration. Nevertheless, according to the procedure used to prepare the serial dilution gravimetrically, the exact concentration of each solution was recorded leading to slight differences between the concentration of each of the three replicates; therefore, the CV measured for the "replicates" that uses the average concentration of each dilution leads to an overestimated CV.

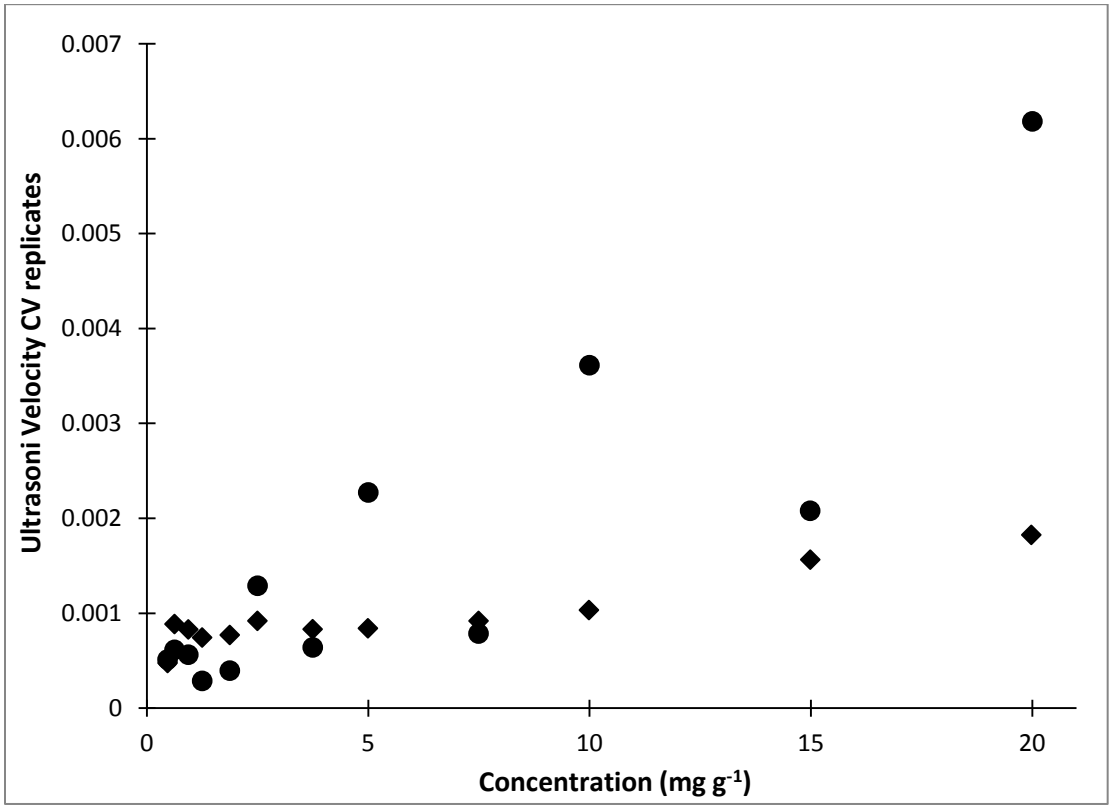

Fig. 5.5. CV of ultrasonic velocity for replicates of hemp seed peptide at different serial dilutions, 1 kDa (◆) and 3 kDa (●).

Ultrasonic attenuation of serial dilutions of hemp seed peptides, 1 kDa and 3 kDa, were found to have a more or less linear relationship against concentration (Fig. 5.6.). However both

peptide serials dilutions presented considerable variation of ultrasonic attenuation values within the concentration range used.

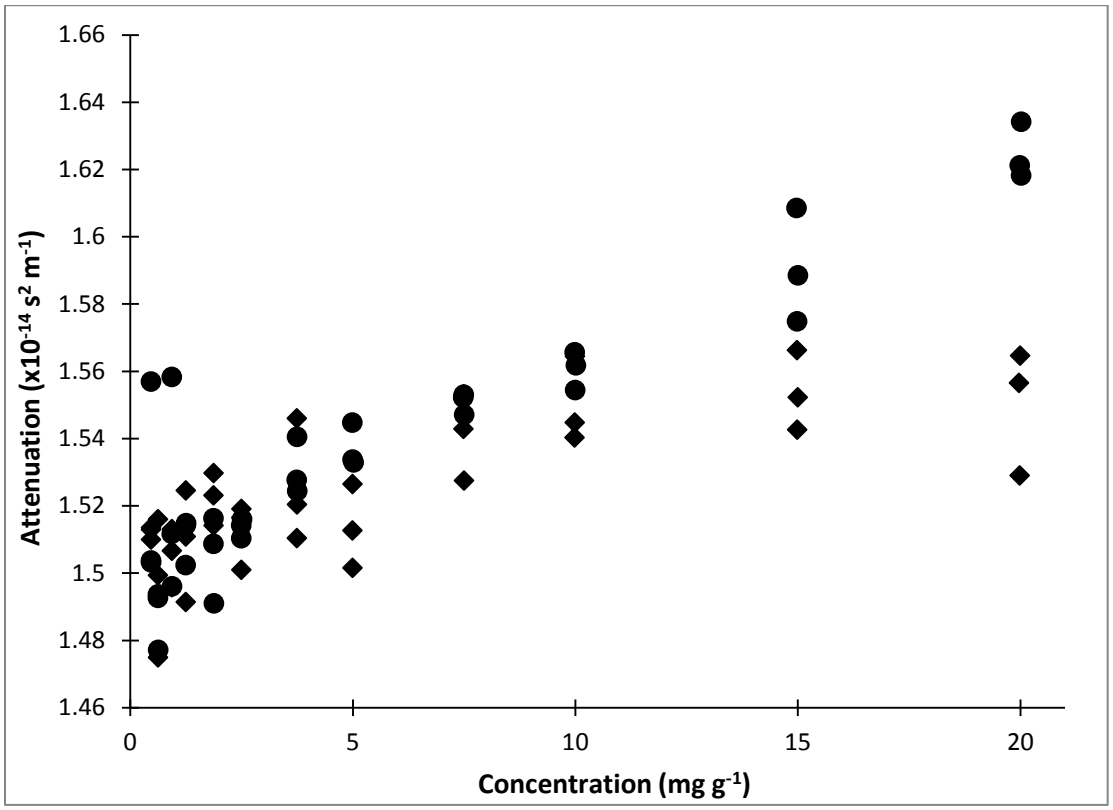

Fig. 5.6. Ultrasonic attenuation of hemp seed peptide serial dilutions, 1 kDa (◆) and 3 kDa (●).

5.2. Density Results

Density (ρ) of the hemp seed peptides serial dilutions was found to have a linear relationship between density and concentration (Fig. 5.7.).

R^2 for the linear regressions of density results of both hemp seed peptides were very good, with values of 0.9998 and 0.9996, respectively, for the 1 kDa and 3 kDa peptides. In order to confirm the linear relationship over all the concentration range of the serial dilutions, linear regressions were calculated for the density results in the lower concentration range (0.45-2.5

mg g^{-1}, Fig. 5.8.) and the higher concentration range (3.75-20 mg g^{-1}, Fig. 5.9.). The linear relation between density and concentration had an R^2 closer to 1 in the higher concentration range, with values of 0.9995 and 0.9994 for 1 kDa and 3 kDa peptides, respectively. A lower R^2 in the lower concentration range was found, with values of 0.9986 and 0.9982 for 1 kDa and 3 kDa, respectively. The slight decrease in R^2 values can be caused by a diminution in precision of the density measurements as the concentration diminished due to limitations of the equipment and experimental errors. This increase in the variation of volumetric determination as the concentration diminishes has been also reported in ionic solutions by Marcus (2006).

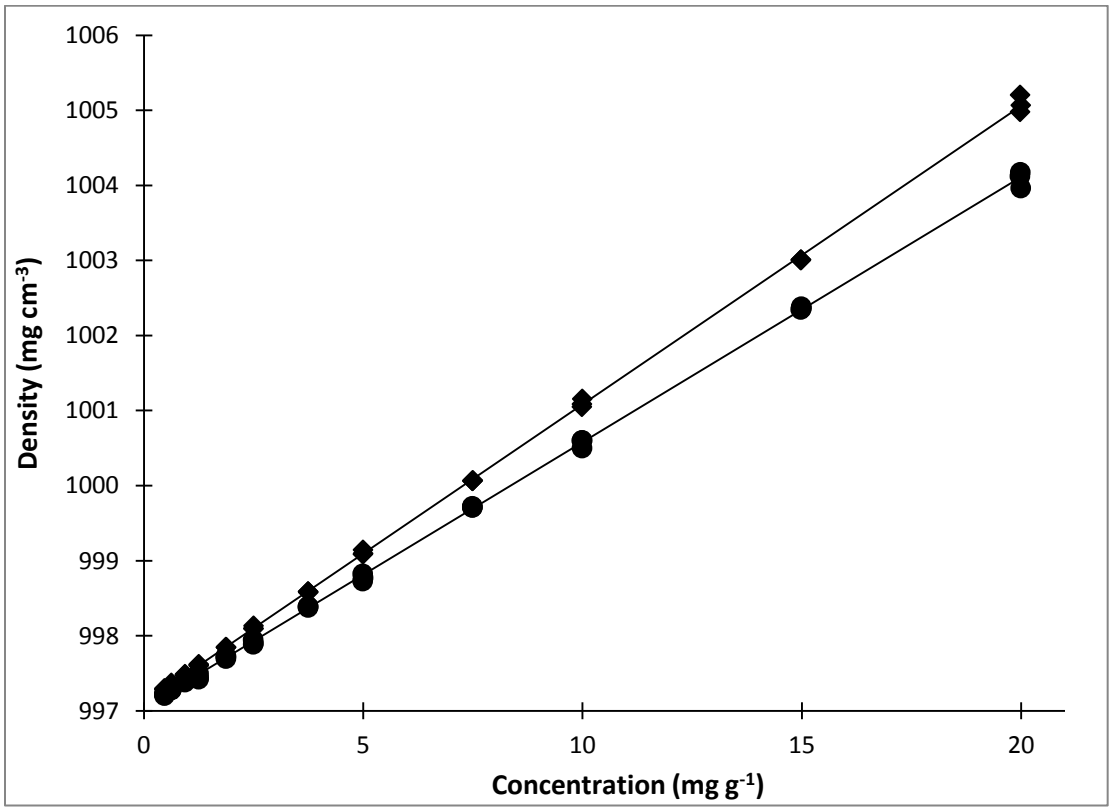

Fig. 5.7. Density of hemp seeds peptide serial dilutions, 1 kDa (◆) and 3 kDa (●).

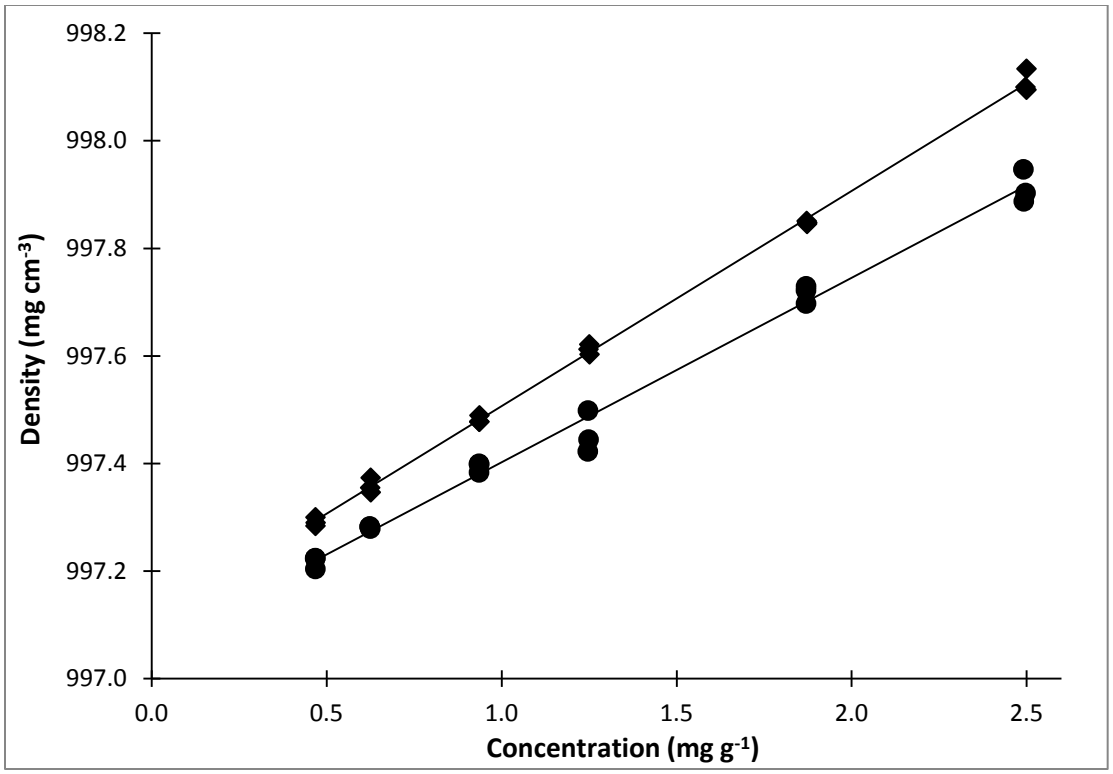

Fig. 5.8. Density of hemp seed peptide serial dilutions at low concentrations, 1 kDa (◆) and 3 kDa (●).

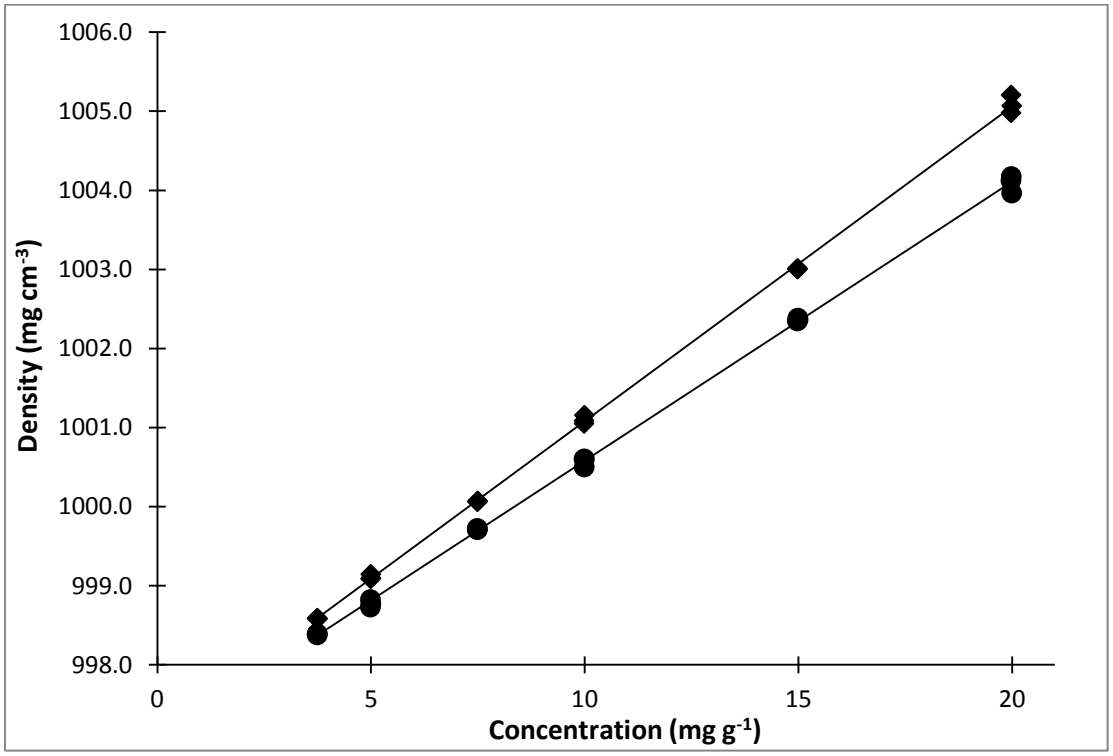

Fig. 5.9. Density of hemp seed peptide serial dilutions at high concentrations, 1 kDa (◆) and 3 kDa (●).

The coefficient of variation (CV) was calculated for all density results of serial dilutions of both hemp seed peptides, and it was calculated between the two subsamples and three replicates. Subsample CV (Fig. 5.10.) values were low in hemp seed peptides, 1 kDa and 3 kDa, with values < 0.009% and < 0.0015%, respectively. Although the subsample CV of 3 kDa peptide solutions includes 2 points with values quite higher than the average, most of the values were lower than 0.0026%. The two values of subsample CV for the 3 kDa peptide solutions out of the average range could possibly be caused by the use of just two subsamples, negatively affecting the precision of the measurement.

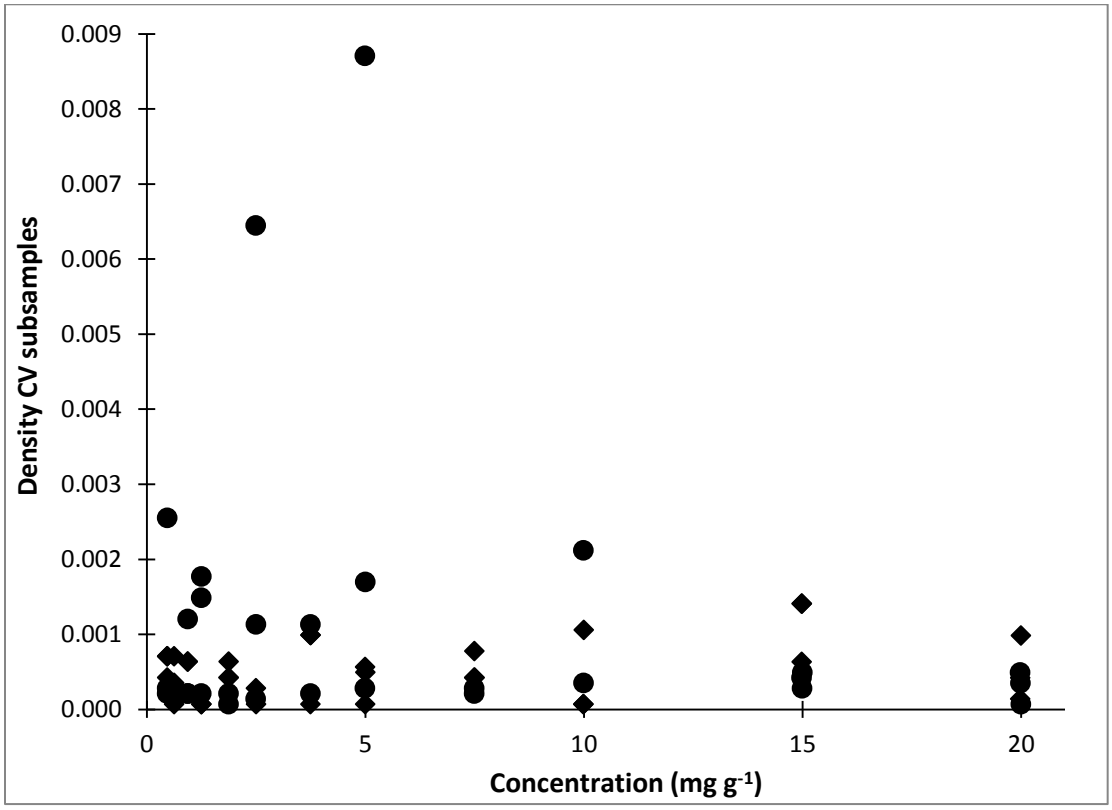

Fig. 5.10. CV of density for subsamples of hemp seed peptide taken at different serial dilutions, 1 kDa (◆) and 3 kDa (●).

Replicate CV values were low for both peptides, with values < 0.0012% and <0.0011%, respectively (Fig.5.11.). Although the CV for the stock solutions for both hemp seed peptides

was a little bit high, most of the values for both peptides were under 0.006%. Nevertheless, as with ultrasonic velocity analyses, the procedure to prepare the serial dilution gravimetrically causes slight differences between the concentrations of the three replicates; therefore, the CV measured for the "replicates " was done using the average of the concentration of each dilution leading to overestimation of the replicate CV.

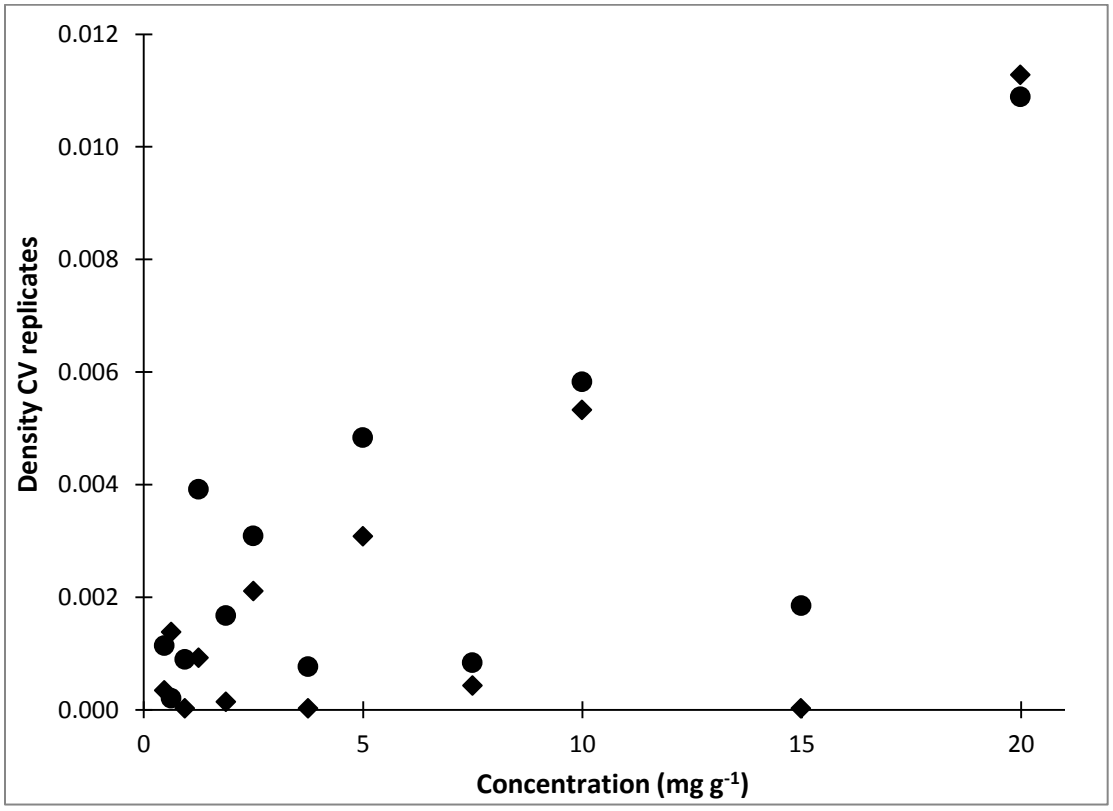

Fig. 5.11. CV of density for replicates of hemp seed peptides taken at different serial dilutions, 1 kDa (◆) and 3 kDa (●).

5.3. Specific Volume

The apparent specific volume, $\bar{V}$, of the hemp seed peptide serial dilutions was calculated from the density measurements as $\bar{V} = \frac{1-(\rho-c)/\rho_0}{c}$ and the values are shown in Fig. 5.12.

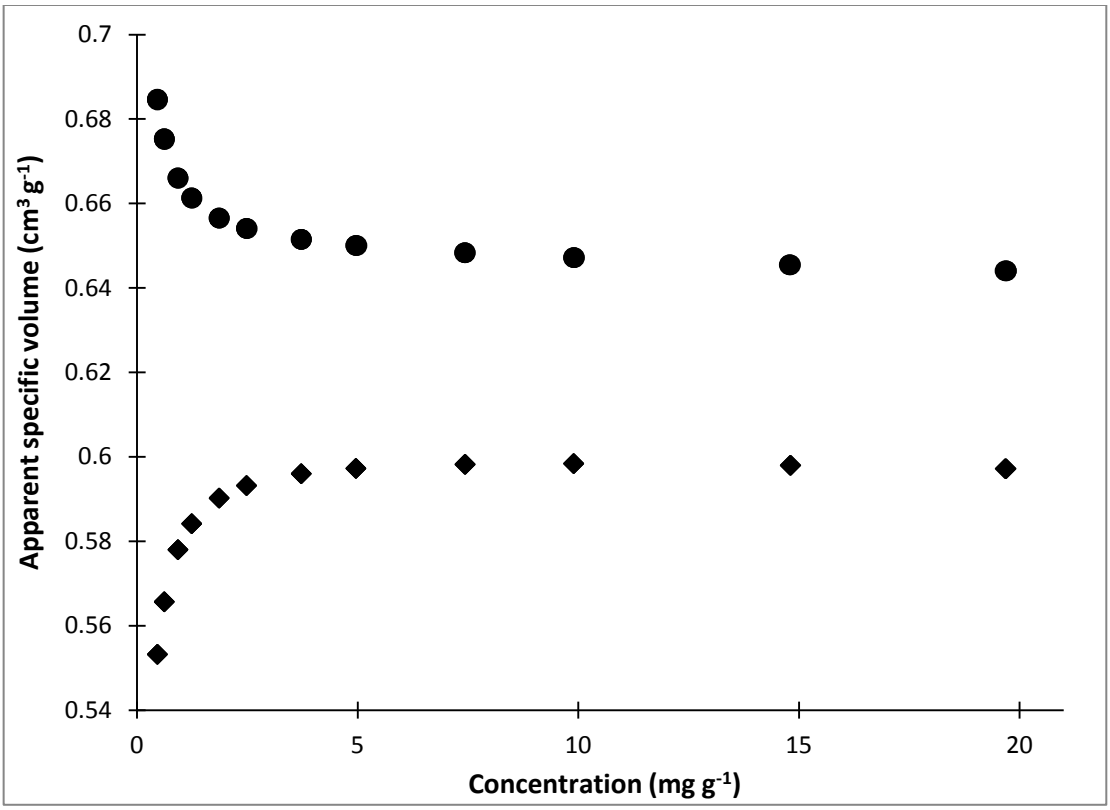

Fig. 5.12. Apparent specific volume of hemp seed peptide serial dilutions, 1 kDa (◆) and 3 kDa (●).

The partial specific volume, $\bar{V}^0$, was calculated by the extrapolation of the apparent specific volume to zero concentration as $\bar{V}^0 = \lim_{c\to 0} \bar{V}$. However, because the apparent specific volume had a rapid decrease for concentrations below 5 mg mL^{-1}, a proper equation to describe the behavior of the experimental data was not found, and in order to approach as much as possible to the correct value, the extrapolation to zero was done using just the two smallest concentration values (as in section 4.3.), although, given the curvature for both peptides, it is possible that even this value would be overestimated. The partial specific volume values are shown in Table 5.1.

Table 5.1. Partial specific volume of hemp seed peptides.

	$\bar{V}^0$ ($cm^3\ g^{-1}$)
Hemp seed peptides 1 kDa	0.516
Hemp seed peptides 3 kDa	0.712

5.4. Compressibility

The adiabatic compressibility coefficient, β_s, was calculated according to the Newton-Laplace equation $\beta_s = \frac{1}{\rho u^2}$, where β_s is calculated as a function of the density and ultrasonic velocity. The relative adiabatic compressibility coefficient, β_s/β_{s0}, which expresses the relation between the adiabatic compressibility coefficient of the solution, β_s, against the adiabatic compressibility coefficient of the solvent β_{s0}, ultra pure water (UPW) for this study, had a linear relationship against concentration.

The apparent adiabatic specific compressibility, $\bar{K}_s$, which express the change in the apparent specific volume as a function of a change of pressure, was calculated as $\bar{K}_s = \beta_{so}\frac{\beta_s/\beta_{so}-(\rho-c)/\rho_0}{c}$ (Fig. 5.13.)

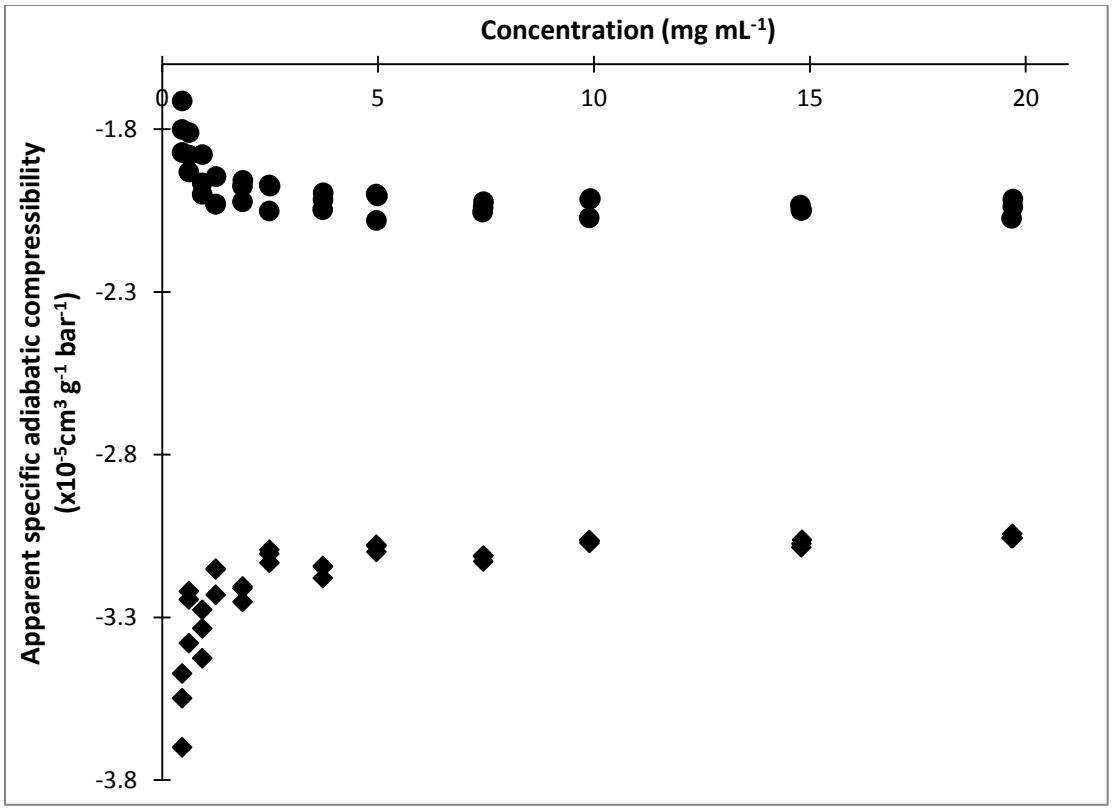

Fig. 5.13. Apparent adiabatic specific compressibility of hemp seed peptide serial dilutions, 1 kDa (◆) and 3 kDa (●).

The partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^0$, which describes the relation between the partial specific adiabatic compressibility, $\bar{K}_S^0$, and partial specific volume, $\bar{V}^0$, was calculated as $\bar{\beta}_S^0 = \frac{\bar{K}_S^0}{\bar{V}^0} = \frac{\beta_{so}}{\bar{V}^o} \lim_{c\to 0} \frac{\beta_s/\beta_{so} - (\rho - c)/\rho_0}{c}$, where the term $\frac{\beta_s/\beta_{so} - (\rho - c)/\rho_0}{c}$ is extrapolated to zero while β_{s0} and $\bar{V}^0$ are constant values. However, as it had occurred with the apparent specific volume, because the term $\frac{\beta_s/\beta_{so} - (\rho - c)/\rho_0}{c}$ had a rapid decrease from concentrations below approximately 3 mg mL^{-1}, a proper equation to describe the behavior of the experimental data was not found, and in order to approach as much as possible to the correct value, the extrapolation to zero was done using just the two smallest concentration values; it is likely that this value is overestimated. The partial specific adiabatic compressibility coefficient $\bar{\beta}_S^0$ values are shown in Table 5.2.

Table 5.2. Partial specific adiabatic compressibility coefficient of hemp seed peptides.

	$\overline{\beta}_s^0$ (x10^{-6} bar^{-1})
Hemp seed peptide 1 kDa	-86.2
Hemp seed peptide 3 kDa	-22.0

5.5. Discussion

Serial dilutions of hemp seed peptides of molecular weights of 1 kDa and 3 kDa had experimental results of density and ultrasonic velocity exhibiting linear relationships against concentration (Fig. 5.1. & 5.7.). According to Pavlovskaya et al. (1992), it is possible to treat the peptide solutions as ideal solutions. However, Murphy et al. (1998) and Sirotkin et al. (2012) pointed out that the protein-solvent interactions in solutions are not ideal and the volumetric properties can help to measure their changes.

Ultrasonic attenuation of both hemp seed peptide serial dilutions had a more or less linear relationship against concentration. However the variation of the ultrasonic attenuation values was considerable within the whole concentration range used. In addition, the values for the 1 kDa hemp seed peptides only slightly changed over the concentration range used, which could be due to the limitation of the attenuation measurements with very small particles (McClements, 1991). Ultrasound attenuation is dependent on the ultrasound frequency and concentration, and is affected by the particle size and conformation transitions, increasing its values as the particle size is bigger (Bryant & McClements, 1999; Corredig et al., 2004; Povey et al., 2011). This is compatible with the ultrasound attenuation values seen for the 1 kDa hemp seed peptides which were lower compared to the values of the 3 kDa hemp seed peptides which have a bigger particle size.

The determination of the volumetric properties of a solute can provide information about the hydration process of the molecule and therefore the solute-solvent interactions (Murphy et al., 1998; Taulier & Chalikian, 2002; Chalikian, 2003; Sirotkin et al., 2012). The solute-solvent interactions between peptides and ultra pure water, can cause the water molecules located in the water shell surrounding the peptide, as well as the water molecules adjacent to this water shell, to have different volumetric properties than bulk water molecules (Chalikian, 2003).

The apparent specific volume of the 1 kDa peptides (Fig.5.12.) was seen to be independent of the concentration in the higher concentration range. Some studies pointed out by Chalikian et al. (1998) showed that the apparent specific volume of very short peptides was independent of the concentration. However, in this study, starting at concentrations between 5 and 3.75 mg mL^{-1}, the apparent specific volume was seen to continuously decrease as the concentration diminished. The total decrease between the highest and the lowest concentration was about 0.044 $cm^3 g^{-1}$. In the 1 kDa hemp seed peptides, the proportion of protein was only about 65%, with another 35% of other components. The high proportion of components like salts and sugars in the 1 kDa hemp seed peptides might contribute to the decrease of the apparent specific volume as the solutions were diluted. As explained in section 4.5. for the KCl solutions, an increase in the electrostriction effect of water molecules that surround electrolytes like salts, as the solution is diluted, might be caused by the reduction of the solute–solute interactions which leads to an increase in disassociation of electrolytes and therefore an increase of water molecules affected by the electrostriction effect. The electrostriction effect is the contraction in the volume of molecules exposed an electric field and in solutions can be caused by solute-solvent interactions (Desnoyers et al., 1965; Marcus, 2011). The charge of solutes, like electrolytes, in aqueous solution causes the water molecules around the electrolyte

to be affected by the electric field of the charge, and the hydrogen bond network breaks, losing the void spaces and causing a compression of the solvent, but not in the electrolyte molecule (Marcus, 2011). The reduction of the apparent specific volume of protein solutions caused by solvation of salts or sugars has been reported by Durchschlag & Jaenicke (1982). Bernhardt & Pauly (1977) reported changes in the apparent specific volume of proteins caused by the electrostriction effect as a result of addition of salts. Another possibility that cannot be excluded, is the possibility that salts bonded to the peptide could dissociate as the solution was diluted, releasing new electrolytes to the solution that enhance the electrostriction effect; in addition, the side chains of peptide where the salts were bonded can then interact with water molecules enhancing as well the electrostriction effect in the peptide solution.

A contrary effect was seen for the change in the apparent specific volume of the 3 kDa peptide which was seen to continuously increase as the solution was diluted. The reason for such a change has not been completely clarified. Bernhardt & Pauly (1975) reported an increase in the apparent specific volume of bovine serum albumin (BSA) and bovine hemoglobin (Hb) as the concentration diminishes in serial dilutions, giving an interpretation related to the moisture of the dry sample and changes in “free” water molecules and water molecules bonded to the protein. In addition, pH changes in the solution as it was diluted were suggested as an explanation. Bull & Bresse (1979) also reported an increase in the apparent specific volume of BSA as the concentration diminished within a certain concentration range. However, the studies have been conducted in proteins, not peptides, at higher concentrations than those used in this study, but all have reported concentration dependence of the apparent specific volume under certain circumstances (Bernhardt & Pauly, 1975; Bull & Bresse, 1979; Durchschlag & Jaenicke, 1982; Apenten et al., 2000; Sirotkin et al., 2012). It is important to point out the impurity of the 3 kDa peptide, which according to Girgih et al. (2011) was 85% protein with 15 % of other

undetermined components, which could include salts and sugars. The increase in the apparent specific volume of the 3 kDa peptide in a serial dilution starting from the first dilution, could have happened as a result of a restructuring of the peptide as the solution was diluted, which could lead to changes in the interaction between the molecules of water and the peptide. For example, changes in the proportion of water molecules in the water shell around the peptide. A decrease in the number of water molecules in the water shell which have a lower specific volume than the water molecules in the bulk water (Murphy et al. 1998; Sirotkin et al. 2012) could cause an increase in the apparent specific volume. Possible slight changes in the pH of the solution as it was diluted could also affect the apparent specific volume (Bernhardt & Pauly, 1975; Sarvazyan et al., 1979; Chalikian et al., 1995).

The partial specific volume of both hemp seed peptides (Table 5.1.) was calculated by extrapolation to zero of the apparent specific volume with values of 0.516 $cm^3 g^{-1}$ and 0.712 $cm^3 g^{-1}$ for the 1 kDa and 3 kDa hemp seed peptides, respectively. However, as explained previously, due to the curvature seen in apparent specific volume for both peptides the values could be overestimated and underestimated for the 1 kDa and 3 kDa hemp seed peptides, respectively. It is important to point out that due to the impurity of both peptides, the values correspond to a mixture of peptides and other components like salts and sugars. Compared with other results found in the literature, the partial specific volume of the 1 kDa peptides is lower than the apparent specific volume values obtained by Chalikian et al. (1994) for the amino acids glycine and alanine at 25°C with values of 0.578 $cm^3 g^{-1}$ and 0.679 $cm^3 g^{-1}$. The lower values for the 1 kDa peptides might be caused by the influence on the partial specific volume of the non-protein components, whose electrostriction effect on the water molecules reduces the partial specific volume as shown in chapter 4 for the partial specific volume of KCl. In the case of the 3 kDa hemp seed peptides, which have a higher protein content than the 1 kDa hemp seed peptide,

the value of the partial specific volume is higher than the values for amino acids like alanine and glycine, and in the range of values obtained for most globular proteins, at 25°C: 0.70 $cm^3 g^{-1}$ to 0.75 $cm^3 g^{-1}$ (Bernhardt & Pauly, 1975; Bull & Bresse, 1979; Durchschlag & Jaenicke, 1982; Gekko & Hasegawa, 1986; Chalikian et al., 1996; Sitorkin et al., 2012).

The apparent specific adiabatic compressibility of both hemp seed peptides (Fig. 5.13.) was seen to follow more or less the same trend seen with the apparent specific volume. The compressibility of proteins and peptides is related with the hydration of the molecules and therefore the electrostriction effect should be taken into account (Pfeiffer et al., 2008; Marcus, 2011). Peptides are chains of amino acids that are related with the primary and secondary (such as α-helix and β-sheets) structure of proteins (Creighton, 1993), and according to Taulier & Chalikian (2002), for small peptides the intrinsic compressibility is usually small. In addition, the content in both hemp seed peptides of other components like salts, whose negative compressibility is caused by the electrostriction effect (Afanas'ev & Tyunina, 2001), as explained in section 4.5. for KCl, contribute negatively to the compressibility of both hemp seed peptides. Therefore, the reduction in the specific compressibility might be caused, as in the apparent specific volume, by an increase of the electrostriction effect as the solution was diluted as explained previously. However, because the compressibility values are more sensitive to intermolecular interaction than the partial specific volume (Kharakoz, 1997), the apparent compressibility could also be influenced by void spaces within the peptide structure, and therefore small changes in the peptide conformation as the solutions were diluted could also influence the decrease or increase in compressibility, specially for the 3 kDa hemp seed peptide.

The partial specific adiabatic compressibility coefficient of both hemp seed peptides (Table 5.2.) was calculated at infinite dilution because, according to Chalikian et al. (1994), the

solute-solute interactions decrease as the concentration is reduced, so that at infinite dilution the partial compressibility is only influenced by the intrinsic compressibility of the solute and its associated solvent molecules. However, as explained previously, the value obtained for the 1 kDa (-86.2 $x10^{-6}$ bar^{-1}), and 3 kDa (-22.0 $x10^{-6}$ bar^{-1}) hemp seed peptides could be overestimated and underestimated, respectively. Chalikian et al. (1996) and Gekko & Hasegawa (1986) obtained the partial compressibility coefficient of several globular proteins which were all positive and in a range of about 1 $x10^{-6}$ bar^{-1} and 11 $x10^{-6}$ bar^{-1}, which confirms, according to Gekko & Hasegawa (1986), that the positive compressibility values indicate a highly compressible interior, and negative compressibility values indicate an incompressible interior like in fibrous proteins and amino acids where the compressibility values are driven specially by the negative compressibility contribution of the hydration of the solute caused by the solute-solvent interactions (Kharakoz & Sarvazyan, 1993; Apenten et al., 2000). This negative compressibility was seen on the confirmed partial specific adiabatic compressibility coefficient values obtained by Chalikian et al. (1998) for tripeptides which were in a range of -1.7 $x10^{-6}$ bar^{-1} and -6.69 $x10^{-6}$ bar^{-1}. In this study, both hemp peptides had negative partial specific adiabatic compressibility coefficients, which therefore indicates both hemp seeds do not have a highly compressible interior and their compressibility is mainly influenced by the hydration. This is compatible with a similar tendency observed between the apparent specific volume and the apparent specific adiabatic compressibility in both hemp seed peptides. However, it is important to mention that all the experimental results describe the volumetric properties of mixtures of hemp seed peptides and other components like salts which could influence importantly the negative compressibility especially in the 1 kDa hemp seed peptides.

6. Dairy Peptides Results and Discussion

The volumetric properties in aqueous solutions of dairy bioactive peptides were characterized by determination of the apparent specific volume and partial specific volume as well as the apparent specific adiabatic compressibility and partial specific adiabatic compressibility based on high precision measurements of the density and ultrasonic velocity of dairy bioactive peptides in aqueous solutions. Three different dairy bioactive peptides, received from Glanbia Nutritionals (Fitchburg, WI, USA), were used to prepare serial dilutions gravimetrically (section 3.2.2.). Dairy peptides A, C and D were obtained by enzymatic hydrolysis of whey protein and were dialyzed. However the protein content is about 90%, and so they contain small amounts of other components like sugars and salts (Table 3.1.). Density and ultrasonic velocity measurements were conducted as a function of peptide concentration at 24.985°C, as explained in section 5.

6.1. Ultrasonic Velocity Results

Ultrasonic velocity (*u*) of serial dilutions of all dairy peptides A, C and D, were found to have a linear relationship against concentration (Fig. 6.1.).

The R^2 values for the linear regression of the ultrasonic velocity of all dairy peptides, A, C and D, were very good, with values of 0.9999, 1.0000, and 0.9998, respectively. In order to confirm the linear relation over the full concentration range of the serial dilutions, linear regressions were calculated for the ultrasonic velocity within the lower concentration range (0.45-2.5 mg g^{-1}, Fig. 6.2.) and the higher concentration range (3.75-20 mg g^{-1}, Fig. 6.3.). The linear relation between ultrasonic velocity and concentration had R^2 values closer to 1 in the

higher concentration range, with values of 0.9998, 1.0000 and 0.9998 for A, C, and D peptides, respectively. This is somewhat higher than the lower concentration range with R^2 values of 0.9980, 0.9977 and 0.9919 for A, C and D peptides, respectively; however, all R^2 values were very good. The slight decrease in R^2 values can be caused by a diminution in precision of the ultrasonic velocity measurements due to a limitation of equipment precision and experimental errors associated with very low concentration solutions.

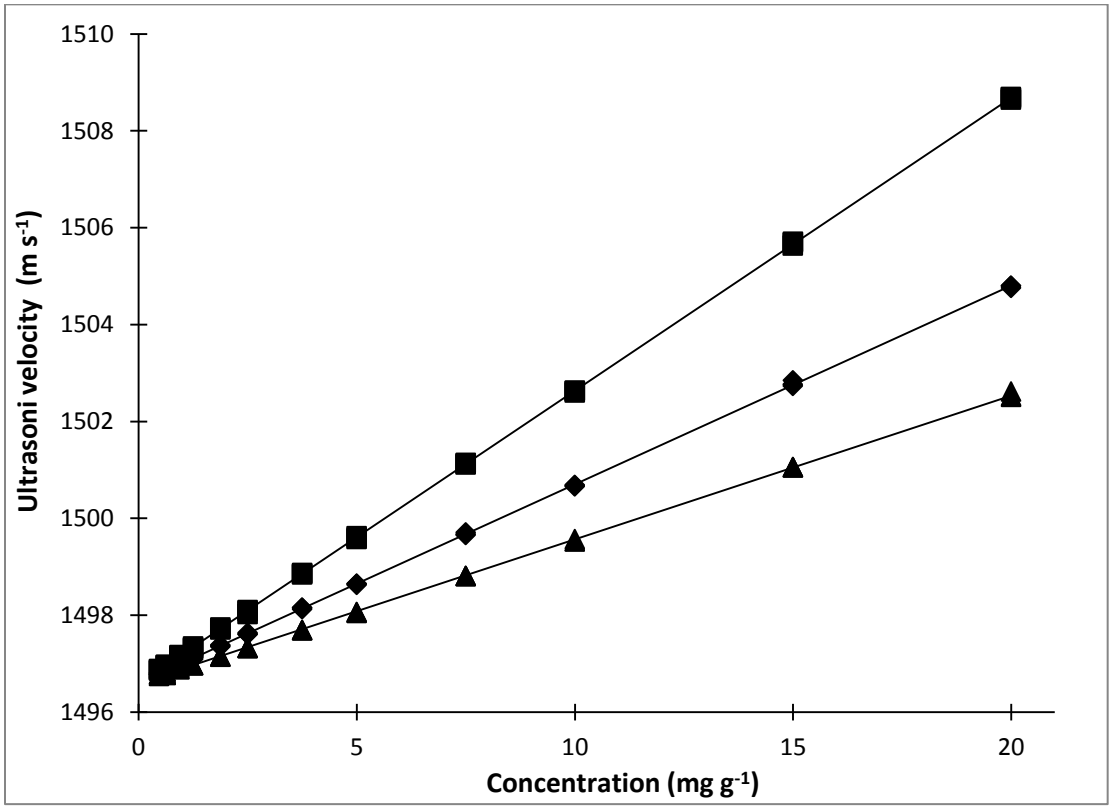

Fig. 6.1. Ultrasonic velocity of dairy peptide serial dilutions, A (◆), C (■), D (▲).

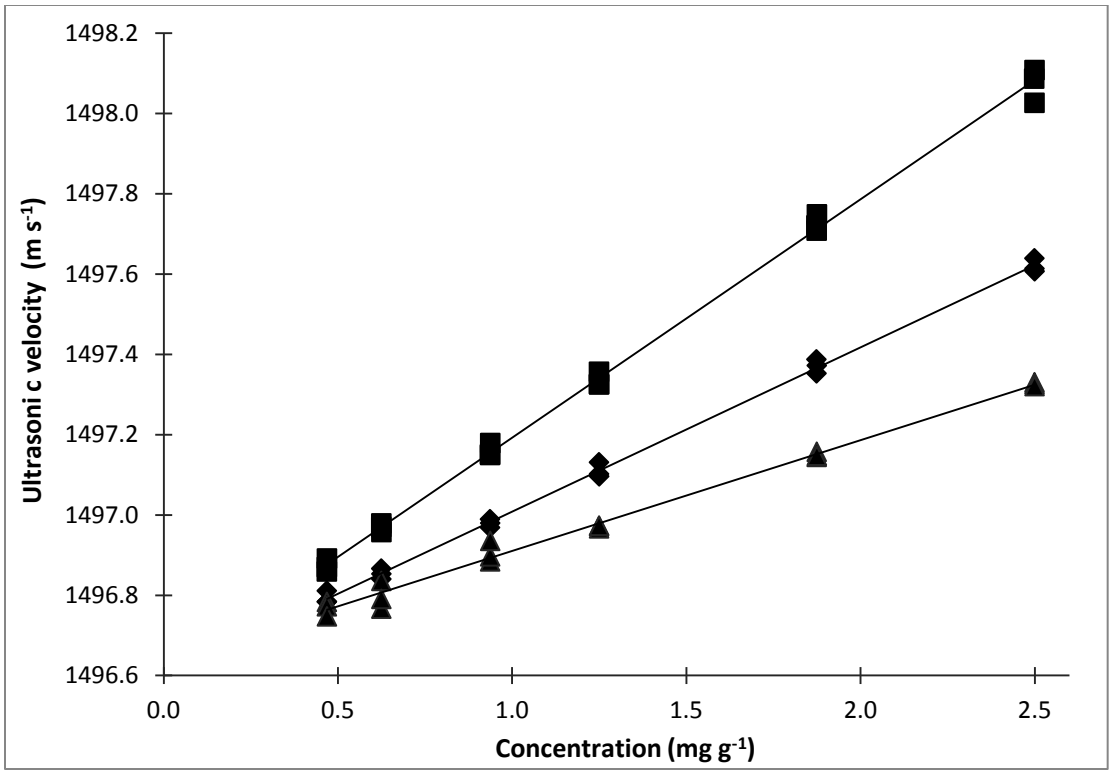

Fig. 6.2. Ultrasonic velocity of dairy peptide serial dilutions at low concentrations, A (◆), C (■), D (▲).

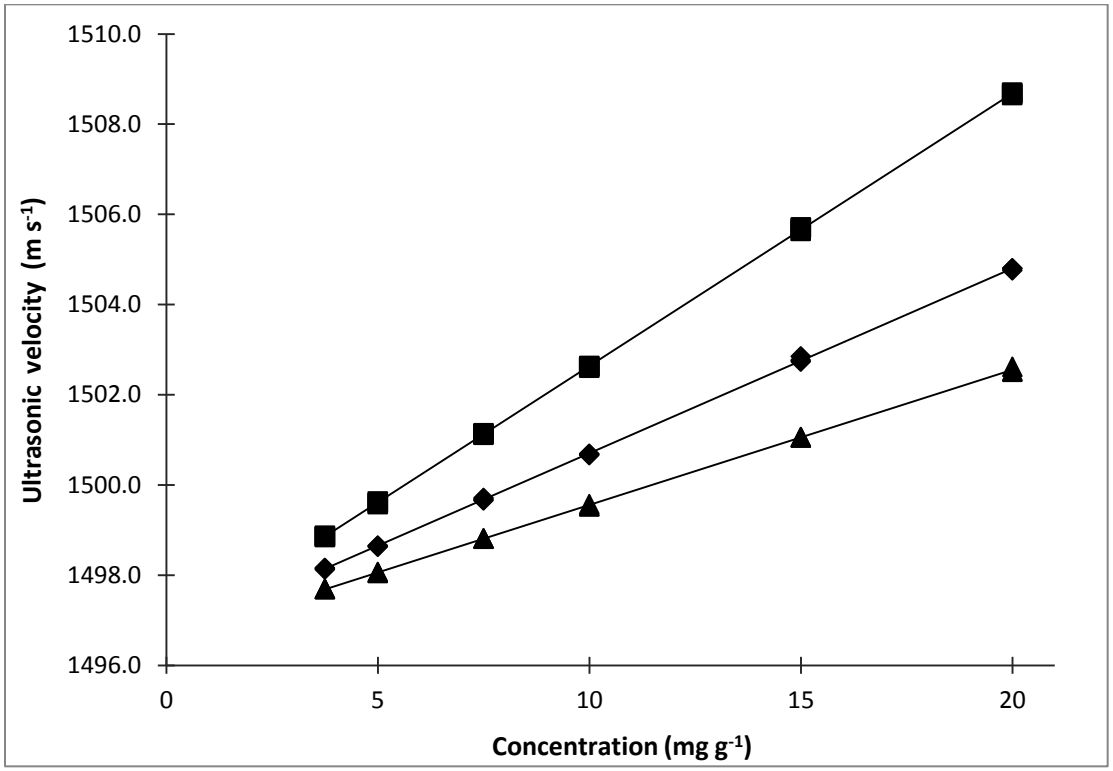

Fig. 6.3. Ultrasonic velocity of dairy peptide serial dilutions at high concentrations, A (◆), C (■), D (▲).

The coefficient of variation (CV) was calculated for the ultrasonic velocity from serial dilutions of all dairy peptides. The CV was calculated for the three subsamples and the three replicates measured. Subsample CV (Fig. 6.4.) values were low for all dairy peptides, with values of $< 0.0025\%$, $< 0.0026\%$ and $< 0.0014\%$, for A, C and D, respectively.

Replicate CV values were also low for the dairy peptides, with values of $< 0.004\%$, $< 0.003\%$ and $< 0.004\%$, for A, C and D, respectively. Nevertheless, as explained for the hemp seed peptides in section 5.1., according to the procedure for preparing the serial dilutions gravimetrically, the exact concentration of each solution was slightly different between the three replicates; therefore, the CV measured for the "replicates" was overestimated.

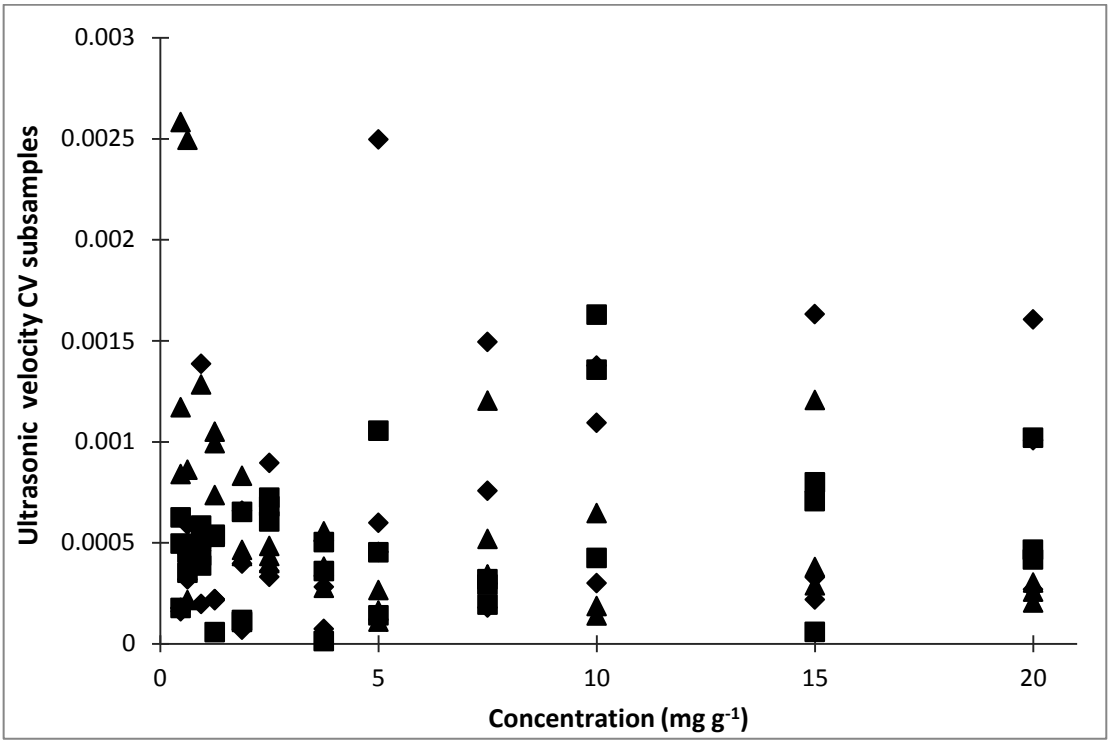

Fig. 6.4. CV for subsamples of ultrasonic velocity of dairy peptide serial dilutions. A (◆), C (■), D (▲).

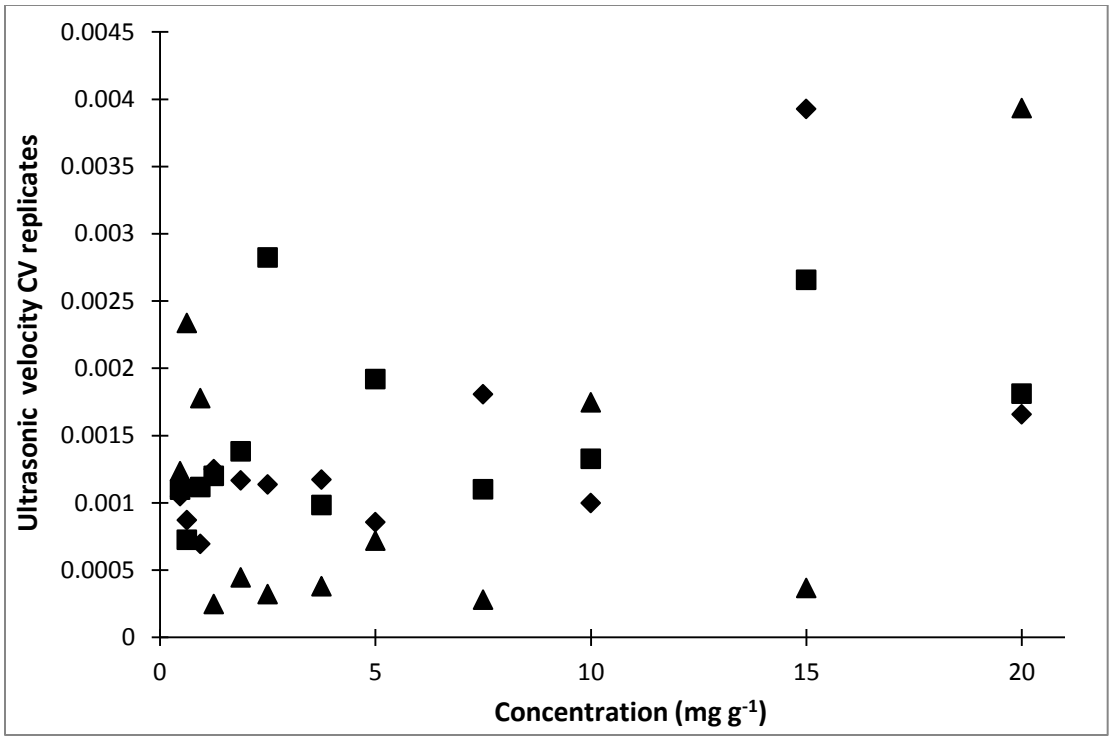

Fig. 6.5. CV for replicates of ultrasonic velocity of dairy peptide serial dilutions. A (◆), C (■), D (▲).

Ultrasonic attenuation of serial dilutions of all dairy peptides A, C and D, were found to have a more or less linear relationship against concentration (Fig. 6.6.). However, at low concentrations the ultrasonic attenuation values presented a higher variation.

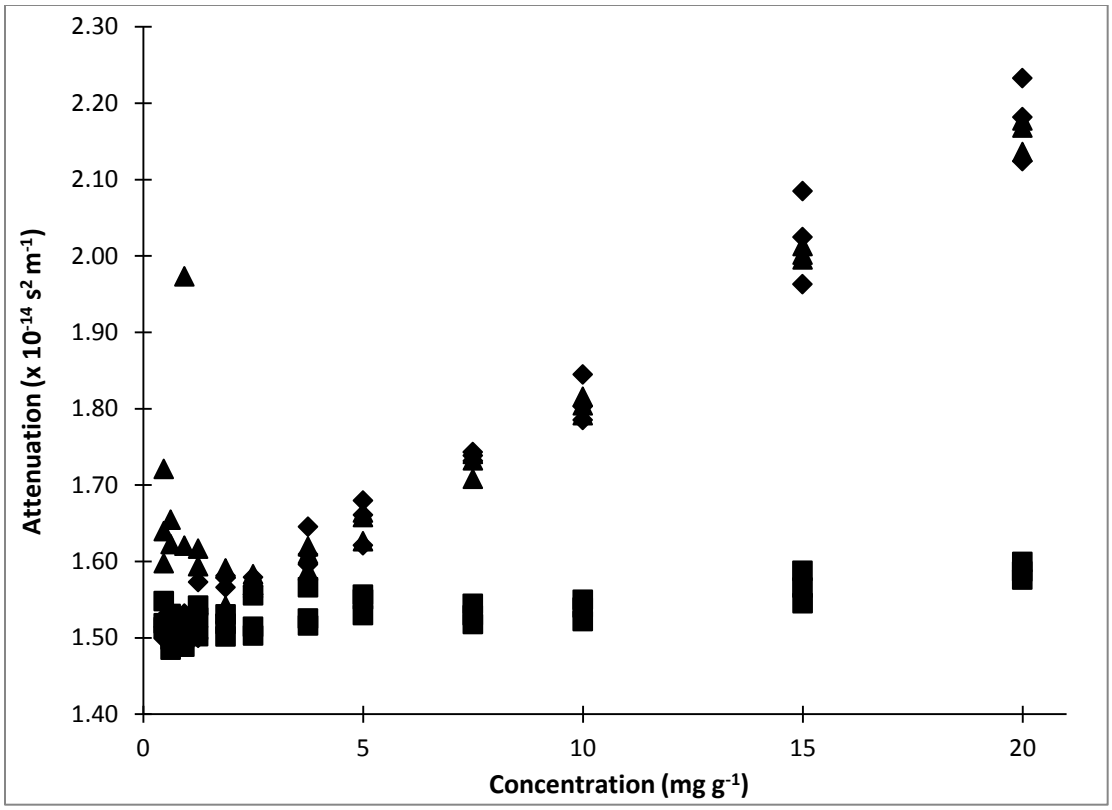

Fig. 6.6. Ultrasonic attenuation of dairy peptide serial dilutions, A (◆), C (■), D (▲).

6.2. Density Results

Density (ρ) of the dairy peptide serial dilutions was found to have a linear relationship against concentration (Fig. 6.7.).

The R^2 values for the linear regression of the density of all dairy peptides, A, C and D, were very good, 0.9999, 0.9997 and 0.9998, respectively. As was done with ultrasonic velocity measurements, in order to confirm the linear relation over all concentration range of the serial dilutions, linear regressions were calculated for density over the lower concentration range (0.45-2.5 mg g^{-1}, Fig. 6.8.) and the higher concentration range (3.75-20 mg g^{-1}, Fig. 6.9.). The linear relation between density and concentration had R^2 values closer to 1 about the higher concentration range, with values of 0.9997, 0.9997 and 0.9999 for dairy peptide A, C and D,

respectively, compared to the lower concentration range with R^2 values of 0.9969, 0.9830 and 0.9846 for dairy peptide A, C and D, respectively. The diminution in the linearity of density values for the lower concentration range was more evident than for the ultrasonic velocity results, although the R^2 values were still good. This increase in the variation of volumetric determination as the concentration diminishes has been also reported in ionic solution by Marcus (2006). The slight decrease in R^2 values can be caused by a diminution in precision of the density measurements due to limitations of equipment precision and experimental errors associated with working with very low concentration solutions.

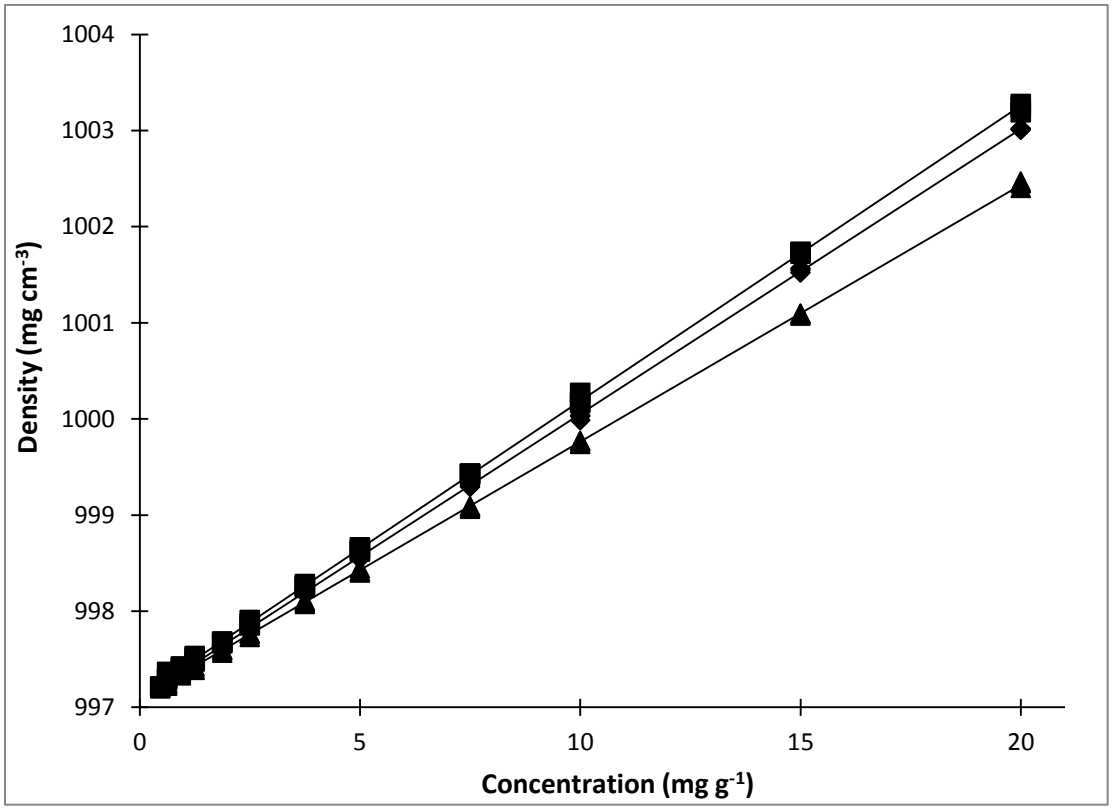

Fig. 6.7. Density of dairy peptide serial dilutions, A (◆), C (■), D (▲).

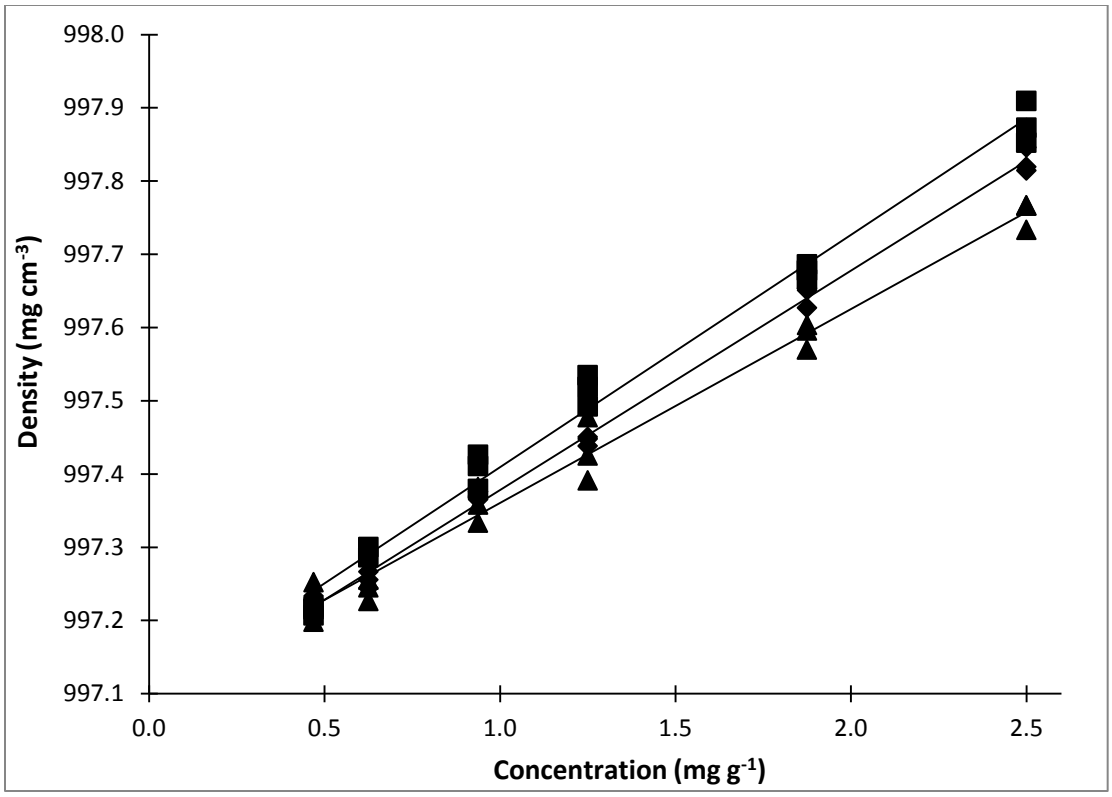

Fig. 6.8. Density of dairy peptide serial dilutions at low concentrations, A (◆), C (■), D (▲).

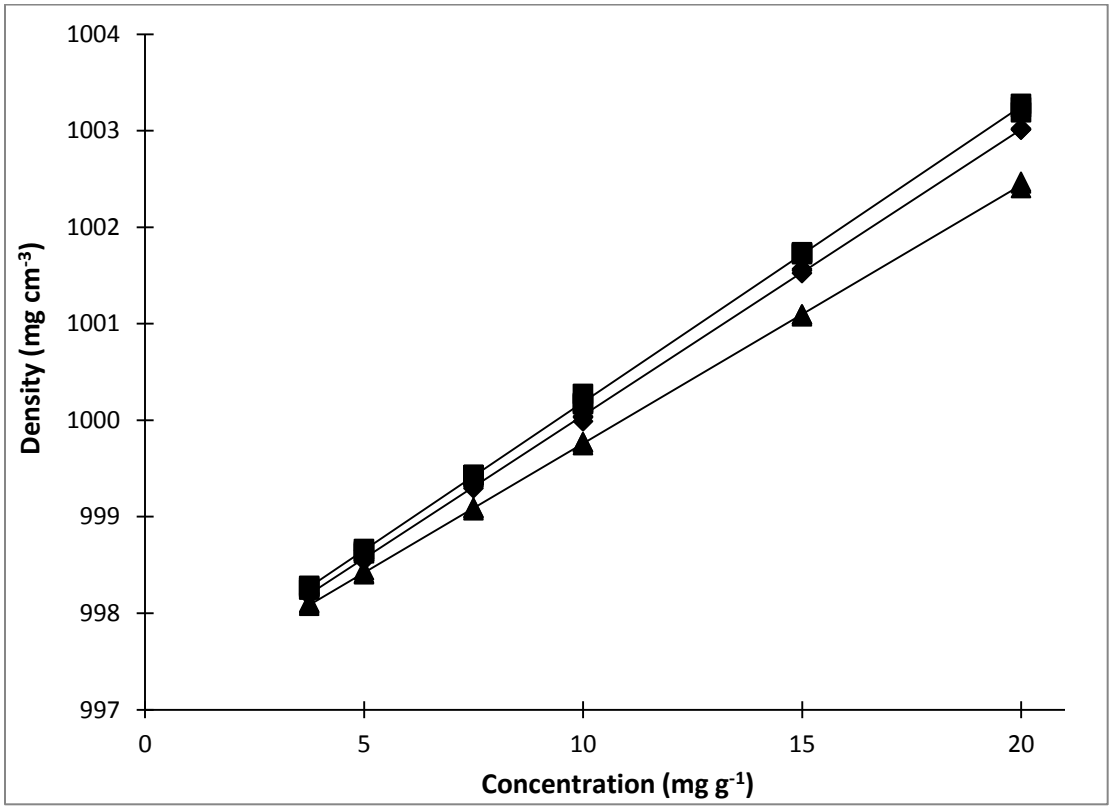

Fig. 6.9. Density of dairy peptide serial dilutions at high concentrations, A (◆), C (■), D (▲).

Serial dilutions were made using ultrapure water for two initial stock solutions at different concentration, 20 mg g^{-1} and 15 mg g^{-1}. Three replicates and three subsamples were used for the analysis; however, as with ultrasonic analyses, due to the preparation being done gravimetrically and the exact weight of the solute and water added being recorded, the concentration of the replicates was slightly different.

The coefficient of variation (CV) was calculated for density results from serial dilutions of all dairy peptides. The CV was calculated between the three subsamples and three replicates. Subsample CV (Fig 6.10.) was generally low for all dairy peptides with values $<$ 0.0064%, $<$ 0.0052% and $<$ 0.004%, respectively, for peptides A, C and D, but with most of the CV values for dairy peptides A and D being lower than 0.004%.

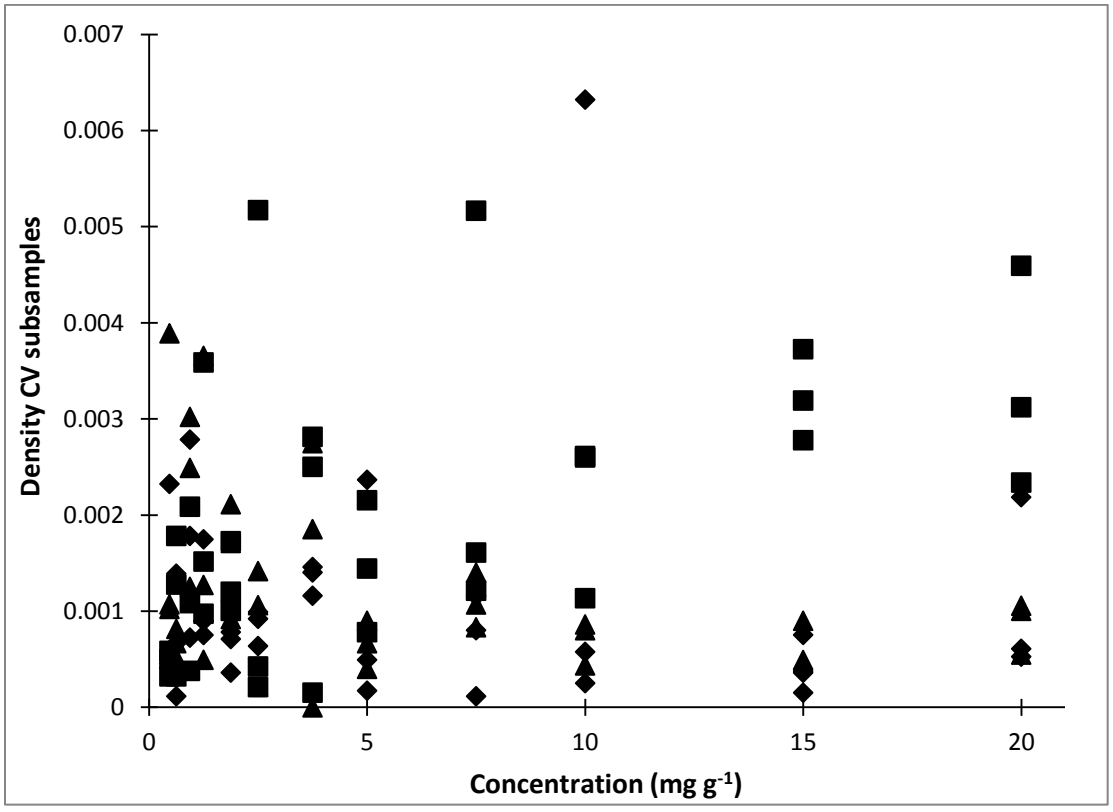

Fig. 6.10. CV of density for subsamples of dairy peptide serial dilutions, A (◆), C (■), D (▲).

CV for replicates (Fig 6.11.) was low for all dairy peptides, with values $<$ 0.006% for A and C, and $<$ 0.0045% for the dairy peptide D. Nevertheless, like in the ultrasonic velocity analyses, the procedure for preparation of the serial dilution was gravimetrically, so that there are slight differences between the concentrations of the three replicates; therefore, the CV measured for the "replicates " is likely an overestimation.

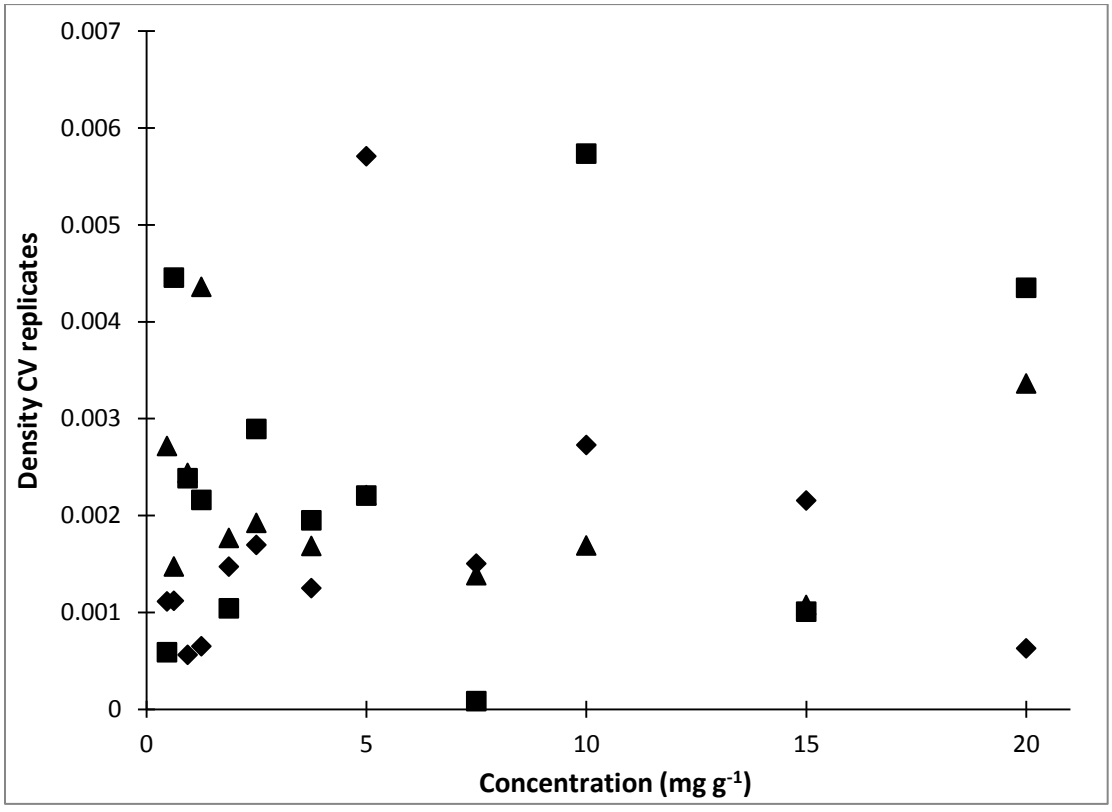

Fig. 6.11. CV of density for replicates of dairy peptide serial dilutions, A (◆), C (■), D **(▲).**

6.3. Specific Volume

The apparent specific volume, $\bar{V}$, of the dairy peptide serial dilutions was calculated from the density measurements of the dairy peptides as $\bar{V} = \frac{1-(\rho-c)/\rho_0}{c}$ (Fig. 6.12.)

The partial specific volume, $\bar{V}^0$, was calculated by the extrapolation of the apparent specific volume to zero concentration as $\bar{V}^0 = \lim_{c\to 0} \bar{V}$. In order to approach as much as possible to the correct value, the extrapolation to zero was done just using the two smallest concentration values (as conducted for hemp seed peptides, Chapter 5). As with those peptides, it is possible that these values for the dairy peptides are overestimated. The partial specific volume values are shown in Table 6.1.

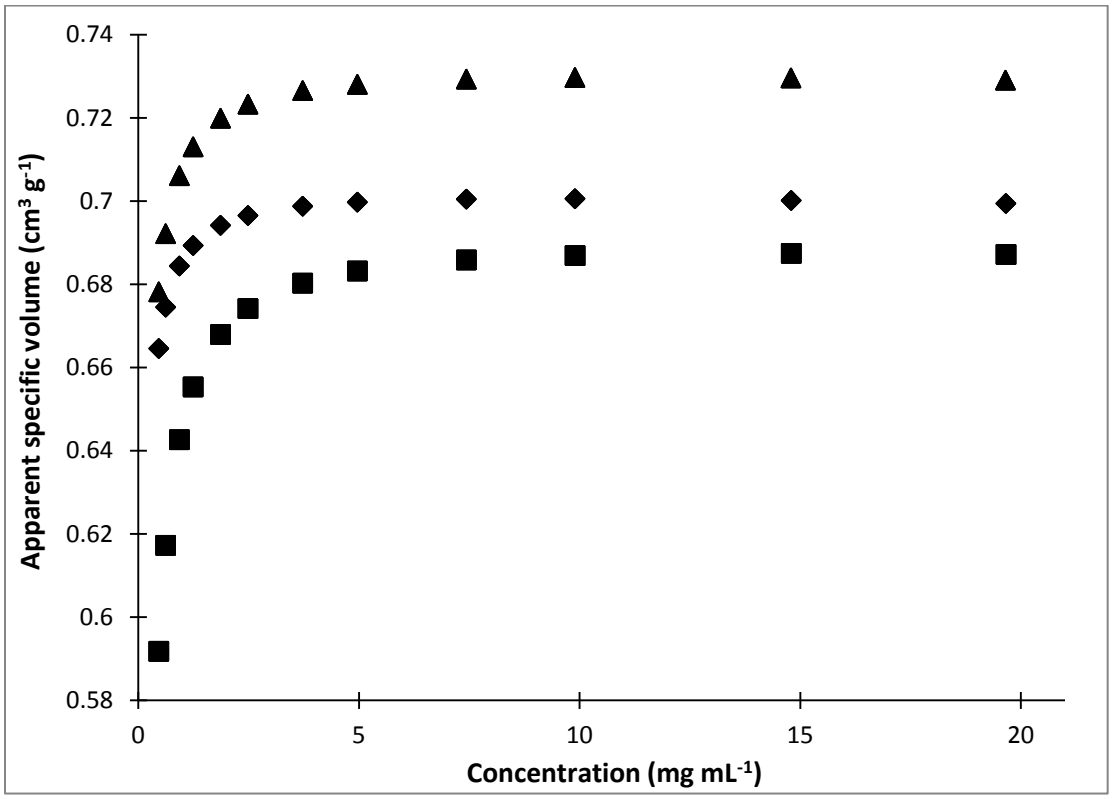

Fig. 6.12. Apparent specific volume of dairy peptides, A, C and D , A (◆), C (■), D (▲).

Table 6.1. Partial specific volume of dairy peptides.

	$\bar{V}^0$ (cm^3 g^{-1})
Dairy peptide A	0.635
Dairy peptide C	0.515
Dairy peptide D	0.636

6.4. Compressibility

The adiabatic compressibility coefficient, β_s, was calculated according to the Newton-Laplace equation $\beta_s = \frac{1}{\rho u^2}$, where β_s is calculated as a function of the density and the ultrasonic velocity. The relative adiabatic compressibility coefficient, β_s/β_{s0}, which expresses the relation between the adiabatic compressibility coefficient of the solution, β_s, against the adiabatic compressibility coefficient of the solvent β_{so}, ultra pure water (UPW) for this study, had a linear relationship against concentration.

The apparent adiabatic specific compressibility, $\overline{K}_s$, which express the change in the apparent specific volume as a function of a change of pressure, was calculated as $\overline{K}_s = \beta_{so} \frac{\beta_s/\beta_{so} - (\rho - c)/\rho_0}{c}$ (Fig. 6.13.).

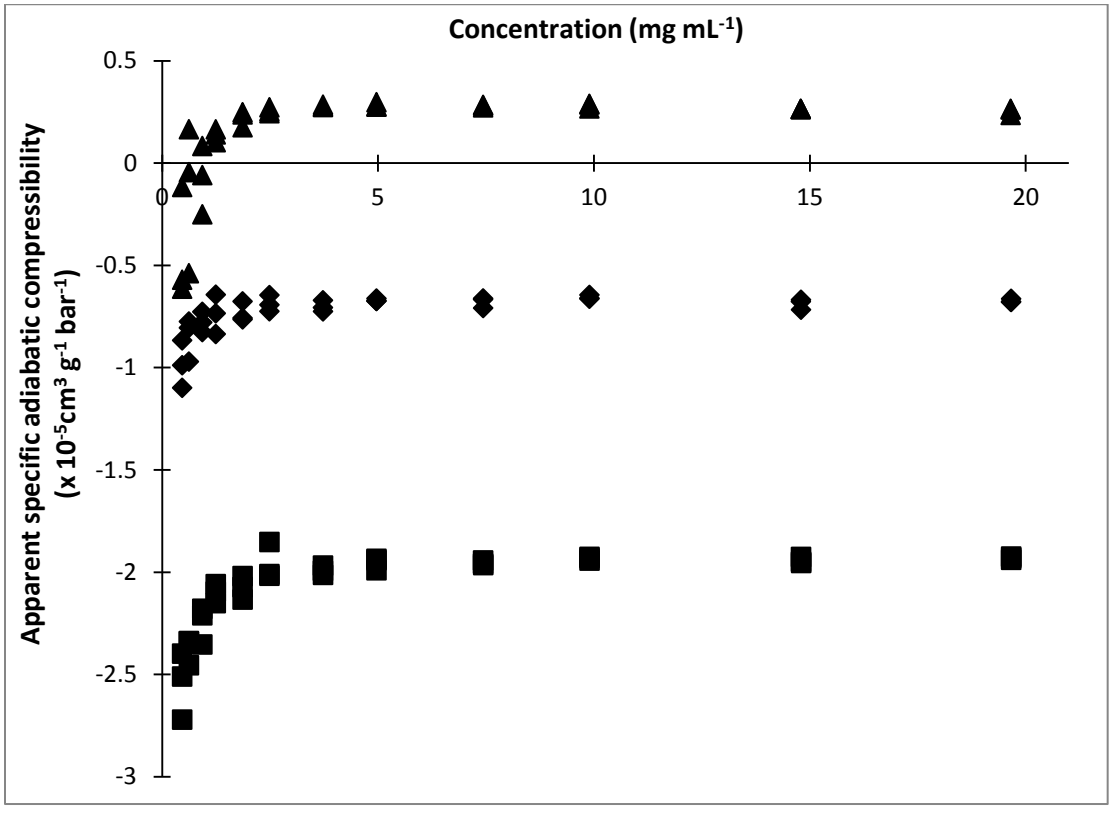

Fig. 6.13. Apparent adiabatic specific compressibility of dairy peptide, A, C and D , A (◆), C (■), D (▲).

The partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^0$, which describes the relation between the partial specific adiabatic compressibility, $\bar{K}_S^0$, and partial specific volume, $\bar{V}^0$, was calculated as $\bar{\beta}_S^0 = \frac{\bar{K}_S^0}{\bar{V}^0} = \frac{\beta_{so}}{\bar{V}^o} \lim_{c\to 0} \frac{\beta_s/\beta_{so} - (\rho - c)/\rho_0}{c}$, where the term $\frac{\beta_s/\beta_{so} - (\rho - c)/\rho_0}{c}$ is extrapolated to zero while β_{s0} and $\bar{V}^0$ are constant values. In order to approach as much as possible to the correct value for $\bar{\beta}_S^0$, the extrapolation to zero was done using just the two smallest concentration values, although it is likely that even this value is overestimated. The partial specific adiabatic compressibility coefficient $\bar{\beta}_S^0$ values are shown in Table 6.2.

Table 6.2. Partial specific adiabatic compressibility coefficient of dairy peptides.

	$\bar{\boldsymbol{\beta}}_{\boldsymbol{S}}^{\boldsymbol{0}}$ (x10^{-6} bar^{-1})
Dairy peptide A	-21.9
Dairy peptide C	-59.0
Dairy peptide D	-20.7

6.5. Discussion

The dairy bioactive peptide A, C and D serial dilutions analysed in this study showed a linear relationship between density and concentration and between ultrasonic velocity and concentration. According to Pavlovskaya et al. (1992) it is possible to treat them as ideal solutions. However as pointed out by Murphy et al. (1998) and Sirotkin et al. (2012) protein solutions cannot be considered ideal because of the dynamics of interactions involved between water molecules and the protein structure; the volumetric properties of these solutions can help to measure the changes of the solute-solvent interactions.

Ultrasonic attenuation of the three dairy peptide serial dilutions had a more or less linear relationship against concentration. However the variation of the ultrasonic attenuation values increased at low concentration, especially of dairy peptide D, which was seen to have an increase of attenuation as the solution was diluted below 2.5 mg g^{-1}. Additionally the attenuation values for dairy peptide C presented just a slight change over the concentration range used, which could be due to the limitation of the attenuation measurements with very small particles (McClements, 1991). The ultrasonic attenuation values can be used to analyse the particle size in suspension as well as the aggregation phenomena, because changes in the organization of the particles affect the way the ultrasound is dissipated (McClements, 1991; Nelson et al., 2001; Mougin et al., 2003; Corredig et al., 2004; Yuno-Ohta & Corredig, 2007; Povey et al., 2011; Wrobel & Time, 2012). The ultrasound attenuation is dependent on the frequency of the ultrasound wave, and on the concentration and the particle size (Mougin et al, 2003). Generally ultrasonic attenuation can be divided into the ultrasound attenuation caused by molecular or chemical relaxation, and the attenuation caused by scattering of ultrasonic waves (Nelson et al., 2001; Mougin et al., 2003; Corredig et al., 2004; Yuno-Ohta & Corredig, 2007; Povey et al., 2011). Ultrasound attenuation is affected by the particle size and any conformation transitions, increasing its value as the particle size is bigger (Bryant & McClements, 1999; Corredig et al., 2004; Povey et al., 2011). The linear decrease of the ultrasonic attenuation of the three dairy peptides as the solutions were diluted might suggest that no aggregation process, which would change the ultrasonic attenuation trend, occurred. Nevertheless, the change in the tendency of dairy peptide D, might indicate certain conformation change in the peptide at concentrations below 2.5 mg g^{-1}, but the variation in the values at these lower concentrations did not permit certainty. In addition, the difference in the

attenuation values between the three diary peptides, might suggest dairy peptides A and D have a similar size while dairy peptide C has a smaller size.

The apparent specific volume of the three dairy peptides A, C and D (Fig. 6.12.), was seen to be independent of concentration in the high concentration range (7.5 – 20 mg mL^{-1}), with just slight changes. However, starting at 5 mg mL^{-1}, a continuous decrease in apparent specific volume was seen, the total decrease between the highest and the lowest concentration solution was about 0.035 cm^3 g^{-1} 0.095 cm^3 g^{-1} 0.050 cm^3 g^{-1} for dairy peptide A, C and D, respectively. The dairy peptides used were dialyzed with a final protein content of about 90% (Table 3.1.), but containing small amounts of salts and sugars "bound" to the peptides. According to Vegarud et al. (2000), the mineral content of milk is present in equilibrium between free ions and complexes with different milk components like proteins, carbohydrates and lipids. Moreover, different whey proteins, like β-lactoglobulin, α-lactalbumin and lactoferrin, and peptides derived from these proteins, have been found to possess mineral binding properties (Vegarud et al., 2000; Thompson et al., 2009), and also the ability to bind small hydrophobic molecules (Creamer & MacGibbon, 1996). Finally, conformational changes in lactoferrin have been reported that are related to the binding and release of iron ions (Chung & Raymond, 1993). The dissociation of these salt complexes has been found to be related with pH and concentration of the solutions (Vegarud et al., 2000; Dalgleish et al., 2005). Therefore, a dissociation of the salt complexes between peptides and salts and sugars as a consequence of the dilution of the dairy peptides solutions to very low concentrations, could lead to an increase in the electrostriction effect due to the increase of water molecules in the water shell surrounding the dissociated salts and peptides.

A second effect might also be involved, this one related with dissociation of the salts that were released from the peptide complexes as the solutions were diluted; this effect is similar to that commented on in section 4.5. and section 5.5. for the KCl serial dilutions and for the 1 kDa hemp seed peptide serial dilutions, in which as the solutions were diluted the solute-solute interactions diminished promoting the dissociation of salt molecules (Marcus, 2005; Marcus, 2006).

A third effect related with the dissociation of peptide-salt complexes might also contribute to the decrease of apparent specific volume. The dissociation of peptide-salt complexes could allow side chains of the amino acids within the peptide structure, where salts had been bound, to interact with water molecules; in addition this dissociation could lead to certain conformational changes in the peptide as has been reported for the lactoferrin protein (Chung & Raymond, 1993).

In summary, the three effects above could lead to an increase in the electrostriction effect and as a consequence a decrease in the apparent specific volume. As was commented in chapters 4 and 5, the electrostriction effect is the contraction in the volume of molecules exposed to an electric field and in solutions can be caused by solute-solvent interactions (Desnoyers et al., 1965; Marcus, 2011). This decrease in the apparent specific volume of protein solutions caused by solvation of salts or sugars has been reported by Durchschlag & Jaenicke (1982). In addition, Bernhardt and Pauly (1977) have also reported on the electrostriction effect causing changes in apparent specific volume as a result of addition of salts to protein solutions. The difference between water molecules involved in the hydration shell and water molecules in the bulk water have been already reported in several papers (Apenten et al., 2000; Chalikian 2003; Chalikian & Filfil, 2003; Bano & Marek 2006; Pfeiffer et al., 2008). Finally, possible slight

changes in the pH of the solution as it was diluted could also affect the apparent specific volume (Bernhardt & Pauly, 1975; Sarvazyan et al., 1979; Chalikian et al., 1995; Vogl et al., 1995).

The partial specific volume of all dairy peptides (Table 6.1.) was calculated by extrapolation to zero of the apparent specific volume with values of 0.635 $cm^3\ g^{-1}$, 0.515 $cm^3\ g^{-1}$ and 0.636 $cm^3\ g^{-1}$ for dairy peptides A, C and D, respectively. However, as explained previously due to the curvature seen in apparent specific volume for all dairy peptides the values could be overestimated. It is important to point out that the values obtained correspond to a mixture of dairy peptides and others components like salts, lactose and lipids that were also present (Table 3.1.). Comparing the values between the three peptides it is possible to see that dairy peptide C showed the lowest partial specific volume, and this could be related to its amino acids sequence. In this case, probably more polar amino acids are exposed to the water molecules, an outcome that also is compatible with the solubility observed for the dairy peptide C which was the most soluble of the three dairy peptides. According to Sirotkin et al. (2012) the hydration of polar amino acids causes a reduction in the specific volume while exposed hydrophobic groups are usually associated with small negative to positive changes in the specific volume. Therefore the dairy peptides D and A, which present similar partial specific volume, should have a similar proportion of polar and hydrophobic groups exposed to the water at infinite dilution. Nevertheless, at higher concentration the difference between the apparent specific volume of peptide A and D was clearly higher (Fig. 6.12.). It is important to mention that the difference in the amino acids content between the three dairy peptides might also affect the amount of salt-peptide complexes formed and dissociated in each dairy peptide, and hence influence the effect associated with these complexes on the partial specific volume. Chalikian et al. (1994) obtained apparent specific values for two amino acids, glycine and alanine, at 25°C with values of 0.578 $cm^3\ g^{-1}$ and 0.679 $cm^3\ g^{-1}$, observing no concentration dependence on the specific volume.

Nevertheless, the influence of the type of amino acids on the partial specific volume, especially of small peptides was seen by Schwitzer & Hedwig (2005) who calculated the partial specific volume of two tripeptides (Gly-Asp-Gly and Gly-Glu-Gly) obtaining values of 0.509 $cm^3\ g^{-1}$ and 0.537 $cm^3\ g^{-1}$. They also found dissociation of the glutamic acid affected the partial specific volume of one of the tripeptides. Hedwig (1994) and Chalikian et al. (1998) have reported the partial specific volume of various glycine tripeptides (Gly-X-Gly) in the ranges of 0.497 $cm^3\ g^{-1}$ to 0.630 $cm^3\ g^{-1}$, and 0.567 $cm^3\ g^{-1}$ to 689 $cm^3\ g^{-1}$, respectively. The large difference in the partial specific volume between the two amino acids and between various tripeptides, which are strong electrolytes, shows the important influence of the amino acid content and charged groups on the partial specific volume, complicating a direct comparison of experimental values with those in the literature. Compared with the literature, the three dairy peptides had lower values than the partial specific volume values obtained for most globular proteins, that at 25°C are in the range between 0.70 $cm^3\ g^{-1}$ and 0.75 $cm^3\ g^{-1}$ (Bernhardt & Pauly, 1975; Bull & Bresse, 1979; Durchschlag & Jaenicke, 1982; Gekko & Hasegawa, 1986; Chalikian et al., 1996; Sitorkin et al., 2012). However, the apparent specific volume values, at the highest concentration (20 mg g^{-1}) for the three dairy peptides (0.699 $cm^3\ g^{-1}$, 0.687 $cm^3\ g^{-1}$, 0.729 $cm^3\ g^{-1}$ respectively for A, C and D) were closer to values reported for globular proteins.

The apparent specific adiabatic compressibility was different between the three dairy peptides. Dairy peptide D was the only one that had a positive compressibility which suggests that it has the higher amount of hydrophobic amino acids that would cause a less extended structure in solution and hence more void spaces and a more compressible interior. According to Gekko & Hasegawa (1986), positive compressibility values indicate a highly compressible interior, and negative compressibility values indicate an incompressible interior, like in fibrous proteins and amino acids, where the compressibility values are driven primarily by the negative

compressibility contribution of the hydration of the solute caused by solute-solvent interactions (Kharakoz & Sarvazyan, 1993; Apenten et al., 2000). However, the apparent specific compressibility of dairy peptide D becomes negative as the concentration decreased. The apparent specific adiabatic compressibility was seen to have negative values for dairy peptides A and C, suggesting these two peptides have a higher amount of hydrophilic amino acids exposed to the water and an incompressible interior. The compressibility results were compatible with the solubility seen for these two peptides, since the dairy peptide C was the one with the highest solubility, while dairy peptide A was slightly less soluble than dairy peptide C but clearly more soluble than dairy peptide D. Hydrophobic amino acids (non-polar) can cause an imperfect packing and a highly compressible interior, and also the hydration contribution decreased when they are exposed to water, while polar amino acids cause a negative hydration contribution to the compressibility (Gekko & Hasegawa, 1986; Chalikian et al., 1996). Therefore, the differences in compressibility suggest differences in amino acid sequence and conformation.

The apparent specific adiabatic compressibility of the three dairy peptides A, C and D, was independent of the peptide concentration in the higher concentration range 5 – 20 mg mL^{-1}. However at a concentration between about 1.875 and 2.5 mg mL^{-1} a decrease of apparent specific adiabatic compressibility started, becoming more pronounced as concentration diminished. The total decrease between the highest and the lowest concentration was about 3.1, 6.1, and 6.9 x 10^{-6} cm^3 g^{-1} bar for dairy peptides A, C and D. The apparent specific adiabatic compressibility of all dairy peptides was seen to follow a similar trend as was seen with the apparent specific volume. The compressibility of proteins and peptides is related with the hydration of the molecules and therefore electrostriction effects should be considered (Pfeiffer et al., 2008; Marcus, 2011). Because peptides have more extended structures than proteins, due to peptides being generally comprised of primary and secondary conformations (such as α-helix

and β-sheets) (Creighton, 1993), they tend to have less void interior spaces than proteins. According to Gekko et al. (2004), compressibility of the void spaces contributes a positive value while the compressibility of the water molecules around the surface contributes a negative value to the partial specific adiabatic compressibility. Therefore, because of the importance of the hydration contribution to compressibility in peptides, the reduction in the specific compressibility might be caused, as in the apparent specific volume, by an increase of the electrostriction effect caused by the three effects described for the apparent specific volume: 1) the presence of peptide-salt complexes (Vegarud et al., 2000) and the dissociation of them as the solution was diluted that caused an increase in the number of water molecules around the dissociated salts which are affected by the electrostriction effect (Afanas'ev & Tyunina, 2001); 2) dissociation of the salt molecules released from the peptides to the solution caused by a decrease of the solute-solute interactions as the solution was diluted (Marcus 2005; Marcus, 2006); 3) the dissociation of peptide-salt complexes could allow side chains of amino acids within the peptide structure, where salts had been bound, to interact with water molecules, as explained previously. In addition, possible conformational changes driven by the release of salts have been reported with the lactoferrin protein (Chung & Raymond, 1993). Therefore, the partial specific adiabatic compressibility is mainly influenced by properties of the water in shell around the solutes as it happened with the apparent specific volume.

Gekko & Hasegawa (1986), Kharakoz & Sarvazyan (1993) and Chalikian et al. (1996) obtained the partial specific adiabatic compressibility coefficient of several globular proteins, which were all positive and in a range from about 1 $x10^{-6}$ bar^{-1} to 11 $x10^{-6}$ bar^{-1}, which according to Gekko & Hasegawa (1986) indicates that these proteins have a highly compressible interior. Pfeiffer et al. (2008) calculated the apparent specific compressibility coefficient of several globular proteins, obtaining positive values between 3 $x10^{-6}$ bar^{-1} and 9 $x10^{-6}$ bar^{-1}, and

obtaining negative values for sugars (glucose -15 $x10^{-6}$ bar^{-1} , sucrose -5.3 $x10^{-6}$ bar^{-1} and raffinose -18.9 $x10^{-6}$ bar^{-1}) and amino acids (glycine -61.2 $x10^{-6}$ bar^{-1}). Kharakoz (1991) and Chalikian et al. (1992) calculated the partial specific adiabatic compressibility of several amino acids, obtaining negative values in a range between -23 $x10^{-6}$ bar^{-1} and -33 $x10^{-6}$ bar^{-1}. Finally, Chalikian et al. (1998) reported the partial specific adiabatic compressibility coefficient for tripeptides which were in a range of -1.7 $x10^{-6}$ bar^{-1} and -6.69 $x10^{-6}$ bar^{-1}, with negative values where the compressibility values are driven primarily by the negative compressibility contribution of the hydration of the solute caused by solute-solvent interactions (Kharakoz & Sarvazyan, 1993; Apenten et al., 2000). Therefore, the partial specific compressibility of the three dairy peptides, at values of A (- 31.9 $x10^{-6}$ bar^{-1}), C (-59.0 $x10^{-6}$ bar^{-1}) and D (-20.7 $x10^{-6}$ bar^{-1}), were below the values of small peptides and about the values of amino acids in solution. Since the, values are, far from the values for globular proteins, I conclude that at infinite dilution conditions, for the three dairy peptides, intrinsic compressibility is small values and the values of the partial specific adiabatic compressibility are driven mainly by the hydration contribution. Although it is important to remember that the partial specific adiabatic compressibility calculated in this study corresponds to a solution of the dairy peptide A, C and D and the dissociated salts and some lactose.

7. Liposomes Results and Discussion

Phosphatidylcholine (PC) liposomes were prepared as model membranes with refined lecithin dispersed in ultrapure water as explained in section 3.2.5. Volumetric properties of liposomes prepared in ultrapure water were characterized by determination of the apparent specific volume and partial specific volume as well as the apparent specific adiabatic compressibility and partial specific adiabatic compressibility based on high precision density and ultrasonic velocity measurements of liposome serial dilutions at 24.985°C. Multilamellar vesicles (MLV) were produced by dispersion of phosphatidylcholine in ultrapure water and further extrusion was performed to create unilamellar vesicles (UV). UV and MLV serial dilutions were prepared as explained in section 3.2.6.

7.1. Particle Size Results

Particle size measurements were applied to suspensions of UV and MLV liposomes to know the particle size and the distribution in order to understand the efficiency of the extrusion method explained in section 3.2.5. in producing homogeneous suspensions of unilamellar vesicles from a multilamellar vesicle suspension. There are several studies which have probed the convenience of the extrusion technique in order to create homogenous suspensions of unilamellar vesicles (Hope et al., 1985; Ramaswami et al., 1992; Hianik et al., 1997; Romanowski et al., 2002; Rybar et al. 2007; Almeida & Pokorny, 2010).

The particle size measured by mean diameter of the UV was 128 nm and 270 nm for MLV (Table 7.1.), confirming the important reduction in size achieved by the extrusion method.

Table 7.1. Liposome particle size.

Replicate	Particle size Mean diameter (nm)	
	MLV	**UV**
R1	279	125
R2	281	131
R3	249	135
Mean	270	128
STD	17.9	4.2
CV	6.7	3.3

The particle size distribution for the MLV (Fig. 7.1.) was seen to be more variable and heterogeneous with a wider particle size range distribution. The particle size distribution for UV liposomes (Fig. 7.2.) was seen to follow a normal distribution as expected, according to MacDonald et al. (1991), who worked with the same kind of extruder.

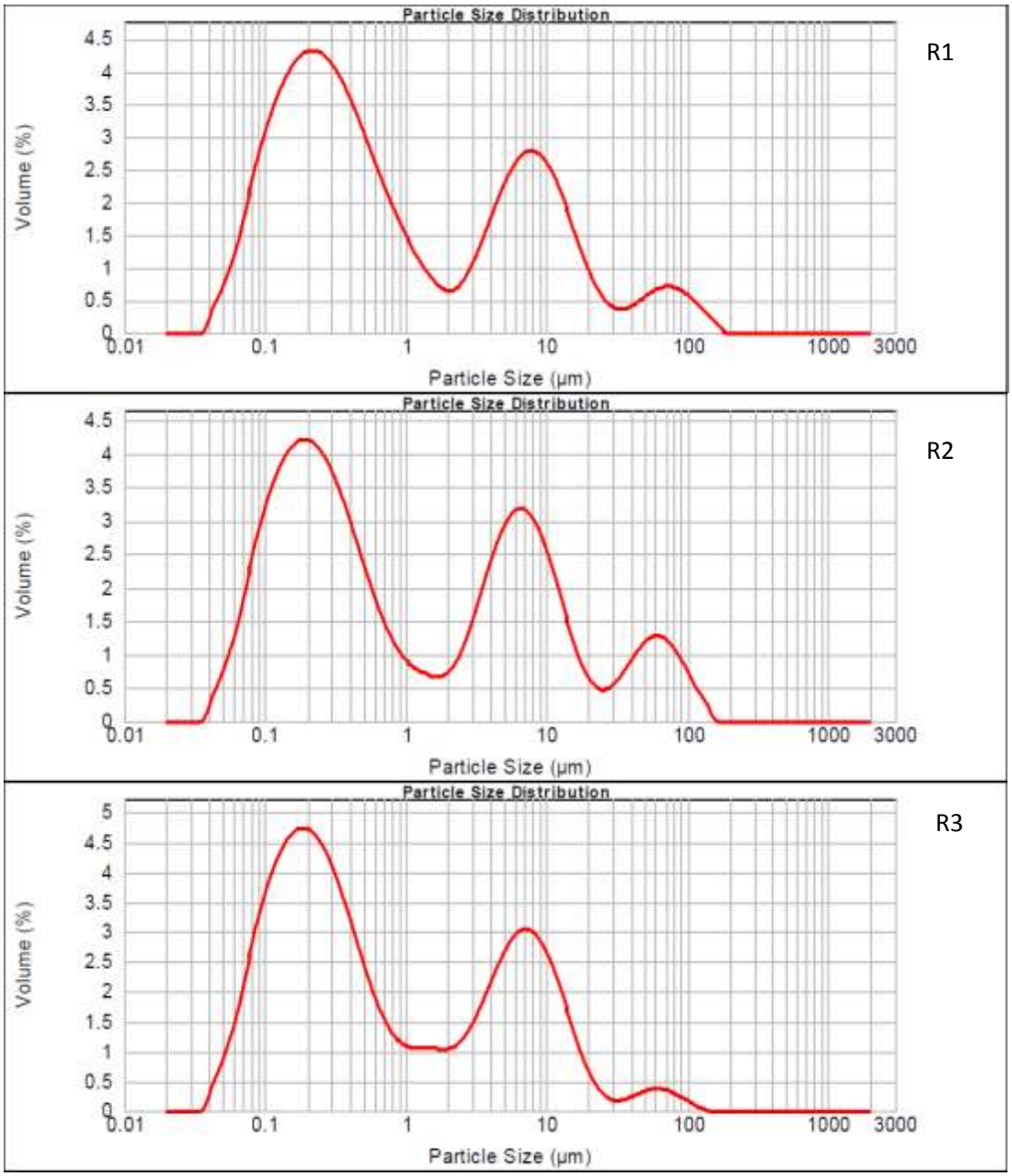

Fig. 7.1. Multilamellar vesicles (MLV) particle size distribution. Replicate 1 (R1), replicate 2 (R2) and replicate 3 (R3).

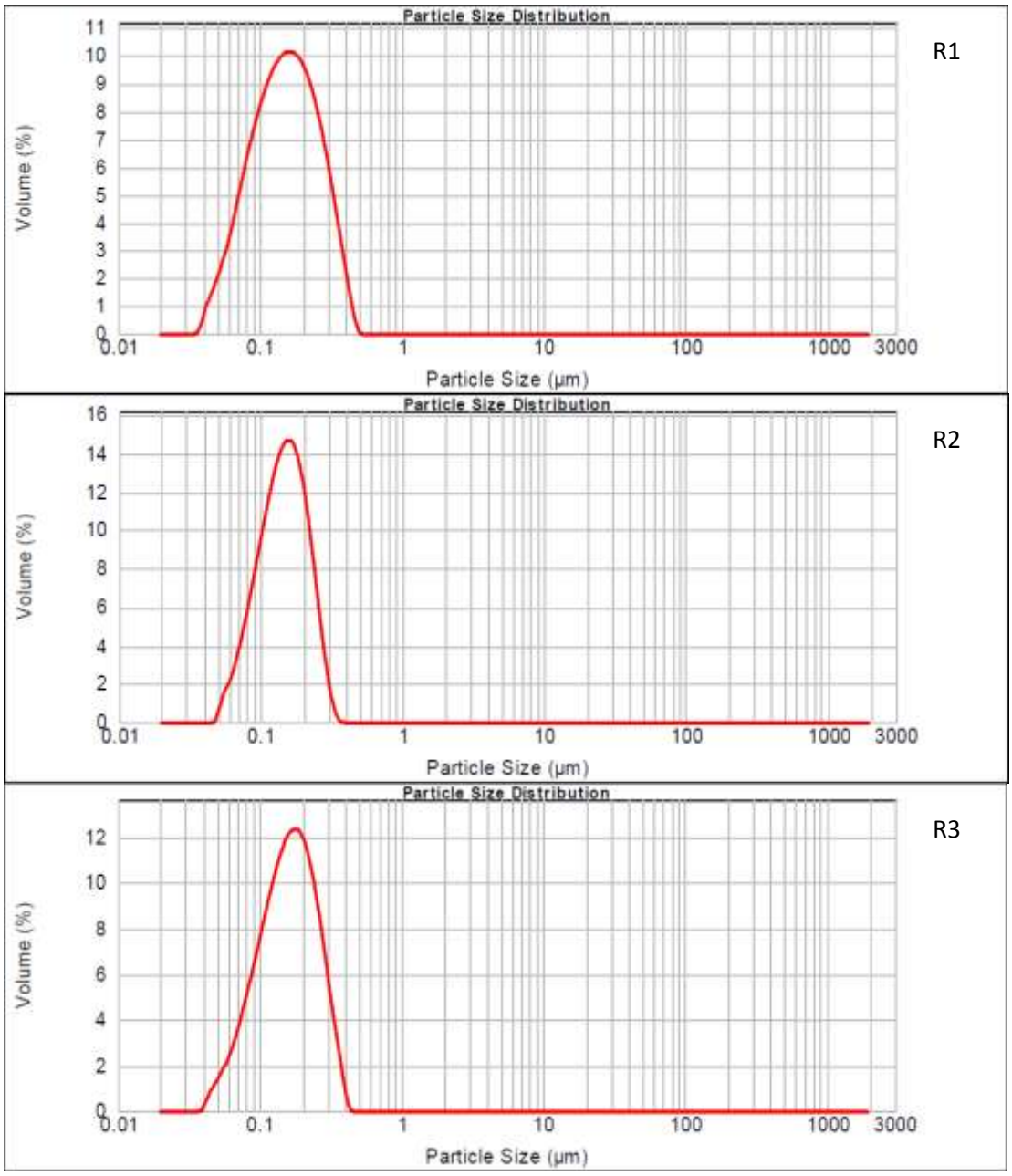

Fig. 7.2. Unilamellar vesicles (UV) particle size distribution. Replicate 1 (R1), replicate 2 (R2) and replicate 3 (R3).

7.2. Ultrasonic Velocity Results

Ultrasonic velocity (*u*) of serial dilutions of both liposomes, UV and MLV, were found to have a linear relationship between ultrasonic velocity and concentration (Fig. 7.3.).

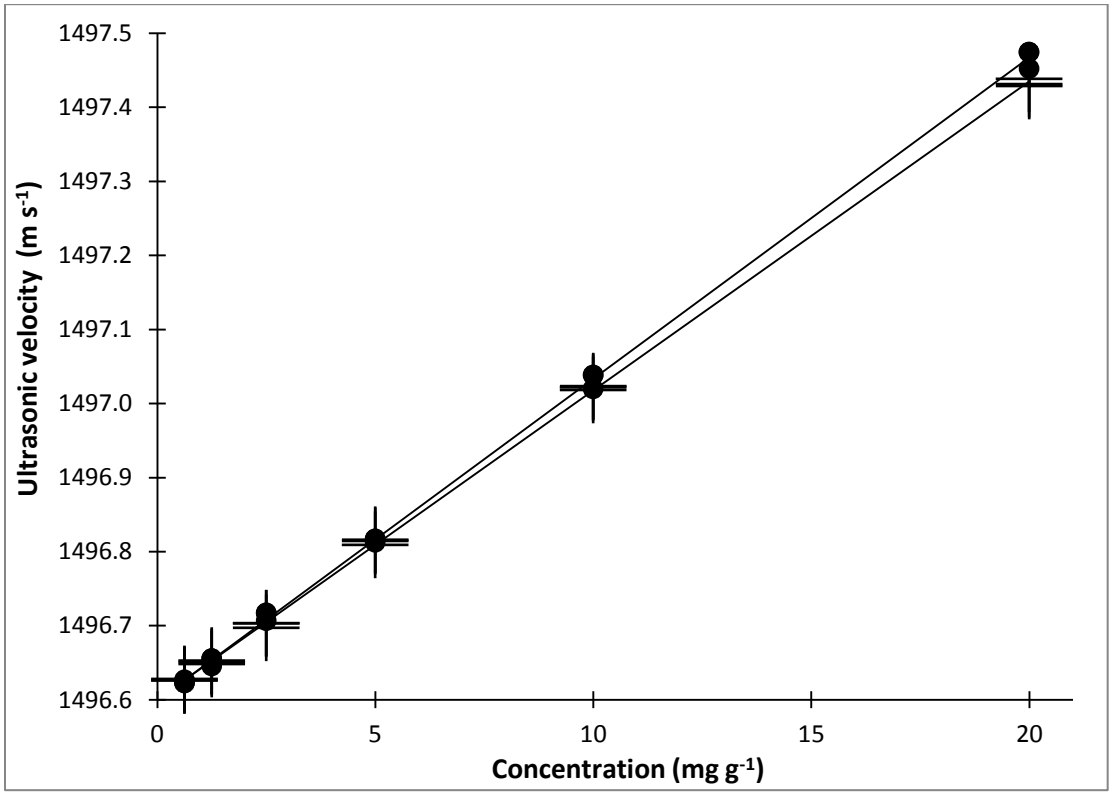

Fig. 7.3. Ultrasonic velocity of serial dilutions of liposomes, UV (┼), MLV (●).

The R^2 values for the linear regression of the ultrasonic velocity of both liposomes, UV and MLV, were very good, with values of 0.9998 and 0.9994, respectively. In order to confirm the linear relation over all the concentration range of the serial dilutions, linear regressions were calculated for the ultrasonic velocity in the lower concentration range (0.45-2.5 mg g^{-1}, Fig. 7.4.) and the higher concentration range (3.75-20 mg g^{-1}, Fig. 7.5.). The linear regression calculated in the high concentration range of the ultrasonic velocity of both liposome serial dilutions, UV and MLV, had R^2 values close to 1 with values of 0.9998 and 0.9991 respectively. The R^2 values corresponding to the lower concentration range (Fig. 7.4.) were not as close to 1, with values of

0.9953 and 0.9875, respectively; however, fits at lower concentrations were also good, confirming the good linear fit over all the concentrations used. The nature of the multilamellar vesicles, which have a wide particle size range due to the preparation method used, could influence the reduction in the linearity of the relationship of ultrasonic velocity and concentration of the MLV.

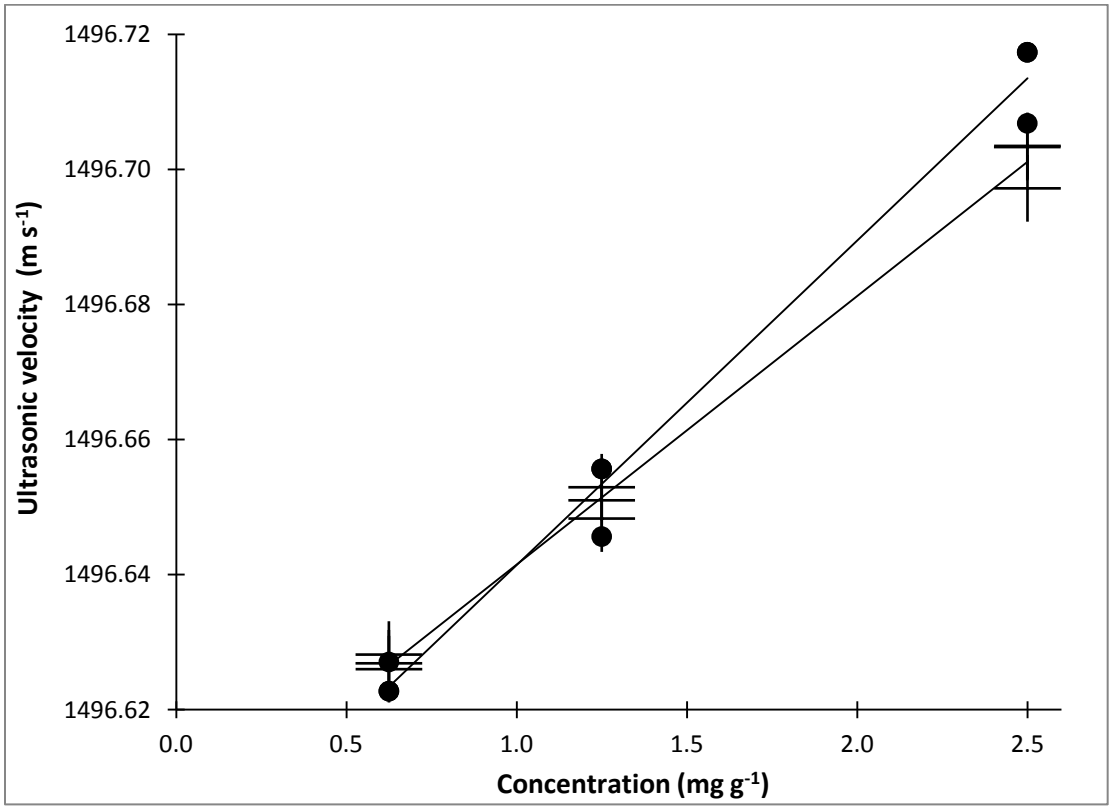

Fig. 7.4. Ultrasonic velocity of serial dilutions of liposomes at low concentrations, UV (┼), MLV (●).

Serial dilutions were made using ultrapure water with an initial stock solution at 20 mg g^{-1} concentration. Three replicates and three subsamples were used for the analysis; however, due to the preparation being done gravimetrically, so that the exact weight of the solute and the water added was recorded, the concentration of each replicate was slightly different.

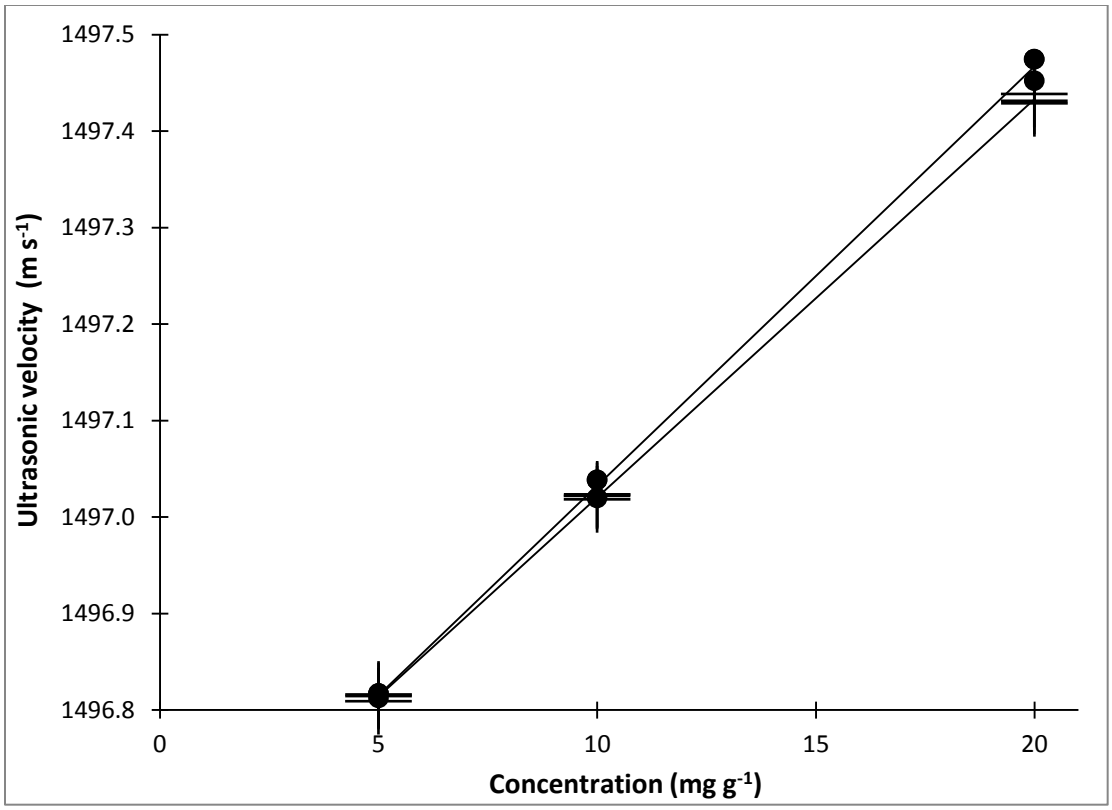

Fig. 7.5. Ultrasonic velocity of serial dilutions of liposomes at high concentrations, UV (┼), MLV (●).

Coefficient of variation (CV) values were calculated for the ultrasonic velocity results from the subsamples and replicates of the serial dilutions of both liposomes. The CV was calculated between subsamples and between replicates. Subsample CV (Fig. 7.6.) values were very low in both liposomes with values of $< 0.0007\%$, and $< 0.0009\%$ for UV and MLV, respectively.

Replicate CV (Fig. 7.7.) values were very low as well in both liposomes with values of $< 0.0004\%$ and $< 0.0009\%$ for UV and MLV, respectively.

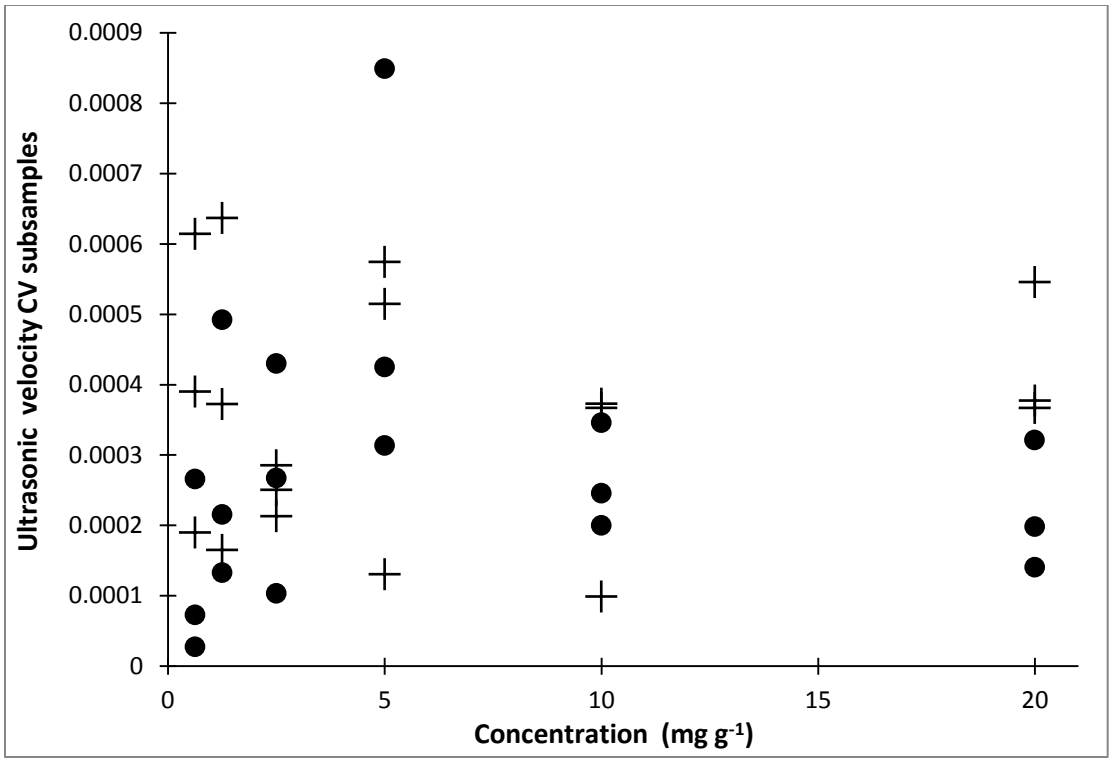

Fig. 7.6. Ultrasonic velocity CV for serial dilutions of subsamples of liposomes, UV (┼), MLV (●).

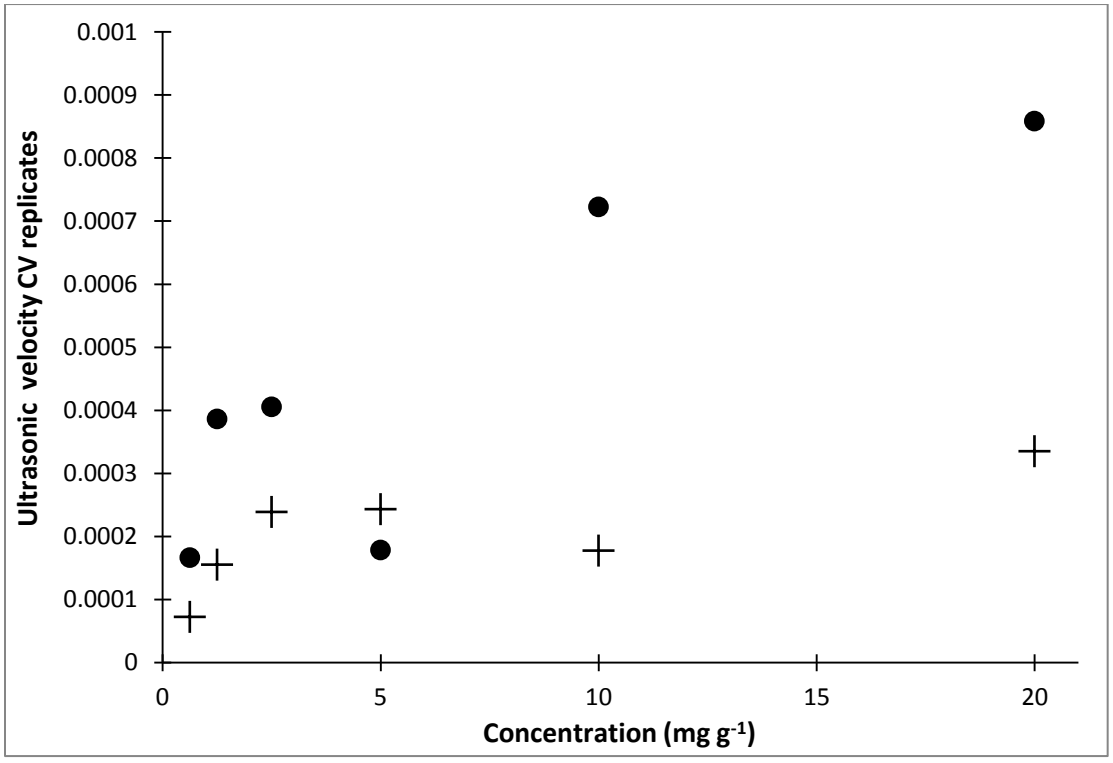

Fig. 7.7. Ultrasonic velocity CV for serial dilutions of replicates of liposomes, UV (┼), MLV (●).

Ultrasonic attenuation of serial dilutions of both liposomes, UV and MLV, were found to have a more or less linear relationship between ultrasonic attenuation and concentration (Fig. 7.8.).

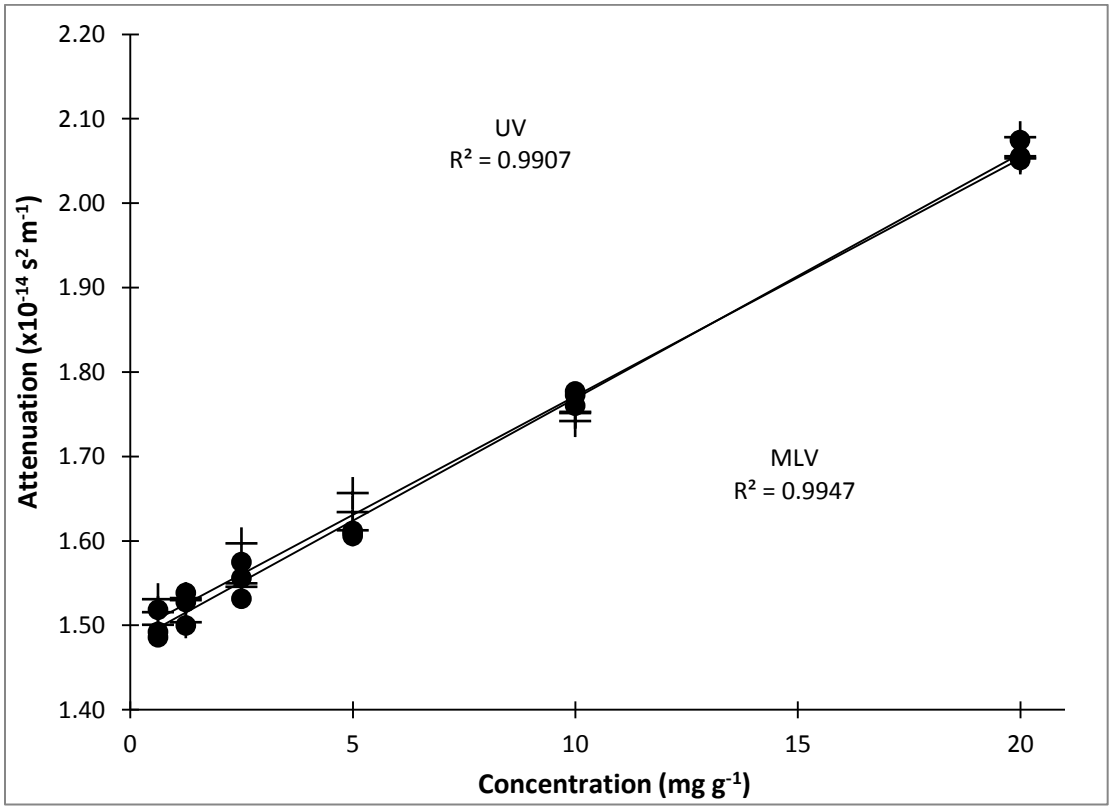

Fig. 7.8. Ultrasonic attenuation of serial dilutions of liposomes, UV (┼), MLV (●).

7.3. Density Results

Density (ρ) of serial dilutions of both liposomes, UV and MLV, was found to have a linear relationship against concentration (Fig. 7.9.).

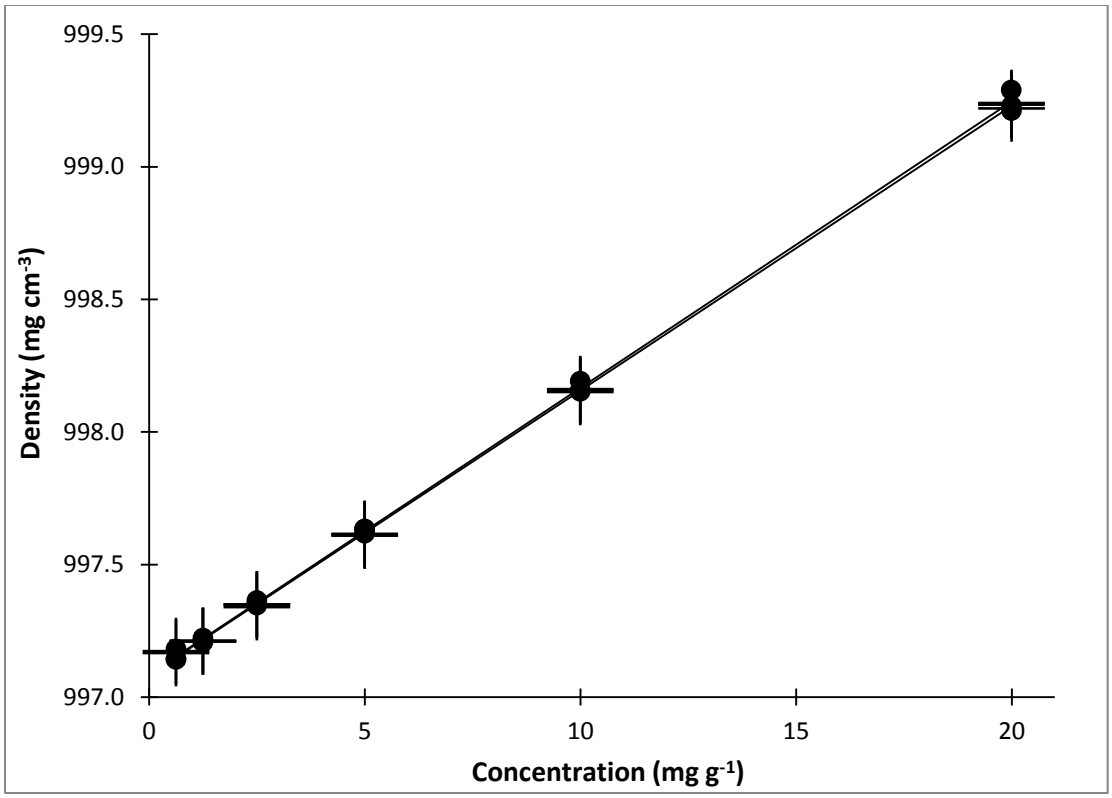

Fig. 7.9. Density of serial dilutions of liposomes, UV (┼), MLV (●).

R^2 values for the linear regression of the density of both liposomes, UV and MLV, were very good, at 0.9998 and 0.9994, respectively. As was done with ultrasonic velocity measurements, in order to confirm the linear relation over the full concentration range of the serial dilutions, linear regressions were calculated for the ultrasonic velocity in the lower concentration range (0.45-2.5 mg g^{-1}, Fig. 7.10.) and the higher concentration range (3.75-20 mg g^{-1}, Fig. 7.11.). The linear relation between density and concentration in the higher concentration range had R^2 values of 0.9999 and 0.9989 for the liposomes UV and MLV, respectively. The R^2 values in the lower concentration range (0.9865 and 0.9782 for liposomes UV and MLV respectively) were not as close to 1, but were still good. The slight decrease in R^2 values can be caused by a diminution in precision of the density measurements due to a limitation of the equipment and experimental errors associated with the use of very low concentration suspensions.

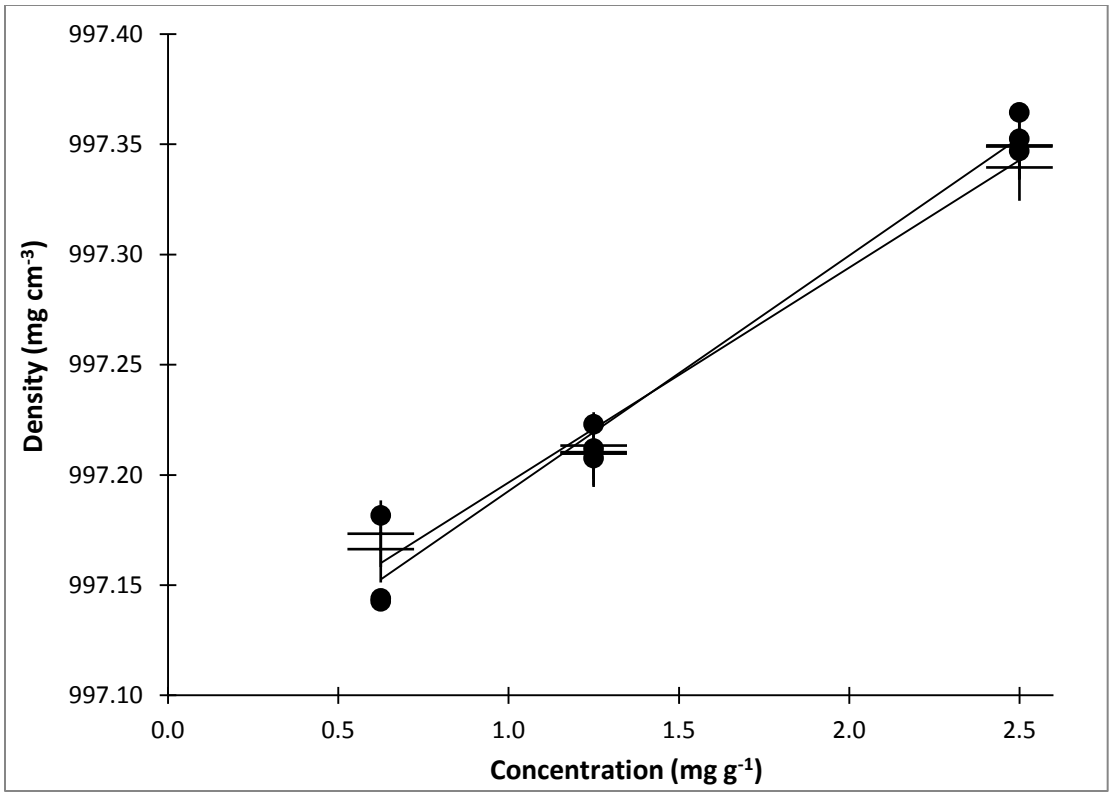

Fig. 7.10. Density of serial dilutions of liposomes at low concentrations, UV (┼), MLV (●).

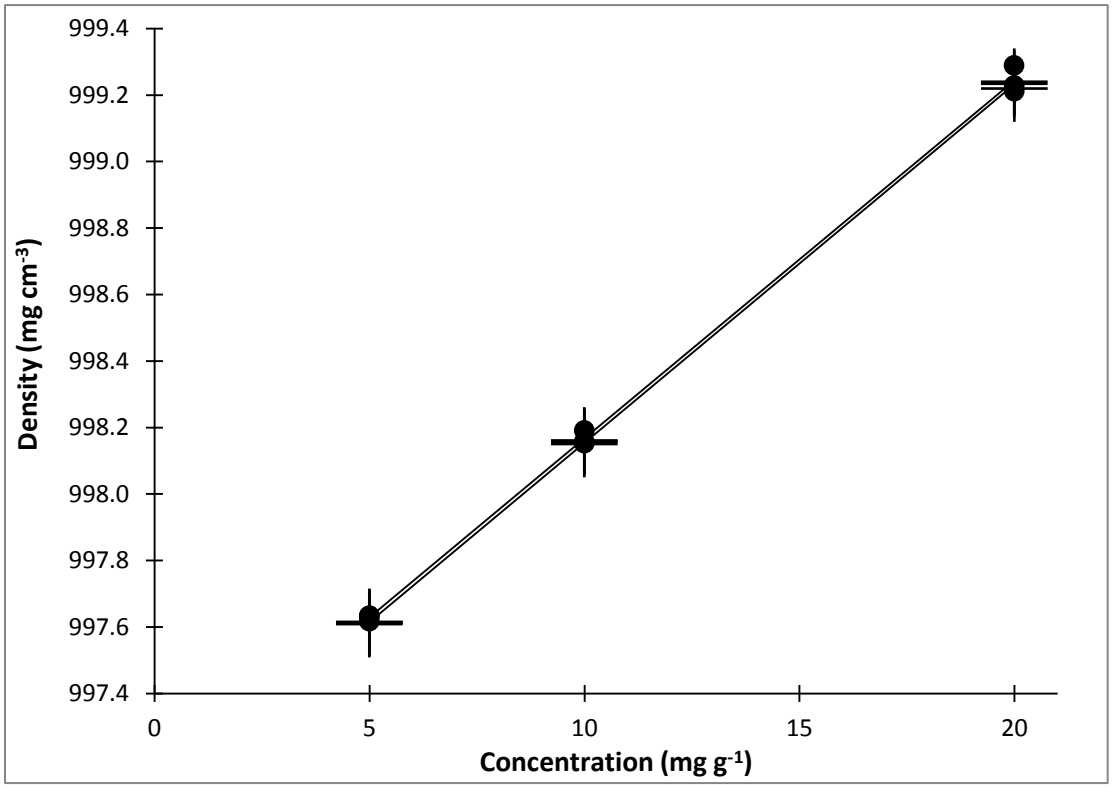

Fig. 7.11. Density of serial dilutions of liposomes at high concentrations, UV (┼), MLV (●).

The coefficient of variation (CV) was calculated for the density results from serial dilutions of both liposomes. Subsample CV (Fig. 7.12.) values were low in suspensions of both liposomes with values < 0.0015% and < 0.0027%, for UV and MLV, respectively, but with most of the CV values for both liposomes being lower than 0.0015%.

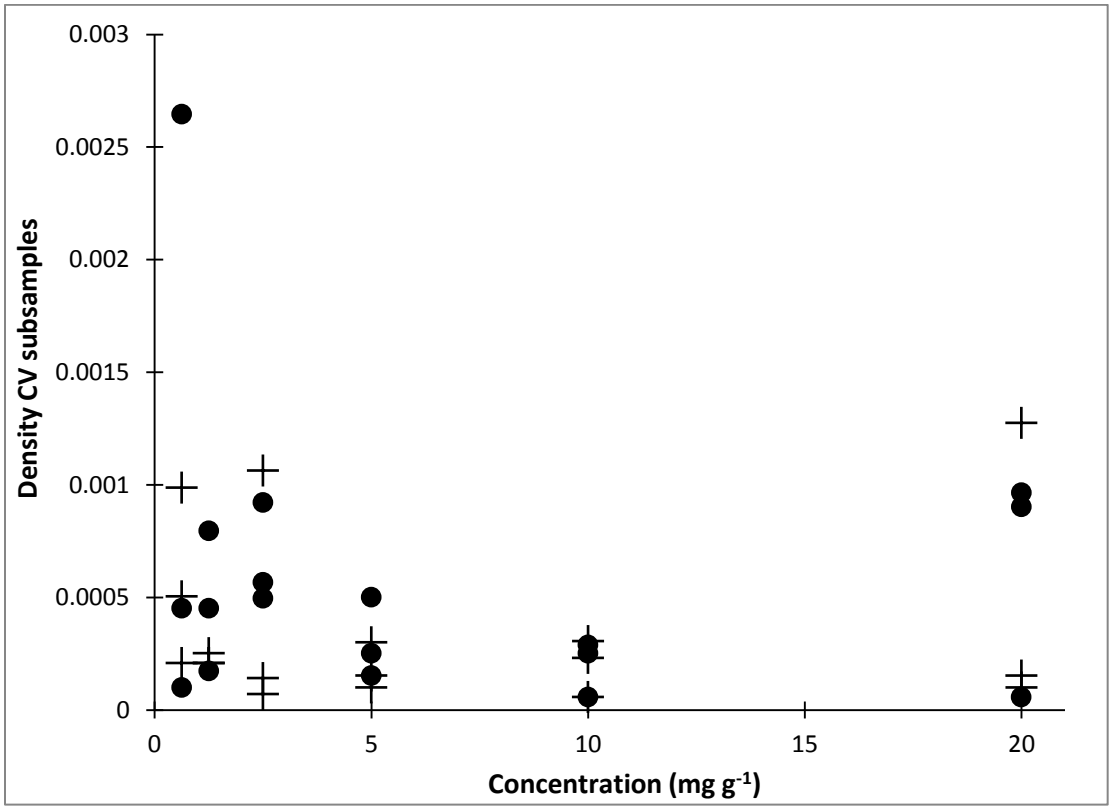

Fig. 7.12. Density CV for serial dilutions of subsamples of liposomes, UV (┼), MLV (●).

Replicate CV (Fig. 7.13.) values were also low in both liposomes, with values <0.0045%. Although the replicate CV values for the stock solutions for both liposomes were a little bit high, most of the values for both liposomes were under 0.0025%. Nevertheless, according to the procedure used to prepare the serial dilution gravimetrically, so that there are slight differences between the concentrations of the three replicates, the CV measured for the "replicates" was done using the average of the concentration of each dilution, leading to overestimation of this value.

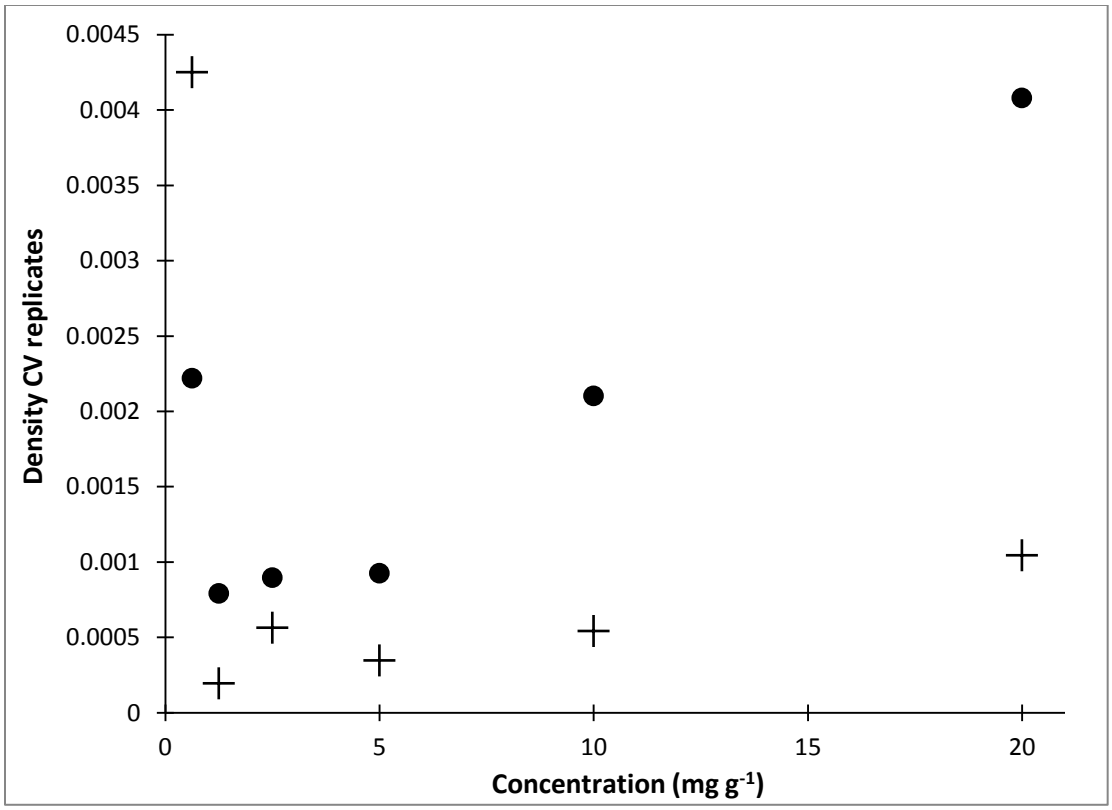

Fig. 7.13. Density CV for serial dilutions of replicates of liposomes, UV (┼), MLV (●).

7.4. Specific Volume

The apparent specific volume, $\bar{V}$, of the liposomes serial dilutions was calculated from the density measurements of the serial dilution as $\bar{V} = \frac{1-(\rho-c)/\rho_0}{c}$ (Fig. 7.14.).

The partial specific volume $\bar{V}^0$ was calculated by the extrapolation of the apparent specific volume to zero concentration as $\bar{V}^0 = \lim_{c\to 0} \bar{V}$. However, because the apparent specific volume had a rapid decrease to concentration values below approximately 5 mg mL^{-1}, the extrapolation to zero was done using just the two smallest concentration values. Because of curvature in Fig. 7.14., it is possible that even this value is overestimated. Partial specific volume values are shown in Table 7.2.

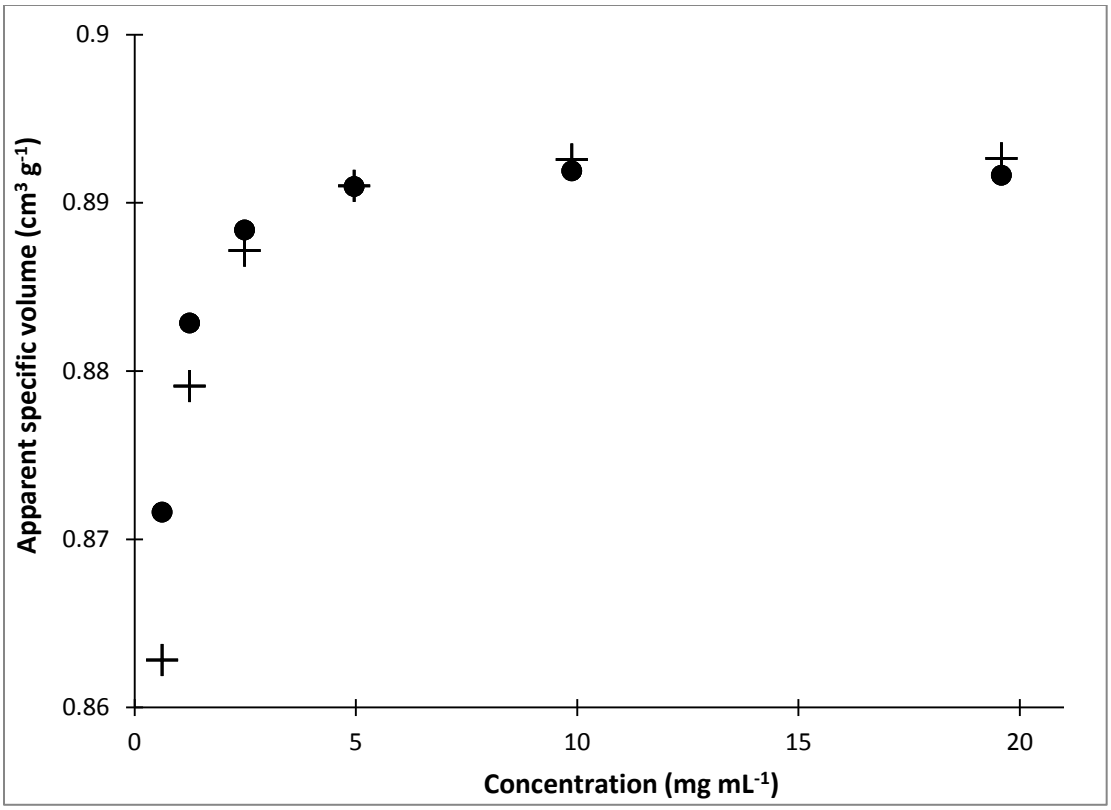

Fig. 7.14. Apparent specific volume of liposomes, UV (┼) and MLV (●).

Table 7.2. Partial specific volume of liposomes.

	$\bar{V}^0$ (cm^3 g^{-1})
Unilamellar vesicles (UV)	0.846
Multilamellar vesicles (MLV)	0.860

7.5. Compressibility

The adiabatic compressibility coefficient, β_s, was calculated according to the Newton-Laplace equation $\beta_s = \frac{1}{\rho u^2}$, where β_s is calculated as a function of the density and ultrasonic velocity. The relative adiabatic compressibility coefficient, β_s/β_{s0}, which expresses the relation between adiabatic compressibility coefficient of the solution, β_s, against the adiabatic

compressibility coefficient of the solvent β_{so}, ultra pure water (UPW) for this study, had a linear relationship against concentration.

The apparent adiabatic specific compressibility, $\overline{K}_s$, which expresses the change in the apparent specific volume as a function of a change in pressure, was calculated as $\overline{K}_s = \beta_{s0}\frac{\beta_s/\beta_{so}-(\rho-c)/\rho_0}{c}$ (Fig. 7.15.).

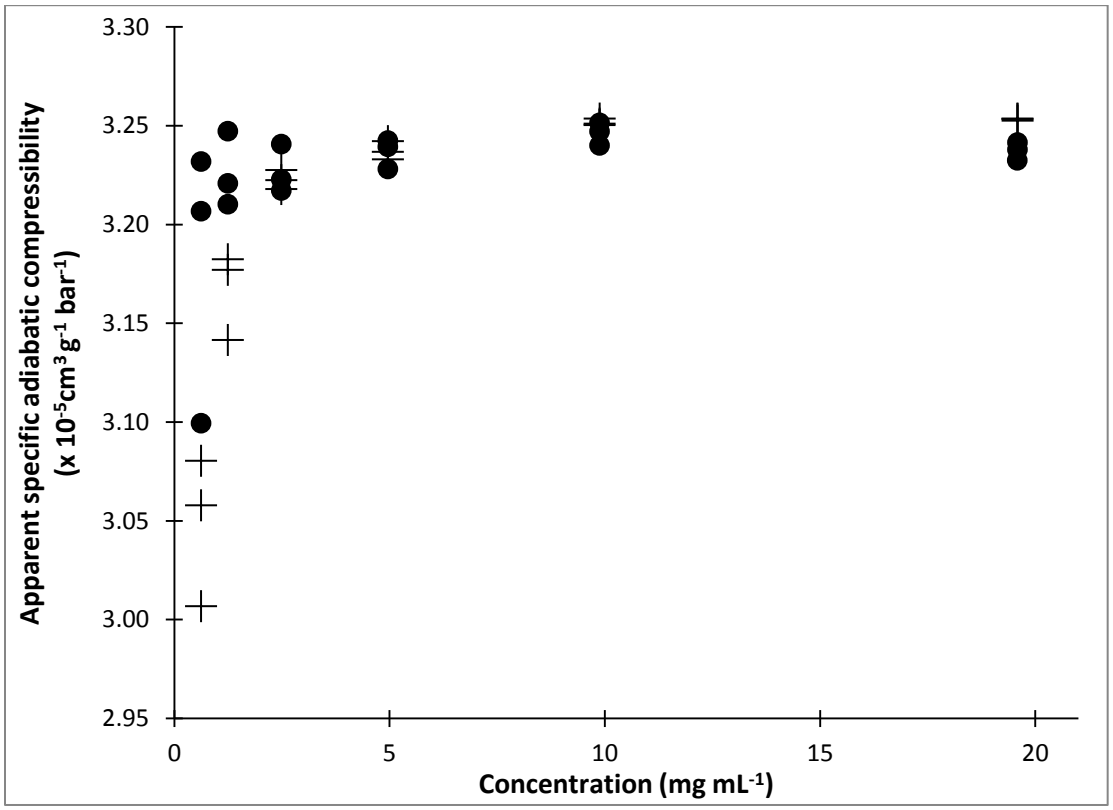

Fig. 7.15. Apparent adiabatic specific compressibility of liposomes, UV (┼) and MLV (●).

The partial specific adiabatic compressibility coefficient, $\bar{\beta}_s^0$, which describes the relation between the partial specific adiabatic compressibility, $\overline{K}_s^0$, and partial specific volume, $\bar{V}^0$, was calculated as $\bar{\beta}_s^0 = \frac{\overline{K}_s^0}{\bar{V}^0} = \frac{\beta_{so}}{\bar{V}^o}\lim_{c\to 0}\frac{\beta_s/\beta_{so}-(\rho-c)/\rho_0}{c}$, where the term $\frac{\beta_s/\beta_{so}-(\rho-c)/\rho_0}{c}$ is extrapolated to zero while β_{s0} and $\bar{V}^0$ are constant values. However, because the term $\frac{\beta_s/\beta_{so}-(\rho-c)/\rho_0}{c}$ had a rapid decrease with decrease in concentration, the extrapolation to zero

was done using just the two smallest concentration values, although it is likely that even this value is overestimated. The partial specific adiabatic compressibility coefficient $\bar{\beta}_S^0$ values are shown in Table 7.3.

Table 7.3. Partial specific adiabatic compressibility coefficient of liposomes.

	$\overline{\boldsymbol{\beta}_{\boldsymbol{S}}^{\boldsymbol{0}}}$ (x 10^{-6} bar^{-1})
Unilamellar vesicles (UV)	34.6
Multilamellar vesicles (MLV)	36.4

7.6. Discussion

Particle size measurements of the unilamellar vesicle (UV) and multilamellar vesicle (MLV) suspensions were done in order to confirm the effectiveness of the extrusion method (MacDonald et al., 1991) for producing unilamellar vesicles (Hope et al., 1984). The use of light scattering methods, like the one used in this study, to measure the particle size of extruded liposomes has already been reported (Ramaswami et al., 1992; Romanowski et al., 2002; Joanne et al., 2009). The results confirmed the extrusion method was able to reduce the particle size in order to get a homogeneous unilamellar vesicle suspension.

The size distribution for the UV suspension was seen to have a normal distribution (Fig. 7.2.) with an average value of about 0.128 µm (Table 7.1.) with particle size varying mainly in the range of 0.05 µm to 0.3 µm compared to the size distribution of the MLV suspension that was seen with a tri-modal distribution (Fig. 7.1.), which had a noticeable heterogeneous particle size varying in the range of 0.05 µm to 100 µm.

Unilamellar vesicles (UV) and multilamelar vesicles serial dilutions analysed in this study were seen to have a linear relationship between density and concentration (Fig. 7.9.) and ultrasonic velocity and concentration (Fig. 7.3.). Density and ultrasonic velocity as well as the volumetric properties, such as the specific volume and the compressibility, have been used to analyse the physical properties of lipid liposomes in aqueous suspensions as well as their interaction with other molecules like proteins or peptides (Colotto et al., 1993; Hianik et al., 1998; Hianik et al., 1999; Krivanek et al., 2000; Koenig & Gawrisch, 2005; Rybar et al., 2007; Uhríková et al., 2007).

Both types of liposome serial dilutions also were found to have a more or less linear relationship between ultrasonic attenuation and concentration with similar ultrasonic attenuation values for both liposomes (Fig. 7.8). The ultrasonic attenuation values can be used to analyse the aggregation phenomena in particle suspensions, because the change in the organization of the particles affects the way the ultrasound is dissipated (McClements, 1991; Nelson et al., 2001; Mougin et al., 2003; Corredig et al., 2004; Yuno-Ohta & Corredig, 2007; Povey et al., 2011; Wrobel & Time, 2012). Generally ultrasonic attenuation can be divided into the ultrasound attenuation caused by molecular or chemical relaxation, and the attenuation caused by scattering of ultrasound (Nelson et al., 2001; Mougin et al., 2003; Corredig et al., 2004; Yuno-Ohta & Corredig, 2007; Povey et al., 2011). The less linear decrease of the ultrasonic attenuation for both liposome as the solution were diluted might suggest there were not any aggregation processes that changed the ultrasonic attenuation trend.

The apparent specific volume of UV and MLV liposome serial dilutions was seen to be independent of concentration at the higher concentration range, but between 5 mg mL^{-1} and 2.5 mg mL^{-1}, a continuous decrease in apparent specific volume was seen; the total decrease

between the highest and the lowest concentration was about 0.030 $cm^3 g^{-1}$ and 0.020 $cm^3 g^{-1}$ for UV and MLV liposomes, respectively. The specific volume of phosphatidylcholine in aqueous suspension has been described as the sum of different contributions (Koenig & Gawrisch, 2005; Uhríková et al., 2007). It is generally subdivided into two segments with different contributions, first the hydration volume, V_H, which is the volume associated with the polar head, and second, the hydrophobic volume, V_C, which is the volume associated with the acyl chains that generally are not in contact with the water (Koenig & Gawrisch, 2005; Uhríková et al., 2007). The specific volume of phosphatidylcholine vesicles is influenced by the anhydrous component, the water enclosed in the vesicles, and the solvated regions of the vesicles where the polar head groups are in contact with the water (Huang & Charlton, 1971). Finally, according to Huang & Charlton (1971), the diffusion of water between the interior and exterior of the vesicles tends to find equilibrium, and hence the authors assumed that the density of water in the interior of the vesicles and the bulk water have the same density. There are several studies in the literature (Hianik et al., 1998; Hianik et al., 1999; Krivanek et al., 2001; Koenig & Gawrisch, 2005; Rybar et al., 2007; Uhríková et al., 2007) where the use of the specific volume has been reported in order to measure the variation in the physical properties of lipid bilayers caused by lipid composition, changes in temperature (associated with the liquid and crystalline phase transition) and interactions with other molecules such as proteins or peptides.

According to Nagle & Wilkinson (1978), the changes in specific volume associated with the hydration of head groups is usually small compared with the change in the specific volume of the hydrophobic segment, V_M, associated with temperature or composition changes. However, in this study, because the composition and temperature were maintained constant, and just the concentration was varied as the suspensions were diluted, the decrease in the apparent specific volume of the UV and MLV liposomes appears to be caused by the increase in

the hydration number of the polar head groups of the liposomes. The dependence of the specific volume on the hydration of phospholipids has been reported by White et al. (1987) and Bechinger & Seelig (1991). Additionally, the reduction in the apparent specific volume was noticeably lower in the MLV liposomes. This smaller reduction can be the result of the multilamellar nature of the MLV liposomes which have a smaller exposed surface to the water compared with the UV liposomes at the same concentration, and therefore a lower amount of head groups exposed to the effect of increased hydration as the suspension was diluted.

The partial specific volume of UV and MLV lipsosmes (Table 7.2.) was calculated by extrapolation to zero of the apparent specific volume with values of 0.846 $cm^3\ g^{-1}$ and 0.860 $cm^3\ g^{-1}$, respectively. However, as explained previously, due to the curvature seen in apparent specific volume for all dairy peptides, the values could be overestimated. Hianik et al. (1998) reported the partial specific volumes at 23°C of planar lipid bilayers composed of different phospholipids to be between 0.92 $cm^3\ g^{-1}$ and 0.98 $cm^3\ g^{-1}$. The large difference of 0.06 $cm^3\ g^{-1}$ between the different bilayers may exemplify the complexity in comparing the values of this study with the literature values due to the sensitivity of the specific volume to difference in the composition of lipid bilayers as well as the temperature of the experiment. Moreover, the phosphatidylcholine used in my study was a mixture of different acyl chains lengths.

The apparent specific adiabatic compressibility of UV and MLV liposomes serial dilutions was seen to be independent of the concentration at the higher concentration range, 5-20 mg mL^{-1}, but between 5 and 2.5 mg mL^{-1}, a continuous decrease in apparent specific compressibility was seen for the UV liposomes while the MLV liposomes had a small decrease. The total decrease between the highest and lowest concentration was about $2.1x10^{-6}\ cm^3\ g^{-1}\ bar^{-1}$ and $0.58x10^{-6}\ cm^3\ g^{-1}\ bar^{-1}$ for UV and MLV liposomes, respectively. According to Gekko et

al. (2004), the compressibility of the void spaces contributes positive values while the compressibility of the water molecules around the surface contributes negative values to the partial specific adiabatic compressibility. Therefore, the positive values indicate a highly compressible interior which is compatible with the highly hydrophobic interior of the liposomes (Gekko et al., 2004; Krivanek et al., 2001). According to Krivanek et al. (2001) the compressibility of phospholipid liposomes is caused by the sum of two contributions, the compressibility of the water inaccessible interior between the two phospholipids layers of the liposome and the compressibility of the water shell that surrounds, in the interior and exterior, the bilayer of the liposome and is affected by the interaction with the polar head groups. The compressibility changes in the liposomes themselves can be caused by changes in the asymmetry of the acyl chains in the interior (Krivanek et al., 2001). Because in this study the composition and temperature were maintained constant, and just the concentrations varied as the suspensions were diluted, the decrease in the apparent specific adiabatic compressibility is likely caused by an increase in hydration of the head groups of the liposomes. According to Rybar et al. (2007), increase in the hydration of liposomes should produce a decrease in the compressibility. Finally an increase of hydration as the cause of the reduction of apparent specific adiabatic compressibility of both types of liposomes is also compatible with the difference in the reduction in compressibility between the MLV and UV liposomes due to the greater exposed surface to the water in the UV liposomes as explained previously with the apparent specific volume.

The partial specific adiabatic compressibility coefficient of UV and MLV lipsosmes (Table 7.3.) was calculated at infinite dilution because at infinite dilution the partial compressibility is only influenced by the hydration and the intrinsic compressibility of the solute (Chalikian et al., 1994). However, as explained previously, the values obtained for the UV (34.6×10^{-6} bar^{-1}) and

MLV (36.4 $x10^{-6}$ bar^{-1}) could be overestimated. Hianik et al. (1998) reported the partial specific adiabatic compressibility coefficient at 23 °C of liposomes composed of different phospholipids between 57 $x10^{-6}$ bar^{-1} and 82 $x10^{-6}$ bar^{-1}; the large difference of 25 $x10^{-6}$ bar^{-1} between the different bilayers may exemplify, as with the partial specific volume, the complexity in comparing the values of this study with the literature values due to the sensitivity of the compressibility to difference in the composition of lipid bilayers as well as the temperature of the experiment. Moreover, the phosphatidylcholine used in my study was a mixture of different acyl chains lengths. Different acyl lengths affect the phase transition temperature between the liquid form phase and the gel solid phase.

8. Liposomes-Dairy Peptides Results and Discussion

High precision ultrasonic velocity and density measurements were used to analyse interactions between dairy bioactive peptides in solution and liposomes, unilamellar vesicles (UV) and multilamellar vesicles (MLV), that were used as model membranes. Dairy bioactive peptides A, C and D were used to prepare solutions at two different concentrations, 20 mg g^{-1} and 15 mg g^{-1}, as explained in section 3.2.10.

Refined Lecithin was used to produce phosphatidylcholine (PC) liposomes as described in section 3.2.10. The liposomes were prepared at 20 mg g^{-1} concentration.

Density and ultrasonic measurements were performed at 24.985°C, as explained in section 3. The temperature was set to maintain the same conditions that had been used in the characterization of the volumetric properties of dairy peptides and liposomes individually. Density and ultrasonic velocity were performed in liposome-dairy peptide mixes over time, in order to understand if the interactions between liposomes and dairy peptides differ over time.

Solutions of liposomes, UV and MLV, and dairy peptides, A, C and D, were prepared with ultrapure water as explained in section 3.2.10.3., and are going to be referred to as “mixes”.

Liposome-dairy peptide mixes were prepared gravimetrically, and the exact concentrations were recorded. Three replicates were used for the analysis, but because the preparation was done gravimetrically and the exact weight was recorded, the concentration of each replicate was slightly different. The mixes had a final concentration of liposomes of 15 mg g^{-1} in all mixes, and 3.75 mg g^{-1} or 5 mg g^{-1} of dairy peptide in order to have a 3-1 and 4-1 ratio between liposomes and dairy peptides. The liposomes-peptides ratio that has been used varies a lot between different interaction studies, but generally a ratio is used where the liposome is in

a higher proportion than the peptide (Ramaswami et al., 1992; Colotto et al., 1993; Creuzenet et al., 1997; Hianik et al., 1997; Krivanek et al., 2001; Romanowski et al., 2002; Hunter et al., 2005; Rybar et al., 2007; Hianik et al., 2011).

Density and ultrasonic velocity were measured as a function of time at 5, 15, 30, 45 and 60 min from the moment the dairy peptide solution was added to the liposome suspension. However because the density and ultrasonic measurements need temperature stabilization within the cell of each equipment prior to the measurement acquisition, this process slightly delays acquisition at a given time. As a result, the exact moment of the measurements varied slightly between samples. Exact times of the measurements were carefully recorded. Mixes were kept in agitation during the whole experiment.

8.1. Ultrasonic Velocity Results

Ultrasonic velocity (u) of the mixes of UV liposomes at 15 mg g^{-1} with dairy peptides A, C and D at 3.75 mg g^{-1}, and with dairy peptides A, C and D at 5 mg g^{-1}, as a function of time are shown in Fig. 8.1. and 8.2.

Ultrasonic velocity of mixes of UV liposomes at 15 mg g^{-1} and dairy peptides A, C and D at 3.75 mg g^{-1} (Fig. 8.1.) didn't show a noticeable change over time. The same behavior without noticeable change over time was seen in the mixes of UV liposomes and dairy peptides C and D at 5 mg g^{-1} (Fig. 8.2.), but the mixes with dairy peptide A at 5 mg g^{-1} showed that ultrasonic velocity had a tendency to increase over time, although the increase was small.

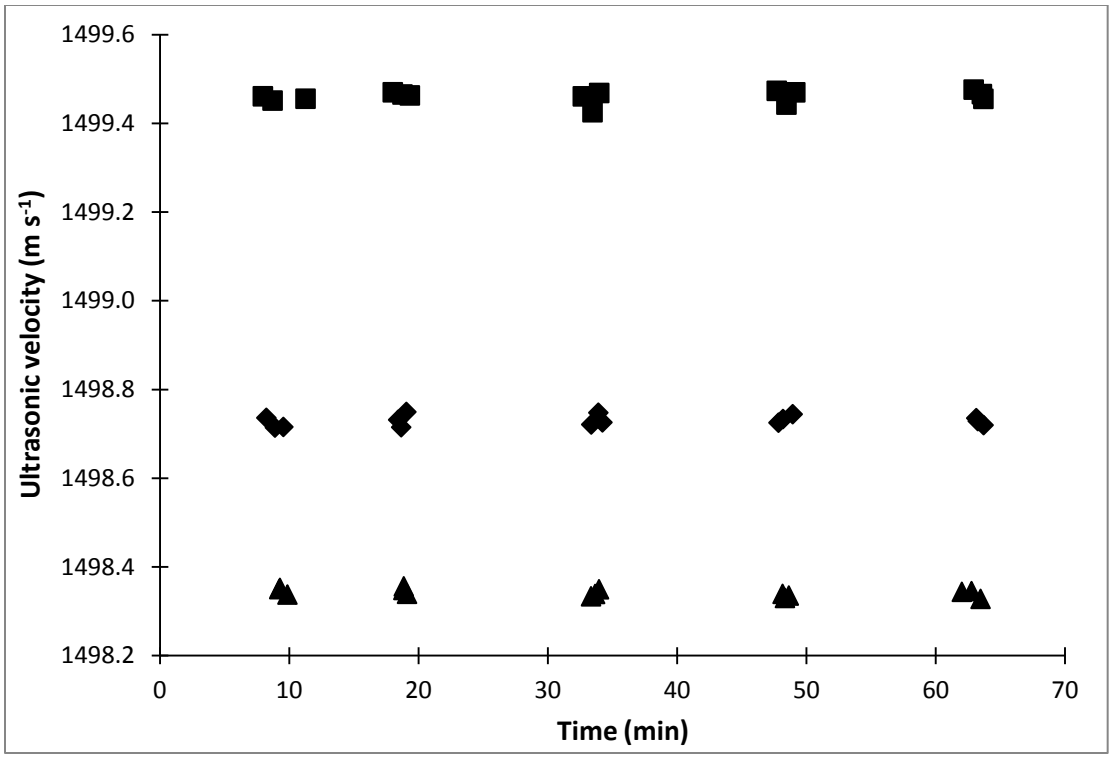

Fig. 8.1. Ultrasonic velocity of mixed solutions of UV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 3.75 mg g^{-1} concentration. UV-A (◆), UV-C (■) and UV-D (▲).

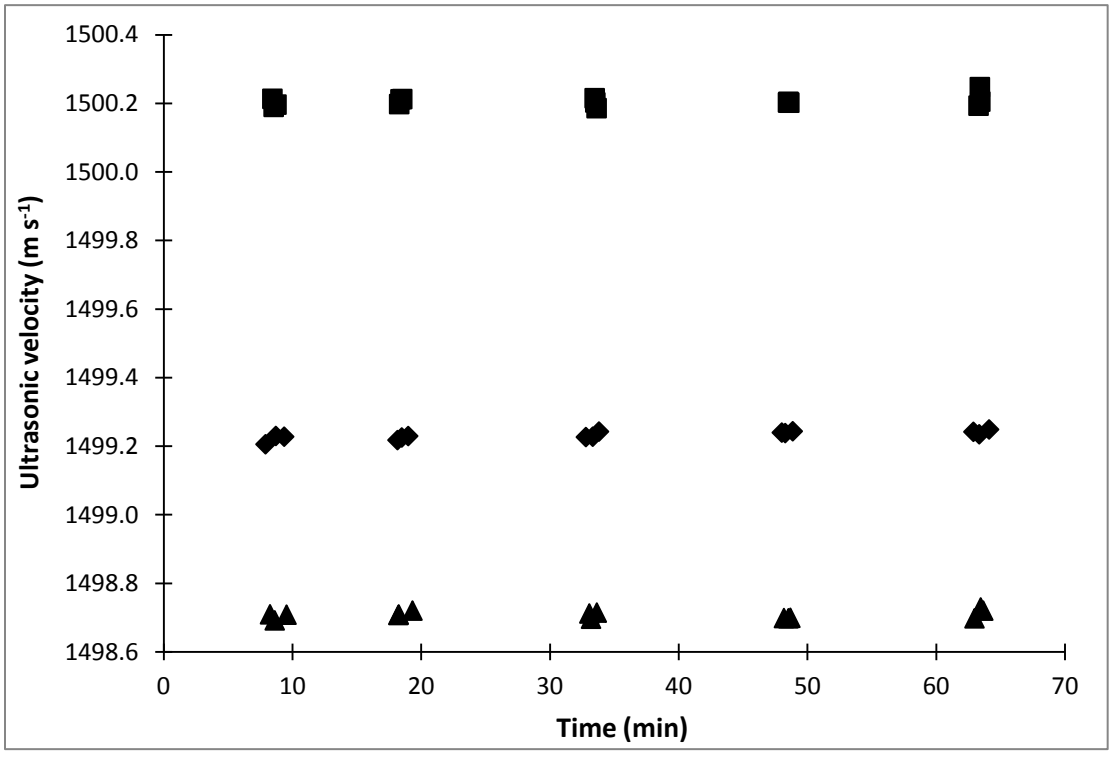

Fig. 8.2. Ultrasonic velocity of mixed solutions of UV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 5 mg g^{-1} concentration. UV-A (◆), UV-C (■) and UV-D (▲).

The ultrasonic velocity of each replicate was independently graphed to analyse differences between replicates, because the three replicates prepared for each mix had slightly different concentration, and also because the measurements were done at slightly different times.

The ultrasonic velocity by replicates graphed independently (Fig. 8.3. and 8.4.) showed no noticeable change over time for mixes of the UV and dairy peptide A, C and D at both concentrations. Although small increases and decreases were seen, the changes were small and it was not possible to have certainty if they were caused by slight differences in the mixes or inherent variation in the measurements. However, for UV and dairy peptide C at 5 mg g^{-1} mixes, a constant increase over time was seen, although this change was also small and within the error limit of the equipment.

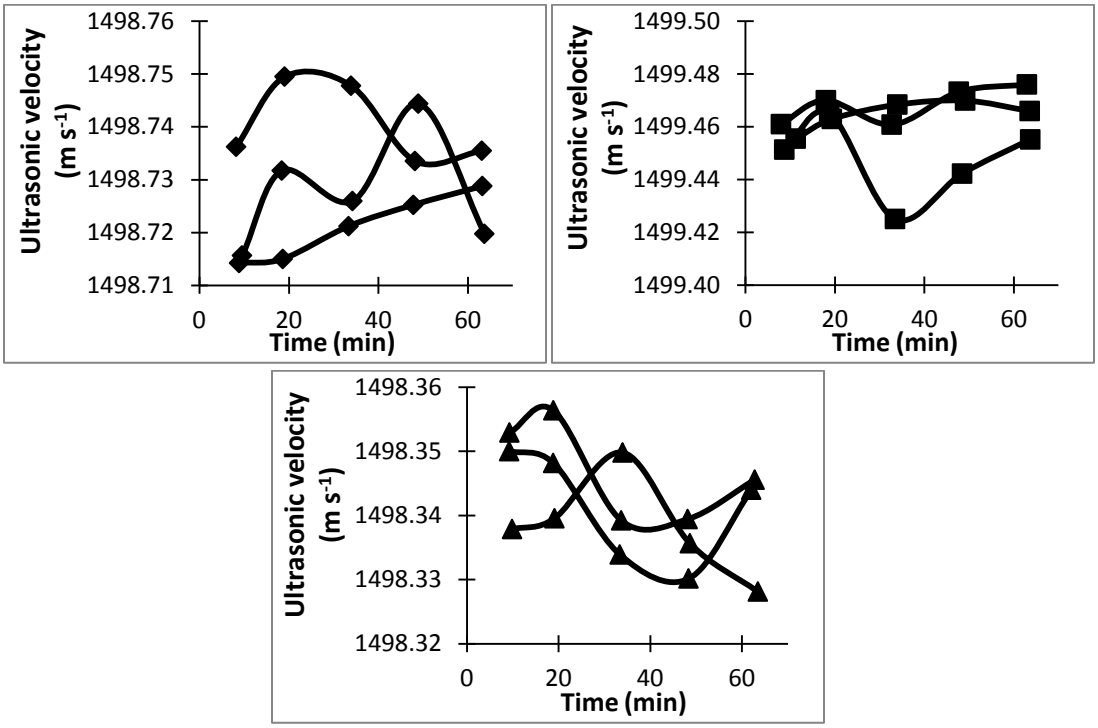

Fig. 8.3. Time dependency of ultrasonic velocity of the three replicates mixes of UV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 3.75 mg g^{-1} concentration. UV-A (◆), UV-C (■) and UV-D (▲).

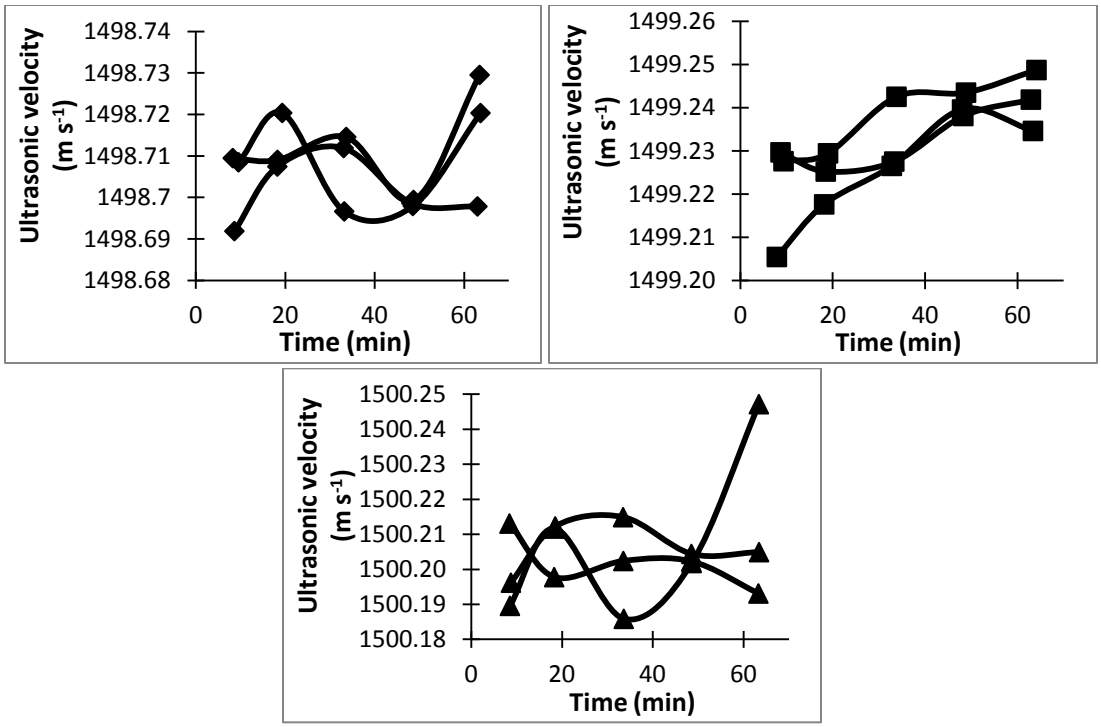

Fig. 8.4. Time dependency of ultrasonic velocity of the three replicates mixes of UV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 5 mg g^{-1} concentration. UV-A (◆), UV-C (■) aUV-D (▲).

Ultrasonic velocity of mixes of MLV liposomes at 15 mg g^{-1} and dairy peptides A, C and D at 3.75 mg g^{-1} (Fig. 8.5.) didn't show noticeable change over time. The same behavior, without noticeable change over time, was seen in the mixes of MLV liposomes and dairy peptides at 5 mg g^{-1} (Fig. 8.6.).

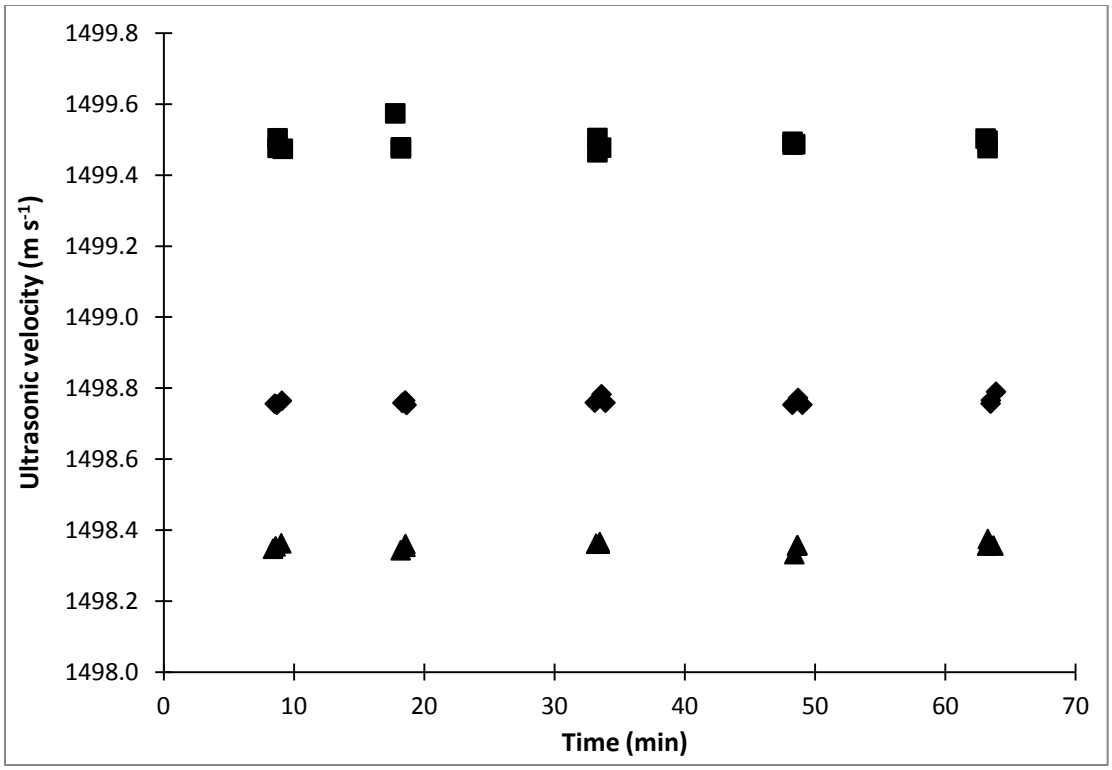

Fig. 8.5. Ultrasonic velocity of mixed solutions of MLV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 3.75 mg g^{-1} concentration. MLV-A (◆), MLV-C (■) and MLV-D (▲).

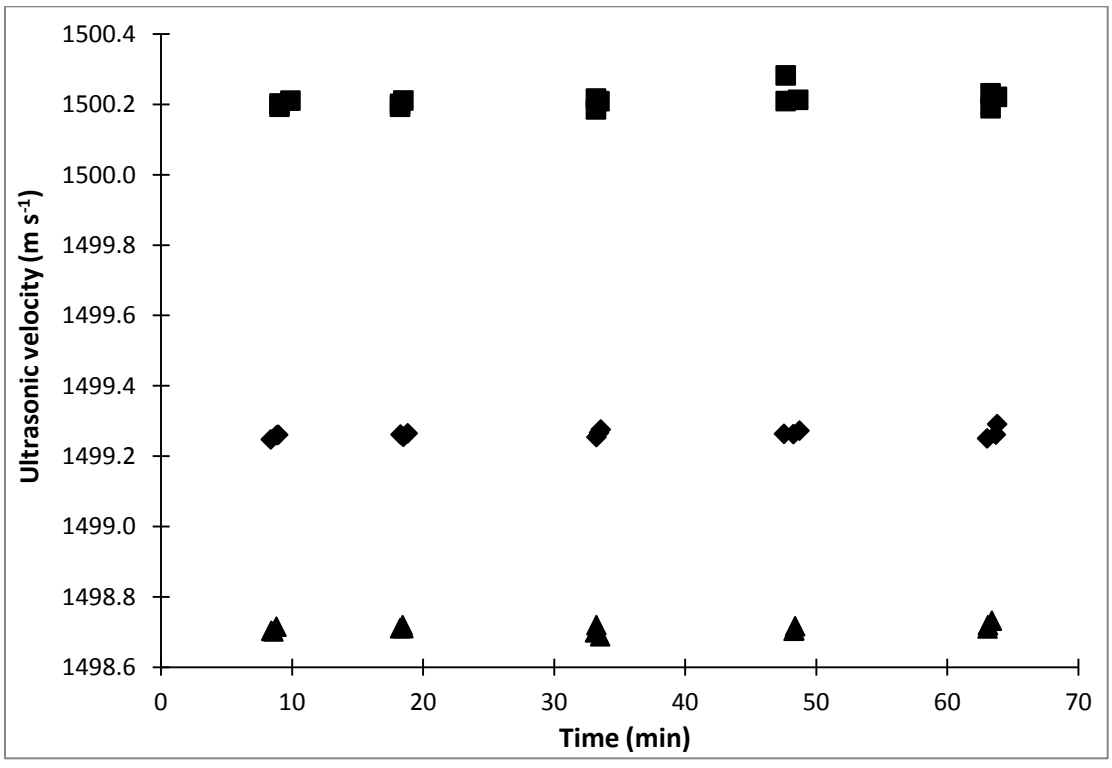

Fig. 8.6. Ultrasonic velocity of mixed solutions of MLV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 5 mg g^{-1} concentration. MLV-A (◆), MLV-C (■) and MLV-D (▲).

As it was done with UV-dairy peptide mixes, because the three replicates prepared for each mix had slightly different concentration and slightly different acquisition times, the behavior of each replicate was independently graphed to analyse for difference between replicates.

The ultrasonic velocity by replicates graphed independently (Fig. 8.7. and 8.8.) showed no noticeable change over time for the mixes of MLV and dairy peptide at both concentrations. Although small increases and decreases were seen, the changes were quite small and it was not possible to be certain if they were caused by changes in the mixes or inherent variation in the ultrasonic velocity measurements.

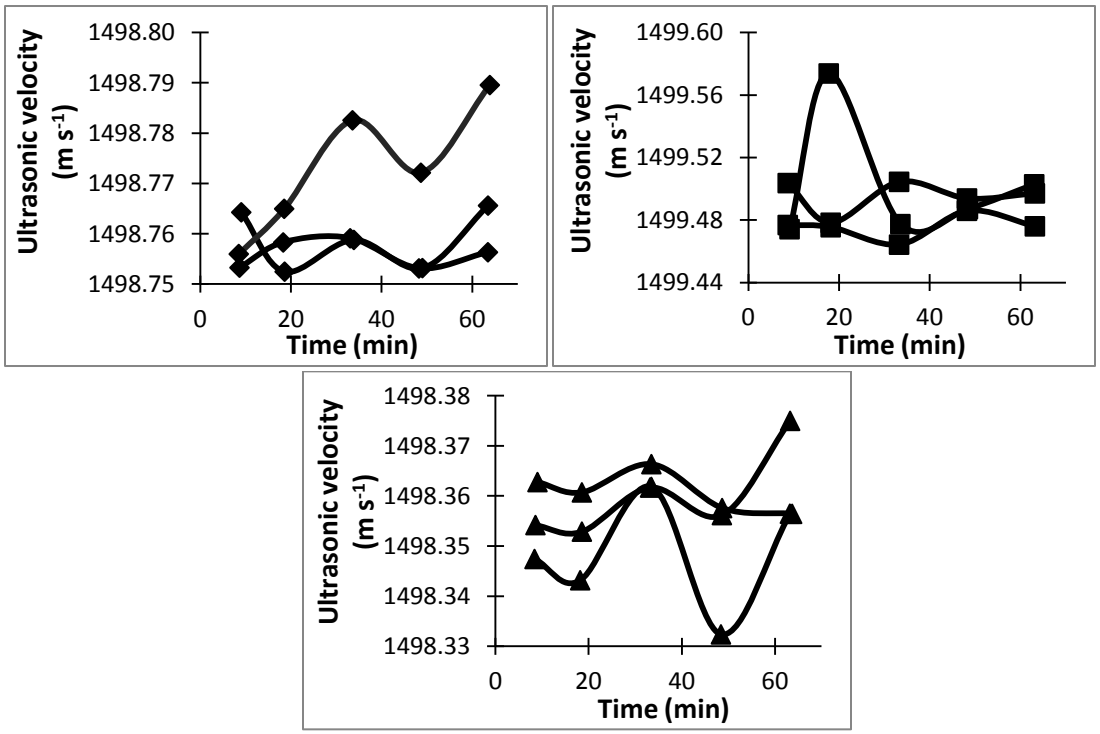

Fig. 8.7. Time dependency of ultrasonic velocity of the three replicates of mixed solutions of MLV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 3.75 mg g^{-1} concentration. MLV-A (◆), MLV-C (■) and MLV-D (▲).

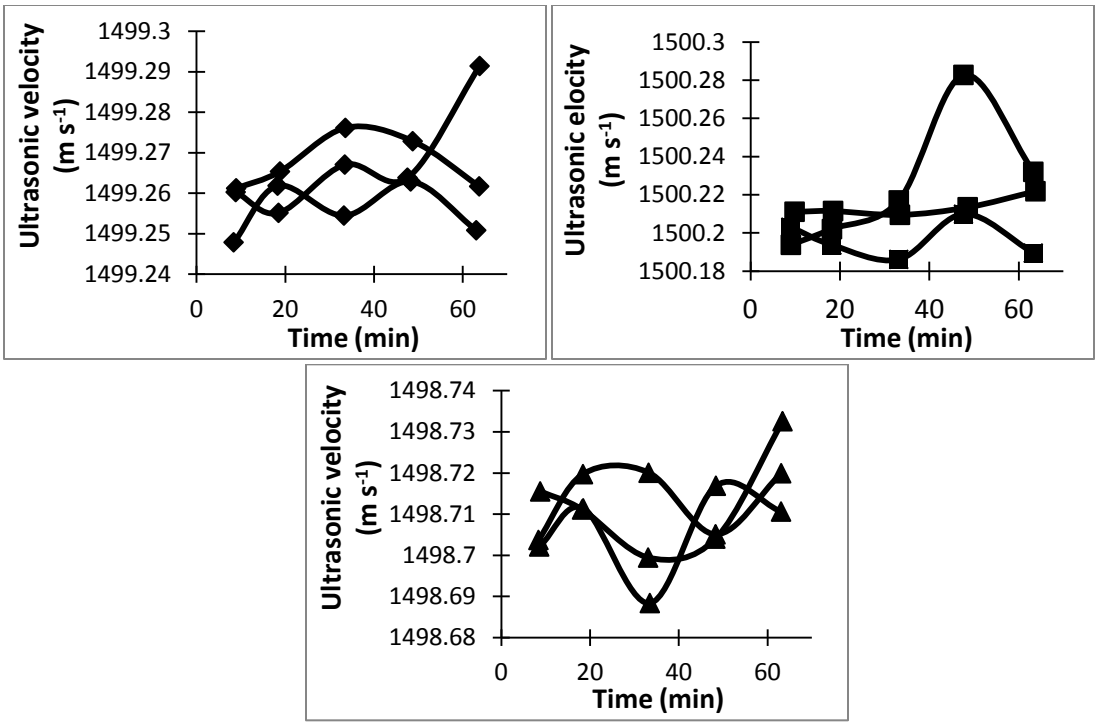

Fig. 8.8. Time dependency of ultrasonic velocity of the three replicates of mixed solutions of MLV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 5 mg g^{-1} concentration. MLV-A (◆), MLV-C (■) and MLV-D (▲).

To evaluate whether the peptides interact with the model membrane, ultrasonic velocity difference of the liposome-dairy peptide mixes was compared with the ultrasonic velocity difference obtained from serial dilutions of liposomes and dairy peptides, the results shown in sections 6. and 7. The ultrasonic velocity difference (ΔU) is calculated by the subtraction of the ultrasonic velocity of the solvent (U_0) from the ultrasonic velocity of the solution (U_S) as $\Delta U = U_S - U_0$. The ultrasonic velocity difference is the value corresponding to the change in ultrasonic velocity of the water caused by addition of the solutes and their interactions with the water molecules in the solution. If the liposomes and dairy peptide in the mixes don't have any interactions, then the volumetric properties should be additive. Ultrasonic velocity of the liposome-dairy peptide mixes were compared with the mathematical simple

addition of the ultrasonic velocity corresponding to the concentration of each material in the mixes (from sections 6. and 7).

Because of slightly different concentrations, and the almost perfect linearity between ultrasonic velocity and concentration, the ultrasonic velocity of the liposomes in solution and dairy peptides in solution were calculated using the linear regression obtained from ultrasonic velocity measurements of serial dilutions of the liposomes and dairy peptides. The sum of values from the liposomes and peptides is going to be called the “additive value”. Hianik et al. (2011) pointed out that the ultrasonic velocity, specific volume and compressibility are additive parameters.

Because the ultrasonic velocity of the mixes was measured as a function of time, and no real differences were apparent, the lowest and the highest values obtained were compared with the theoretical additive value. Error bars, based on the variability of the ultrasonic velocity measurements of ultrapure water in both cells of the Resoscan System (TF Instruments Inc., Germany) (Fig. 8.9.), were assigned to the additive value.

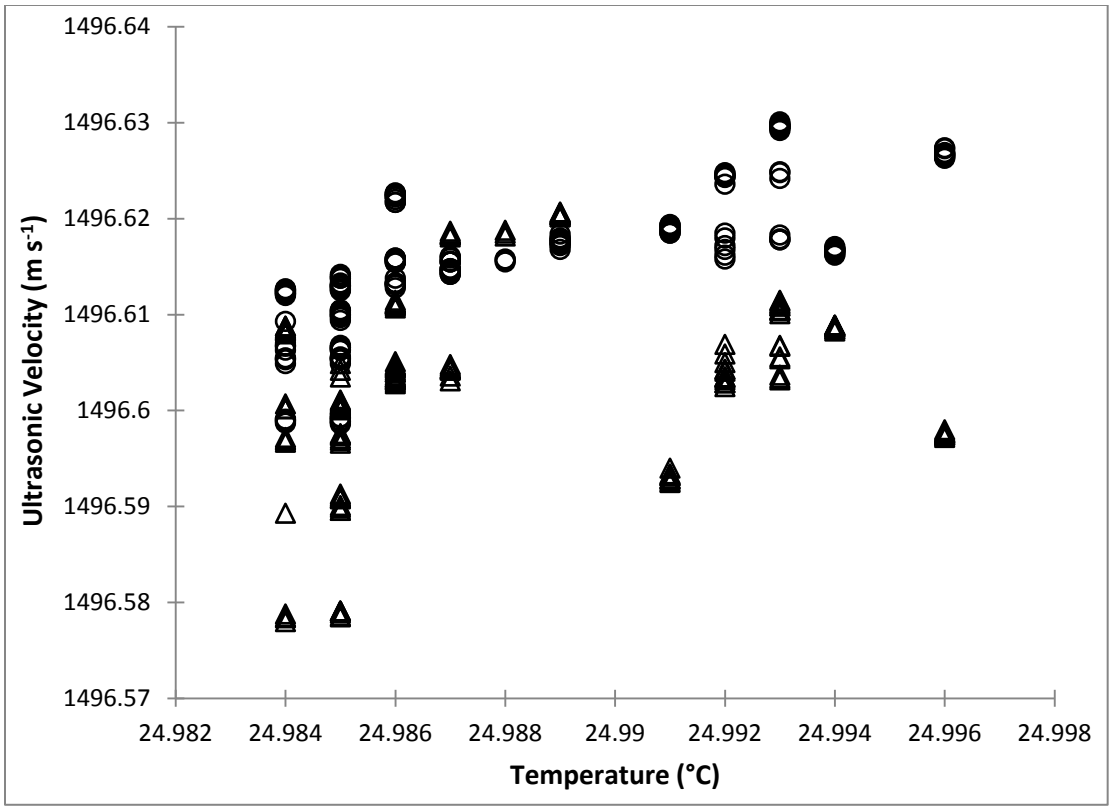

Fig. 8.9. Variation of ultrasonic velocity measurements of ultrapure water in both cells of the ResoScan System (TF Instruments Inc., Germany). Cell 1 (○), cell 2 (△).

The lowest and highest values of ultrasonic velocity of mixes of UV liposomes and dairy peptides A and C at 3.75 mg g^{-1} (Fig. 8.10.) were lower than the additive values calculated. The ultrasonic velocity additive values of mixes of UV and dairy peptide D at 3.75 mg g^{-1} (Fig. 8.10.) were between the highest and the lowest ultrasonic velocity. The same behavior was seen for mixes of UV and dairy peptide A, C and D at 5 mg g^{-1} (Fig. 8.11.). Therefore, dairy peptides A and C interact with the UV liposomes, whereas dairy peptide D does not.

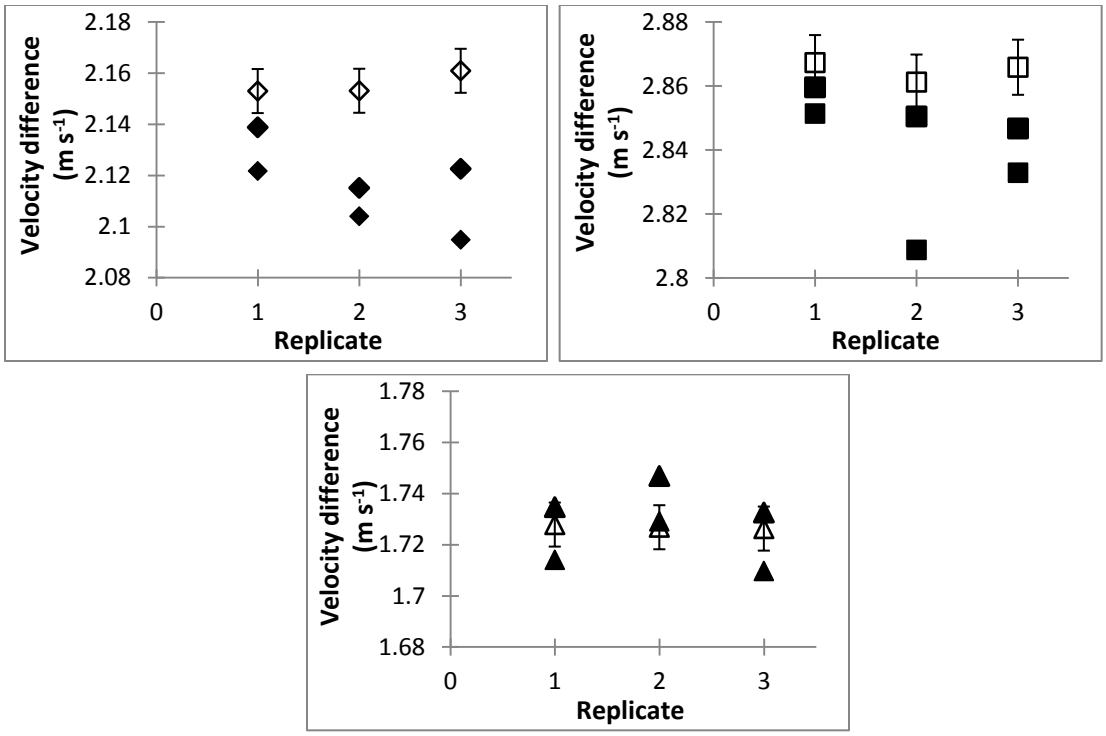

Fig. 8.10. Additive value compared with the experimental lowest and highest values of ultrasonic velocity difference of the three replicates mixes of UV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 3.75 mg g^{-1}. UV-A (◆), UV-C (■) and UV-D (▲).

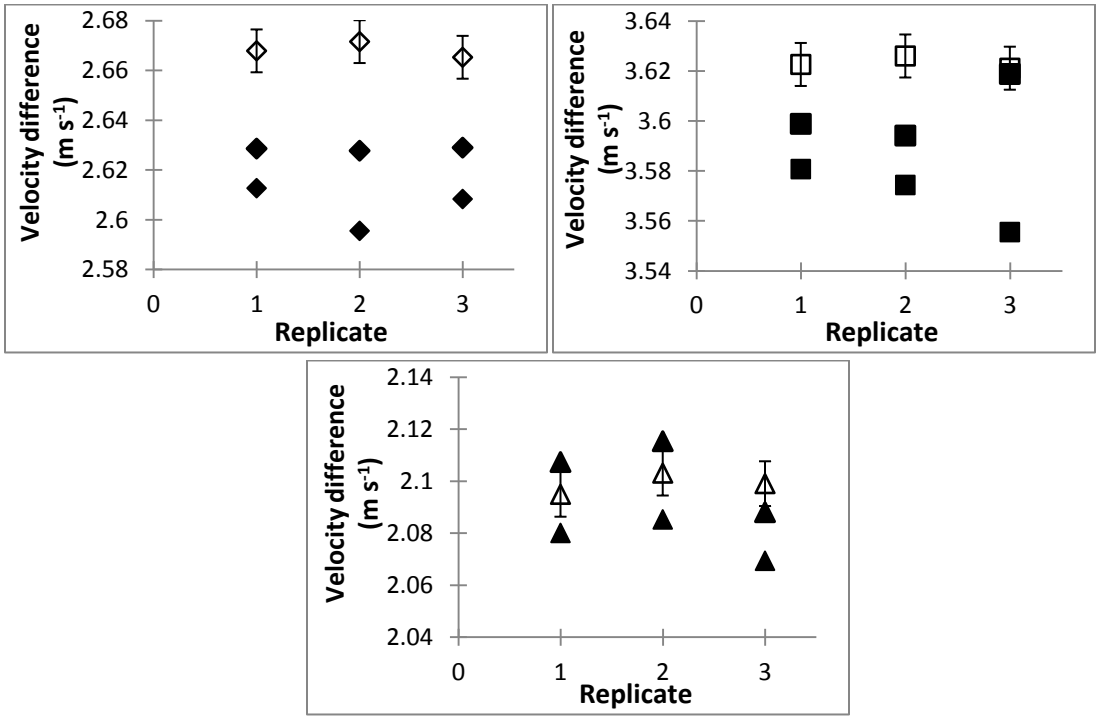

Fig. 8.11. Additive value compared with the experimental lowest and highest values of ultrasonic velocity difference of the three replicates mixes of UV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 5 mg g^{-1}. UV-A (◆), UV-C (■) and UV-D (▲).

The additive values of ultrasonic velocity of mixes of MLV liposomes and dairy peptide A and D at 3.75 mg g^{-1} and 5 mg g^{-1} (Fig. 8.12.) were higher than the experimental values (lowest and highest), therefore, dairy peptides A and D interact with MLV liposomes. For the mixes of MLV liposomes and dairy peptide C, the additive values were higher than the experimental values (lowest and highest) in one replicate at 3.75 mg g^{-1}, and in two replicates at 5 mg g^{-1} (Fig. 8.13.), suggesting that there might be a certain interaction, but this was not clear from the ultrasonic velocity results.

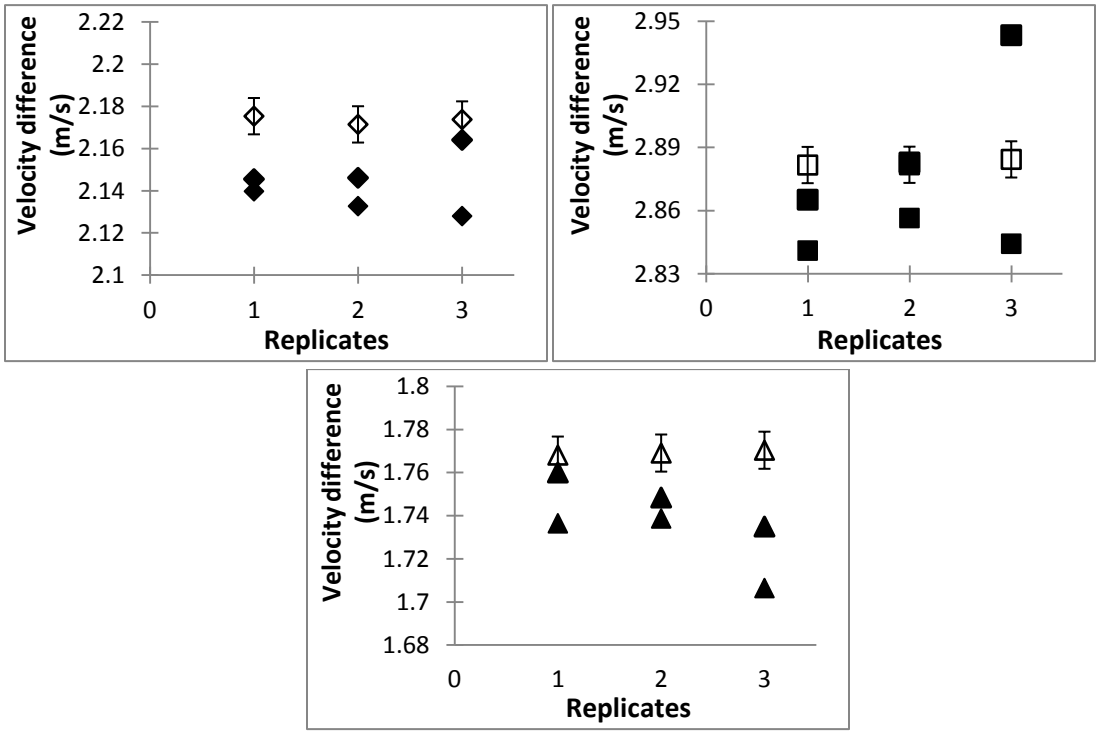

Fig. 8.12. Additive value compared with the experimental lowest and highest values of ultrasonic velocity difference of the three replicates of MLV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 3.75 mg g^{-1}. MLV-A (◆), MLV-C (■) and MLV-D (▲).

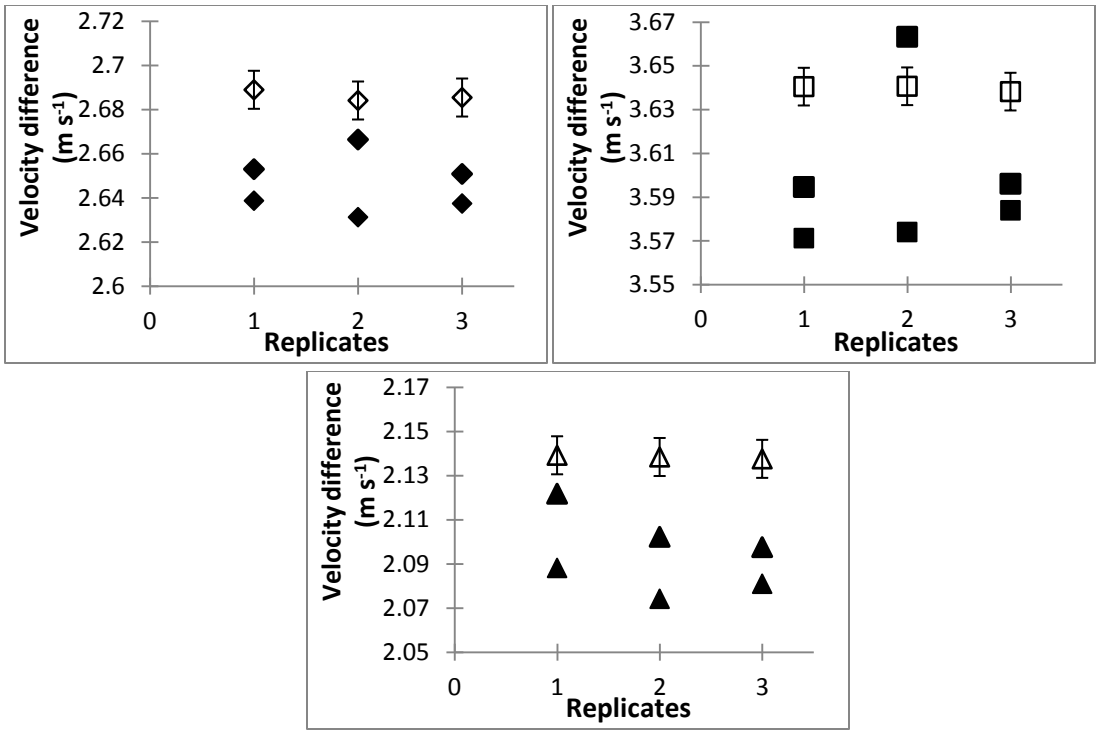

Fig. 8.13. Additive value compared with the experimental lowest and highest values of ultrasonic velocity difference of the three replicates of MLV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 5mg g^{-1}. MLV-A (◆), MLV-C (■) and MLV-D (▲).

8.2. Density Results

Density (ρ) of the mixes of UV liposomes at 15 mg g^{-1} with dairy peptides A, C and D at 3.75 mg g^{-1}, and with dairy peptides A, C and D 5 mg g^{-1}, as a function of time are shown in Fig. 8.14. and 8.15., respectively. In the case of the mixes of UV liposomes and dairy peptide A just two replicates were analysed.

Density of UV liposomes at 15 mg g^{-1} and dairy peptides A, C and D at 3.75 mg g^{-1} mixes (Fig. 8.14.) was seen to increase over time, especially with dairy peptide D. Nevertheless, variability in the density results did not allow a good determination for a value of the specific increase. The same tendency of increase over time was seen in the mixes of UV liposomes and dairy peptides A, C and D at 5 mg g^{-1} (Fig. 8.15.), although the increase was a little bit smaller.

Because the three replicates prepared for each mixed solution had slightly different concentration, as explained previously, and also because the measurements were done at slightly different times, the behavior of each replicate was independently graphed in order to analyse the difference according to a given replicate.

Density by replicates graphed independently (Fig. 8.16. and 8.17.) showed a small tendency to increase over time for the UV and dairy peptide A, C and D at 3.75 mg g^{-1}. Although a tendency to increase was evident over the whole time, 65 min, the change included variation in density within the 65 min of measurements that did not allow certainty in concluding whether the tendency to increase is real or just caused by the inherent variability of the measurements. A similar conclusion could be drawn for the mixes of UV with dairy peptides at a concentration of 5 mg g^{-1} (Fig. 8.17.).

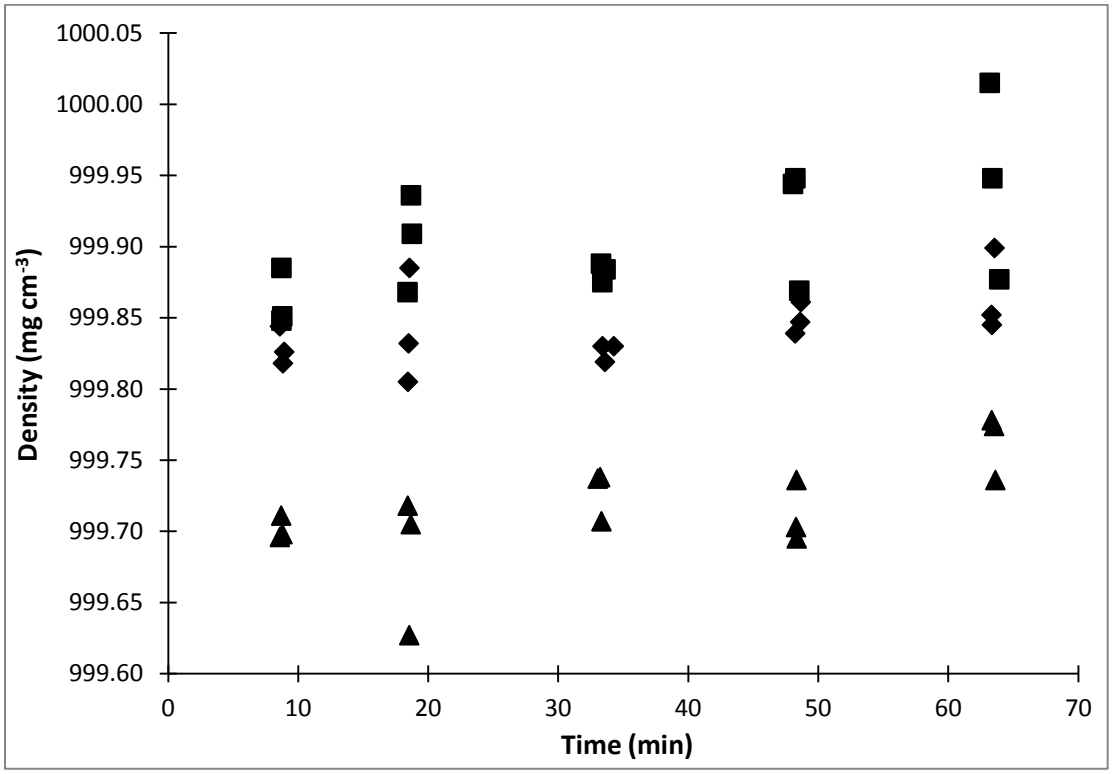

Fig. 8.14. Density of mixes of UV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 3.75 mg g^{-1} concentration. UV-A (◆), UV-C (■) and UV-D (▲).

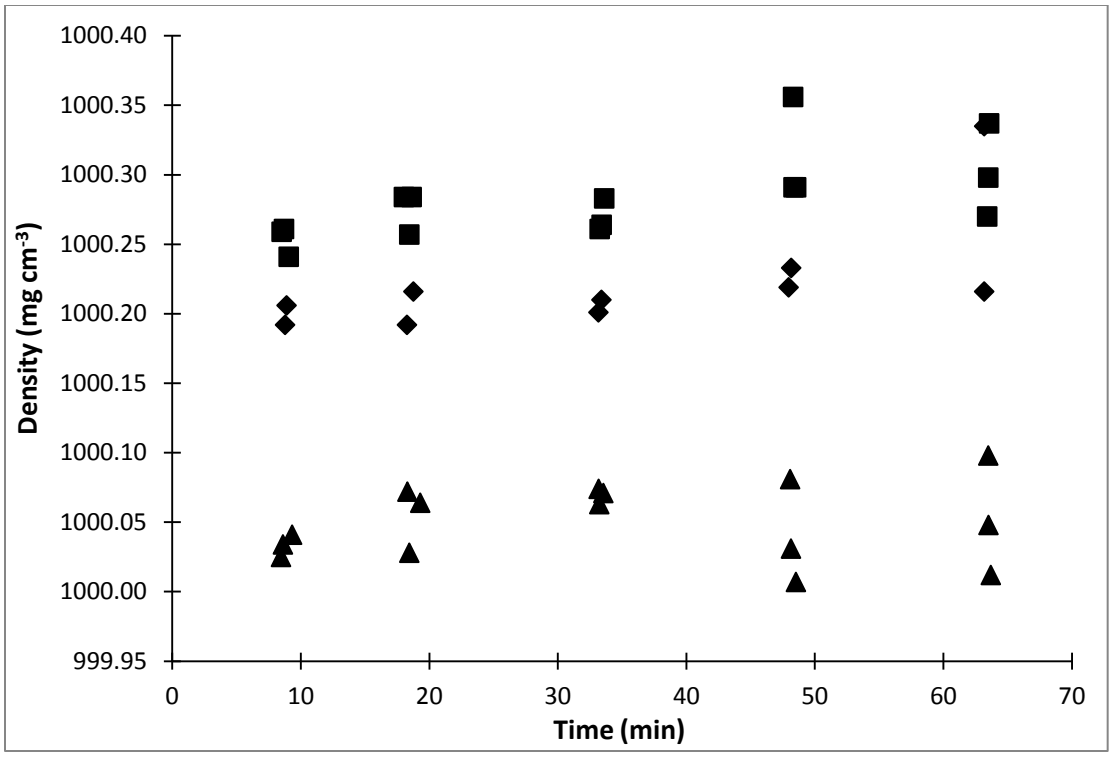

Fig. 8.15. Density of mixed solutions of UV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 5 mg g^{-1} concentration. UV-A (◆), UV-C (■) and UV-D (▲).

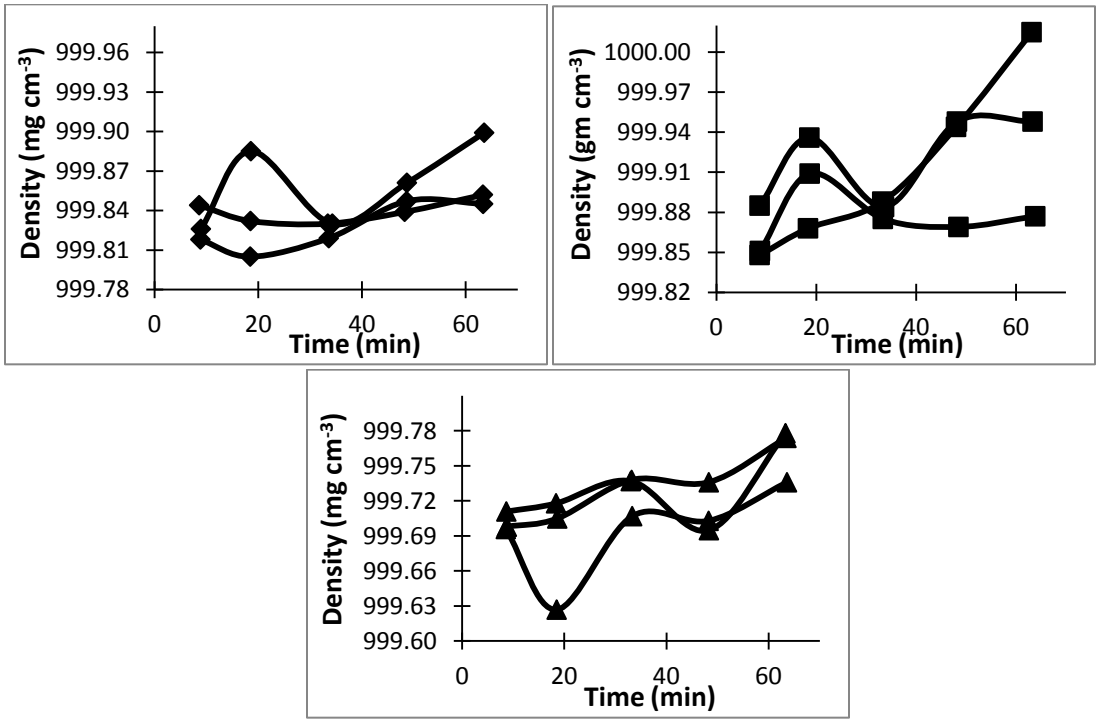

Fig. 8.16. Time dependency of density of the three replicate mixes of UV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 3.75 mg g^{-1} concentration. UV-A (◆), UV-C (■) and UV-D (▲).

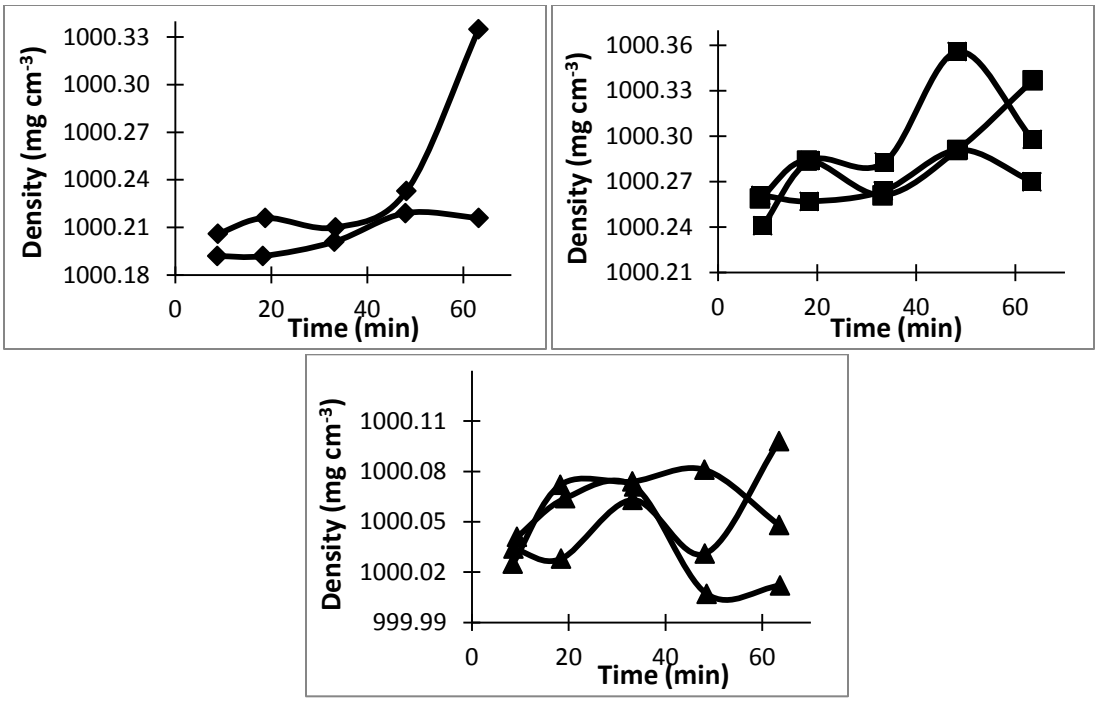

Fig. 8.17. Time dependency of density of the three replicate mixes of UV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 5 mg g^{-1} concentration. UV-A (◆), UV-C (■) and UV-D (▲).

Density (ρ) of the mixes of MLV liposomes at 15 mg g^{-1} with dairy peptides A, C and D at 3.75 mg g^{-1} and with dairy peptides A, C and D at 5 mg g^{-1}, as a function of time are shown in Fig. 8.18. and 8.19.

Density of mixed solutions of MLV liposomes at 15 mg g^{-1} and dairy peptides A, C and D at 3.75 mg g^{-1} (Fig. 8.18.) was seen to have an increasing-decreasing tendency through the 65 min of the experiment. Increase tendency was seen after the first measurement and changing to opposite tendency for the final two measurements. The behavior was more evident for the mix with dairy peptide D. Variability in the density results did not allow a reliable determination of the value of the specific increase. The same increasing tendency over time was seen in the mixes of MLV liposomes and dairy peptides D at 5 mg g^{-1} (Fig. 8.19.). However, MLV liposomes and dairy peptide A and C at 5 mg g^{-1} mixes were seen with increasing tendency of density over time, a tendency which was more evident for the mix with dairy peptide A.

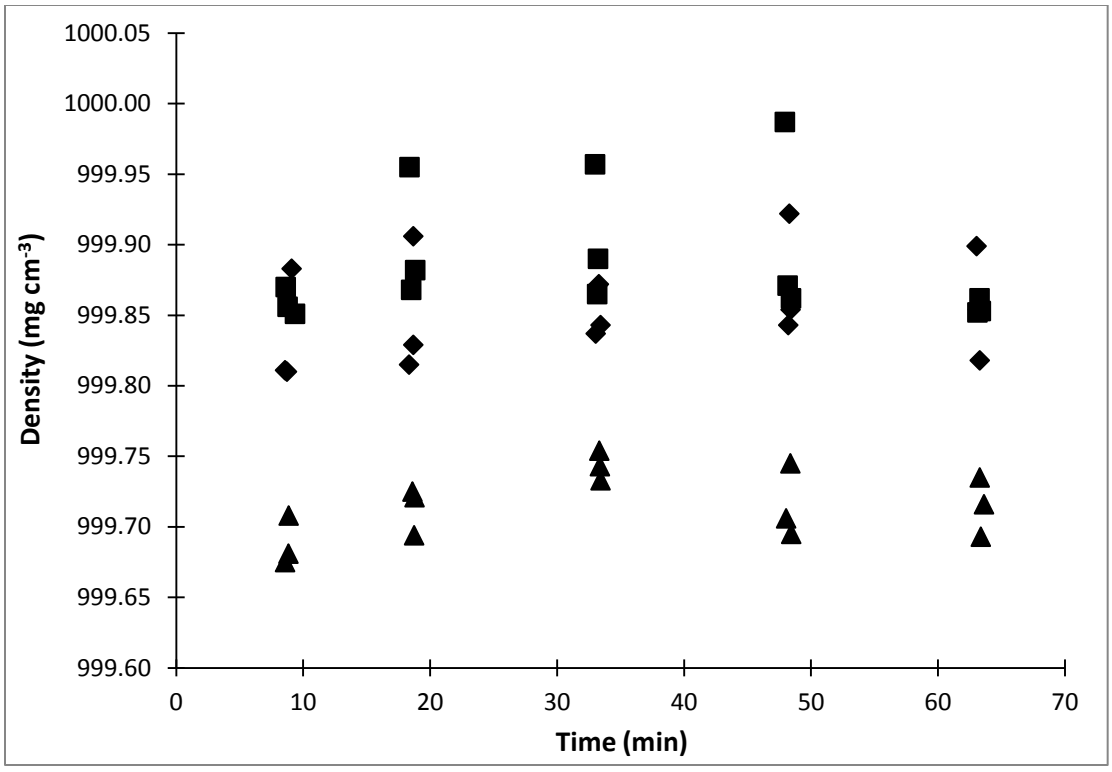

Fig. 8.18. Density of mixes of MLV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 3.75 mg g^{-1} concentration. MLV-A (◆), MLV-C (■) and MLV-D (▲).

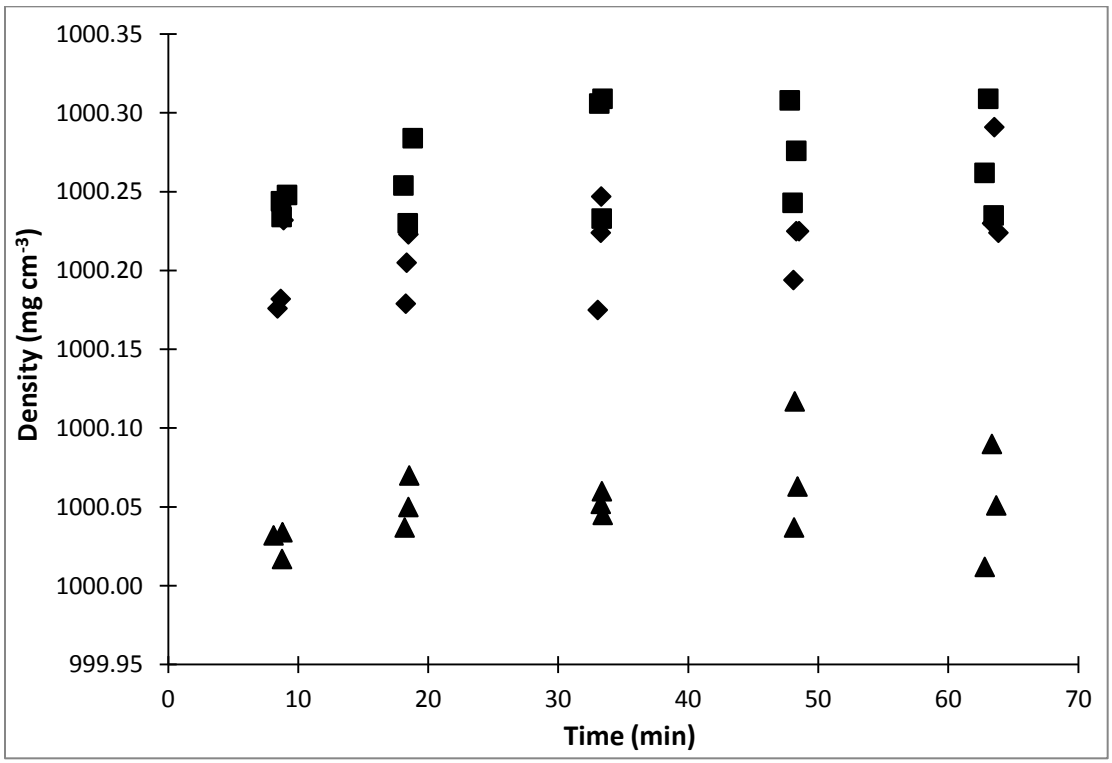

Fig. 8.19. Density of mixes of MLV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 5mgg^{-1} concentration. MLV-A (◆), MLV-C (■) and MLV-D (▲).

As has been done with previous experiments, because the three replicates prepared for each mixed solution had slightly different concentration and also because the measurements were done at slightly different times, the behavior of each replicate was graphed independently.

Density of the mixes of MLV and dairy peptide A, C and D at 3.75 mg g^{-1} by replicates graphed independently (Fig. 8.20.) showed the same increase-decrease tendency through time for dairy peptides C and D, but a rising tendency for replicates was seen for dairy peptide A.

Density of the MLV and dairy peptide A, C and D at 5 mg g^{-1} mixes by replicates graphed independently (Fig. 8.21.) showed again an increase-decrease tendency in one replicate of dairy peptide C, but it didn´t showed any constant tendency between replicates for dairy peptides A and D.

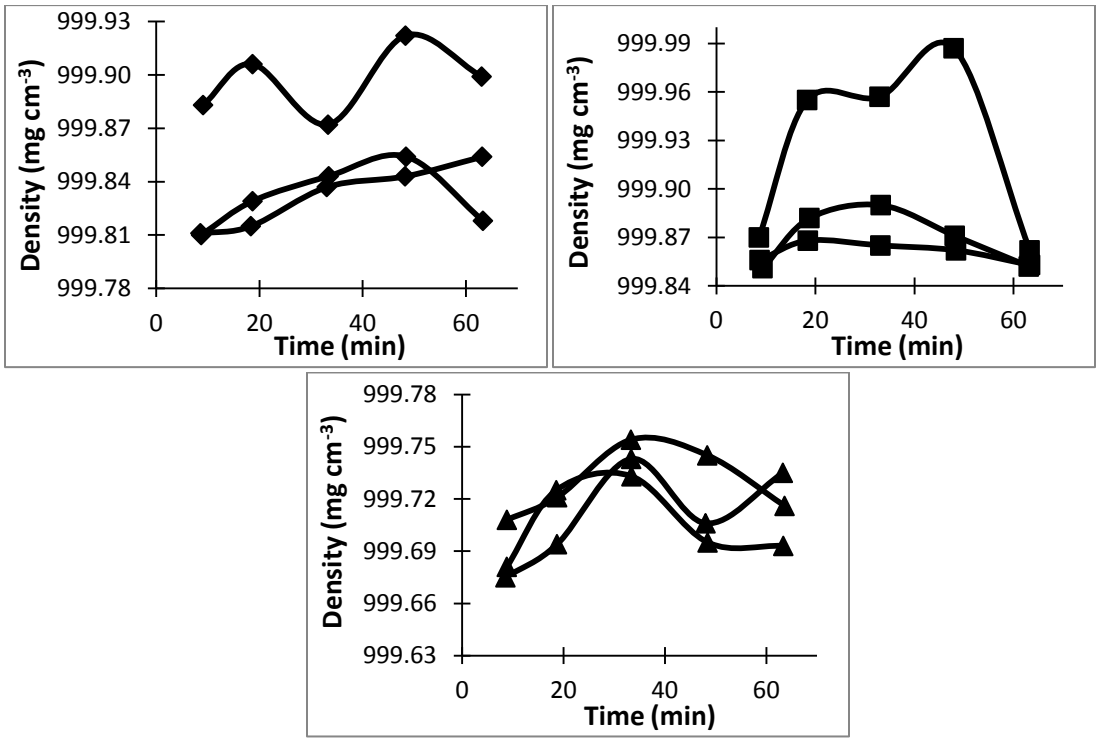

Fig. 8.20. Density of the three replicate mixes of MLV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 3.75 mg g^{-1}. MLV-A (◆), MLV-C (■) and MLV-D (▲).

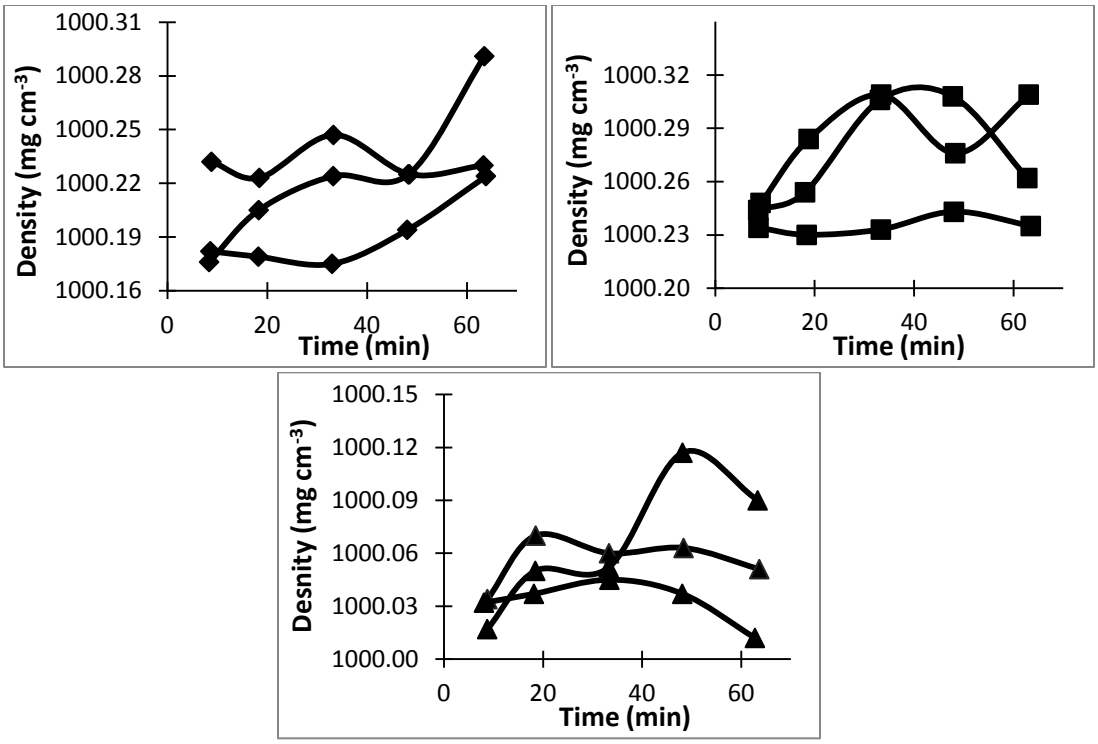

Fig. 8.21. Density of the three replicate mixes of MLV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 5 mg g^{-1}. MLV-A (◆), MLV-C (■) and MLV-D (▲).

As was done with ultrasonic velocity results, density of the mixes of liposomes and dairy peptides were compared with density results obtained from liposome serial dilutions and dairy peptide serial dilutions, the results shown in sections 6. and 7. The density difference ($\Delta\rho$) is calculated by the subtraction of the density of the solvent (ρ_0) from the density of the solution (ρ_S) as $\Delta\rho = \rho_S - \rho_0$. The density difference is the value corresponding to the change in density of the water caused by addition of the solutes and their interactions with the water molecules in the solution. This assumes that the volumetric properties of the liposomes and dairy peptide in the mixes should be additive if they don't have any interaction. Density of the liposomes-dairy peptide mixes was compared with the mathematical simple addition of the density corresponding to the concentration of each component in the mixes.

Density of liposomes in solution and dairy peptides in solution was calculated using the linear regression obtained from the density measurements conducted on serial dilutions of the liposomes and dairy peptides and is going to be called the “additive value”. Error bars, based on the variability of density measurements of ultrapure water in the DMA 5000 density meter (Fig. 8.22.), were added to the means of the additive value.

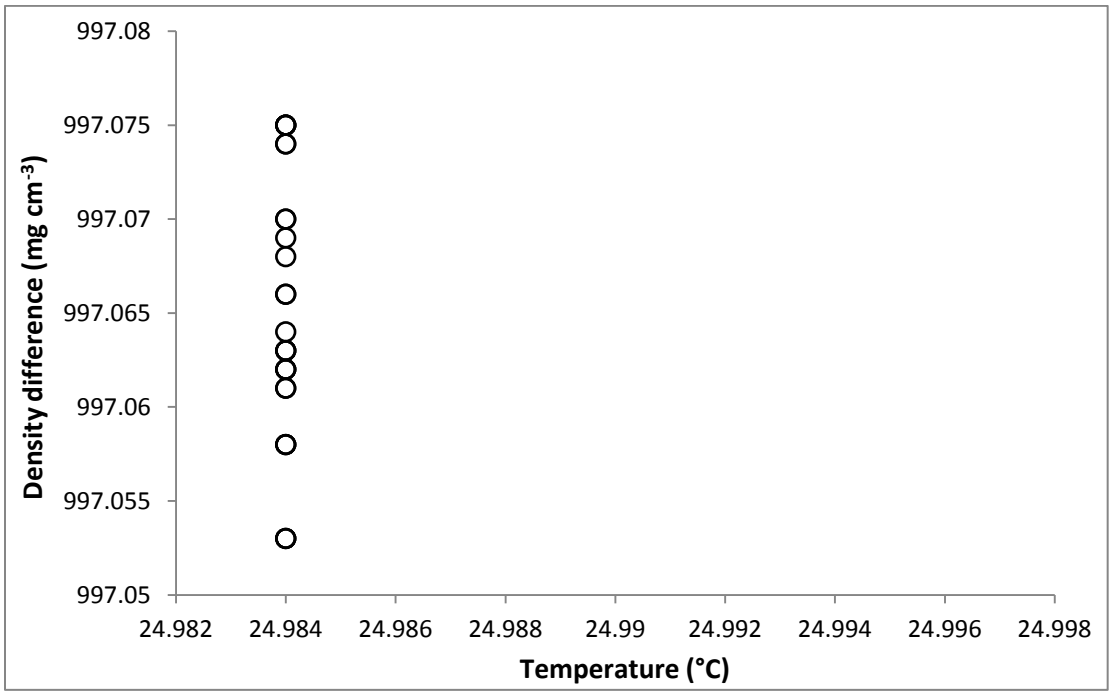

Fig. 8.22. Variation of density measurements of ultrapure water in the density meter DMA 5000 (Anton Paar, Austria).

Because density of the mixed solutions was measured as a function of time, the lowest and the highest values obtained were selected to be compared with the additive value.

Density additive values of mixes of UV-dairy peptide A, C and D at 3.75 mg g^{-1} (Fig. 8.23.) were between the lowest and highest values measured, although for dairy peptide A and C the additive values of density were closer to the lowest values. Similar behavior was seen for density additive values of UV-dairy peptide A, C and D at 5 mg g^{-1} mixes (Fig. 8.24.).

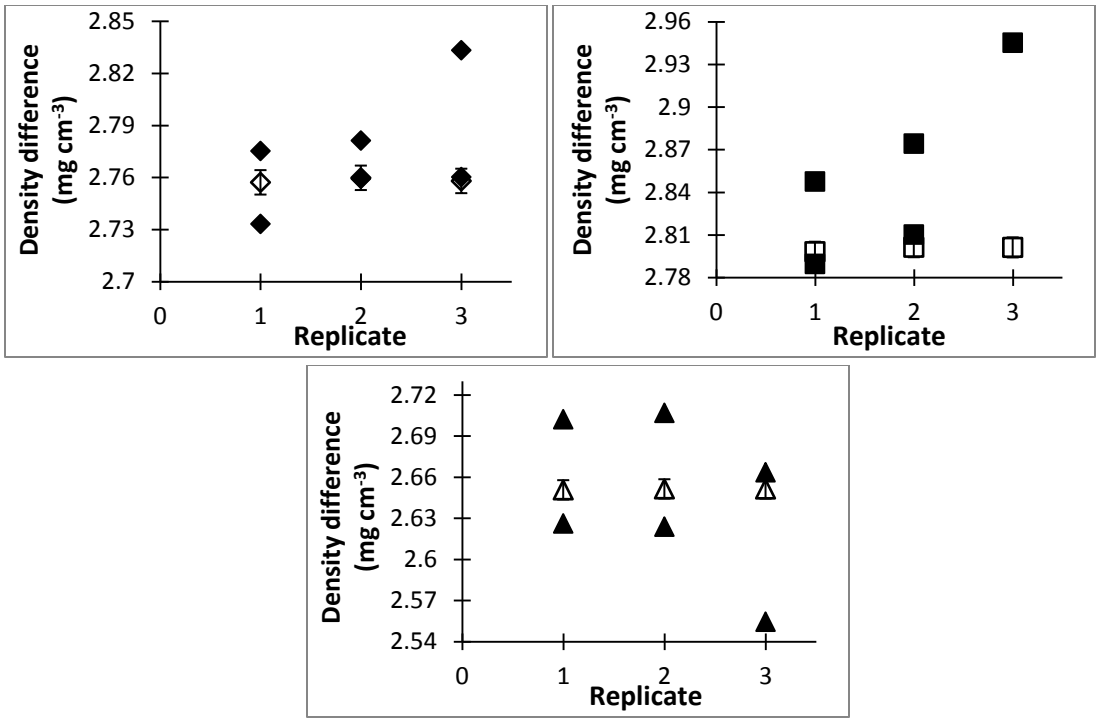

Fig. 8.23. Additive value compared with the experimental lowest and highest values of density difference of the three replicate mixes of UV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 3.75 mg g^{-1}. MLV-A (◆), MLV-C (■) and MLV-D (▲).

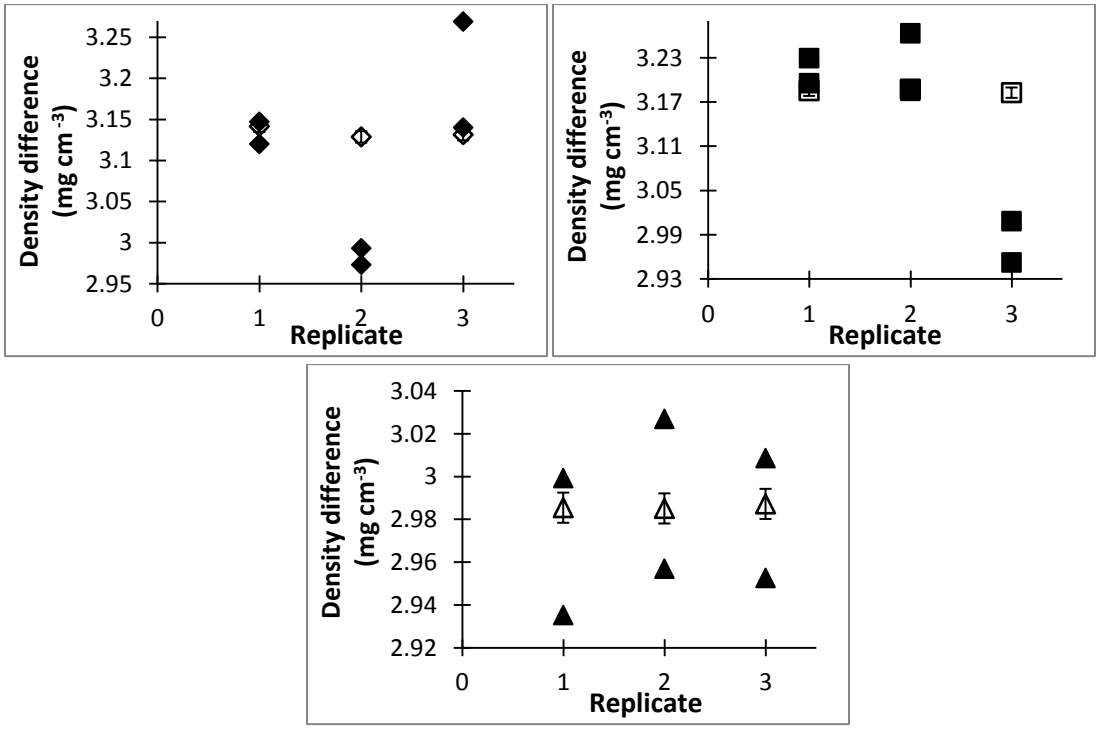

Fig. 8.24. Additive value compared with the experimental lowest and highest values of density difference of the three replicate mixes of UV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 5mg g^{-1}. MLV-A (◆), MLV-C (■) and MLV-D (▲).

Density additive values of mixes of MLV-dairy peptide A and D at 3.75 mg g^{-1} (Fig. 8.25.) were between lowest and highest values measured in two of the three replicates, but density additive values for dairy peptide C were higher than the lowest and highest values in two replicates.

Density additive values of mixes at 5 mg g^{-1} dairy peptide concentration (Fig. 8.26.) were between the highest and the lowest densities.

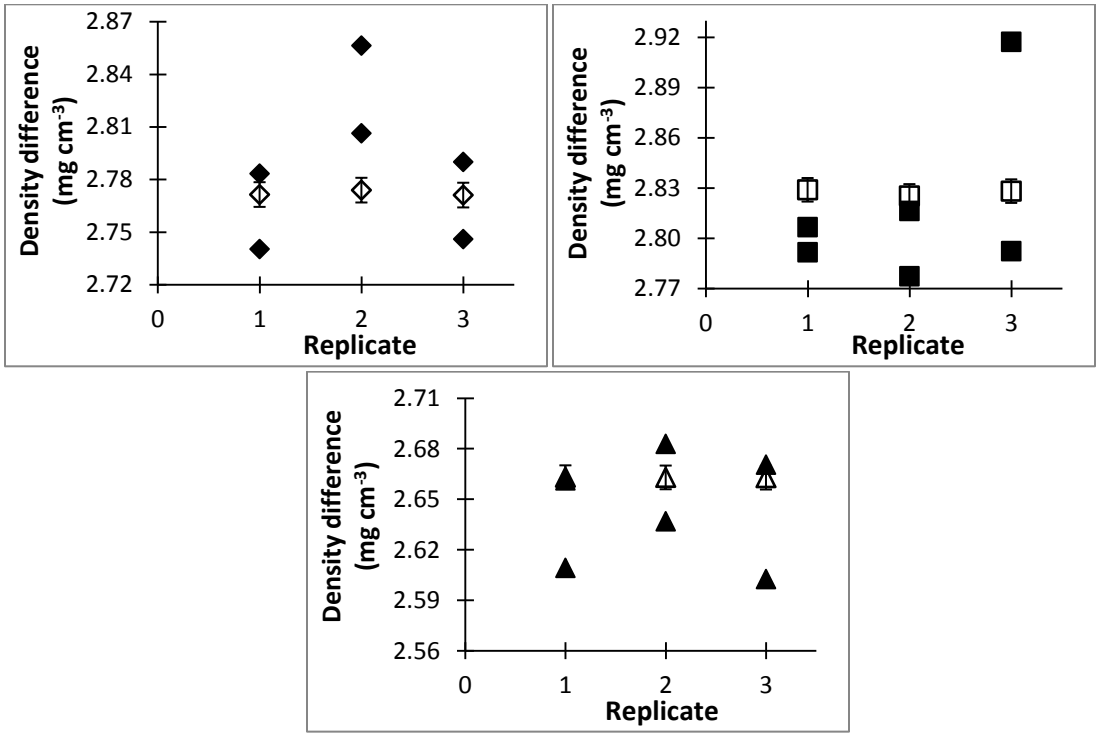

Fig. 8.25. Additive value compared with the experimental lowest and highest values of density difference of the three replicate mixes of MLV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 3.75 mg g^{-1}. MLV-A (◆), MLV-C (■) and MLV-D (▲).

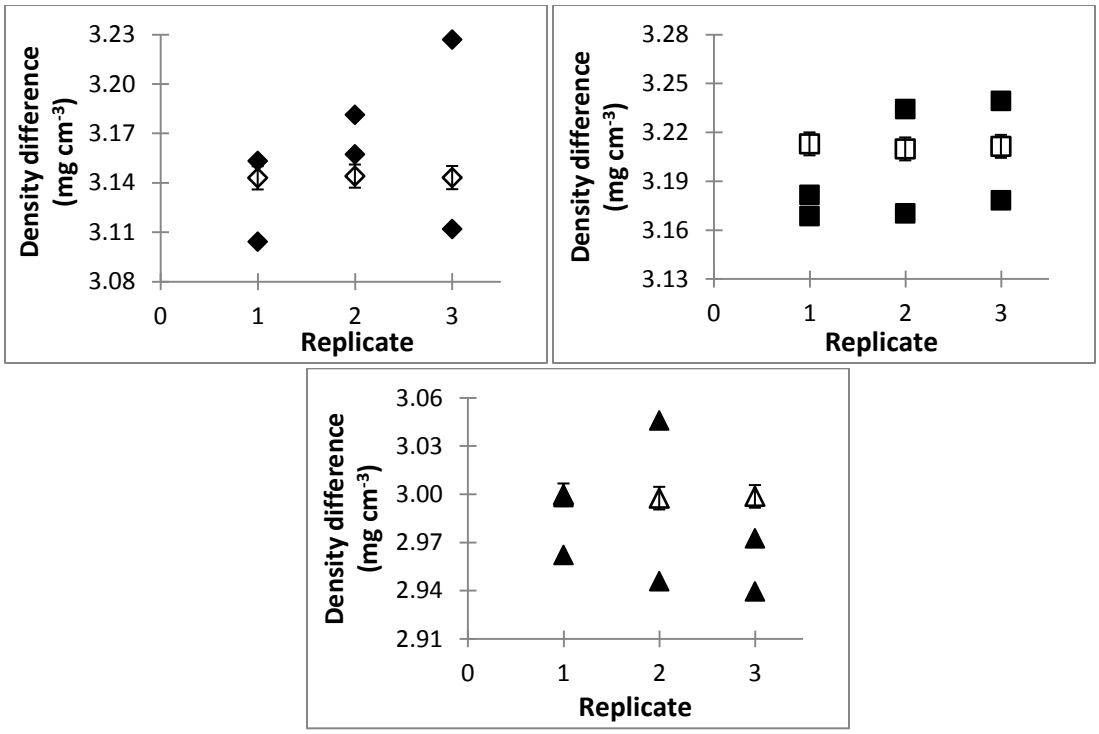

Fig. 8.26. Additive value compared with the experimental lowest and highest values of density difference of the three replicate mixes of MLV liposomes at 15 mg g^{-1} and dairy peptide, A, C and D at 5 mg g^{-1}. MLV-A (◆), MLV-C (■) and MLV-D (▲).

8.3. Discussion

The interactions between cell membrane and peptides are very important regarding the physiological function of peptides (Mozsolits et al., 1999; Hohlweg et al., 2012). Many bioactive peptides interact with the cell membrane as part of their mechanism of exerting their function or in getting transported across the cell membrane (Ramaswami et al., 1992; Creuzenet et al., 1997; Hianik et al., 1997; Krivanek et al., 2001, Romanowski et al., 2002; Hunter et al., 2005; Matos et al., 2008; Orioni et al., 2009). Ultrasonic velocity and density measurements and determination of compressibility has been reported to be very sensitive to the changes involved in protein–liposome and peptide-liposome interactions (Hianik et al., 1997; Hianik et al., 1999; Krivanek et al., 2001; Krivanek et al., 2002; Rybar et al., 2007; Hianik et al., 2011).

8.3.1. Ultrasonic Velocity and Density over Time

Ultrasonic velocity of UV liposomes and dairy peptide mixed solutions did not show any clear change or tendency through the time of the experiment, but a slight tendency to increase in UV-dairy peptide A at 5 mg g^{-1} mixes was observed. Generally, ultrasonic velocity variation over time was small and it was not possible to clearly differentiate time dependent changes from inherent variation in the measurements. Kinetics of peptide-liposome interactions have been reported to be related to the amino acid sequence of the peptide, which is related to peptide hydrophobicity; other important factors that influenced the peptide-liposome interactions are the length of the peptide and the nature of the lipid bilayers (Creuzenet et al., 1997). Mozsolits et al. (1999) reported interaction times for different bioactive peptides interacting with a phosphatidylcholine monolayer to be in the range between 5 and 40 min. Zorko & Langel (2005) reported times between 5 to 60 min for different peptides to penetrate the cell membrane.

Ultrasonic velocity of MLV liposomes and dairy peptides mixes showed similar results to UV-dairy peptides mixes, without any clear change or tendency over time. Analysis of the ultrasonic velocity by replicate also didn´t show any clear change or tendency over time.

Density of the UV liposome-dairy peptide mixes showed a slight tendency to increase over time for UV-dairy peptide mixes at 3.75 and 5 mg g^{-1}, and this tendency was more evident for UV-dairy peptides at 3.75 mg g^{-1}. Density changes over time for MLV liposome-dairy peptide mixes at 3.75 mg g^{-1} showed an increase after the first measurement and the opposite behavior in the last two measurements. The same behavior was seen for the density of MLV liposome-dairy peptide D mixes at 5 mg g^{-1}, while the density of MLV liposome-dairy peptide A mixes and liposome-dairy peptide C mixes at 5 mg g^{-1} showed an increasing tendency. Density of

liposomes-dairy peptide mixes had several results that suggest density could change over time, and additionally that changes in density over time showed a different tendency depending on the unilamellar (UV) or multilamellar (MLV) nature of the liposomes. However, it was not possible to determine with certainty how much of the change was due to inherent variability of the measurements.

8.3.2. Comparison of Ultrasonic Velocity and Density Additive Values with Experimental Values of the Liposomes-Dairy Peptide Mixes

The comparison between lowest and highest experimental ultrasonic velocity values of UV-dairy peptide mixes with the ultrasonic velocity additive value, showed that for dairy peptide A and C the additive values were higher than all ultrasonic measurements of the mixes, suggesting certain interactions between the liposomes and dairy peptides A and C. Krivanek et al. (2002) studied the protein-liposome interactions and reported that the interaction caused a decrease in the ultrasonic velocity, caused by a decreased hydration shell of the peptides and liposomes due to their interaction causing water molecules to reorder. Hianik et al. (1997) found that bioactive peptides that interact with liposomes caused a decrease in ultrasonic velocity at 24°C, but also found that the change in the ultrasonic velocity is influenced by the structural order of the liposomes, which is disrupted by the peptides and affected by the temperature. The mixes of UV liposomes and dairy peptide D showed the additive values of ultrasonic velocity were within the range of the experimental ultrasonic velocity measured for the mixes, implying that there is no evidence of interaction between UV liposomes and dairy peptide D. Depending on the peptide amino acid sequence and the charge of the peptides, the interactions between peptides and liposomes can be caused by hydrophobic effects or electrostatic forces (Creuzenet et al., 1997; Seelig, 2004; Khandelia et al., 2008; Yu et al., 2009). Conformational changes in the

peptides, which can change the hydration of the peptides, as a result of the interaction with lipid bilayer have been reported (Seelig, 2004; Sabaté et al., 2005; Hianik et al., 2011).

Comparison of MLV-dairy peptide mixes showed that additive ultrasonic velocity values were generally higher than ultrasonic velocity measured for the mixes of the three dairy peptides, which suggests that interactions between MLV liposomes and dairy peptides A, C and D cause a decrease in the extent of the water shell around the liposome and dairy peptide as a consequence of the interaction between them. A similar decrease in ultrasonic velocity was reported by Krivanek et al. (2002), who also suggested an aggregation effect between the liposomes could be involved as a consequence of the interaction between liposomes and peptides. Disturbance in the lipid bilayer as a consequence of the interaction with peptides has been reported (Colotto et al., 1993; Hianik et al., 1997, Krivanek et al., 2001; Rybar et al., 2007; Matos et al., 2008; Orioni et al., 2009) and might be also related with changes in the physical properties of the liposomes.

Contrary to what was seen with ultrasonic velocity, density additive values of UV-dairy peptides and MLV-dairy peptides mixes were generally within the range of density values measured for the mixes, giving no evidence, based on density changes, of interaction between liposomes and peptides.

Based on the comparison between the additive values of ultrasonic velocity and the experimental values of the mixed solutions it is possible to suggest that certain interactions take place between UV and MLV liposomes and dairy peptides in aqueous solution, except for UV and dairy peptide D. Additionally, differences in amino acid sequence and structural differences between dairy peptides A, C and D, that have been commented on in section 6.5., will affect their interactions with liposomes. However assessments of density differences of liposome-dairy peptide mixes did not show differences between the peptides.

9. Conclusions and Recommendations

9.1. Conclusions

The volumetric properties of three dairy bioactive peptides and two hemp seed bioactive peptides and two types of liposomes were determined and discussed. Based on the ultrasonic velocity and density measurements, the apparent specific volume and apparent specific adiabatic compressibility were determined, as well as the partial specific volume and partial specific adiabatic compressibility from extrapolation to zero concentration.

The preliminary analysis conducted with KCl serial dilutions showed the good precision achievable in the ultrasonic velocity measurements with the ResoScan System (TF Instruments Inc., Germany) and employing a gravimetrical method to prepare the solutions, which has the advantage of providing high accuracy for the concentration of the solutions. The coefficient of variation of the ultrasonic velocity measurements between subsamples was very low, varying between $< 0.0005\%$ and $< 0.005\%$. The coefficient of variation of replicates, which assesses mainly the precision in the preparation of the samples, was $< 0.0035\%$. The apparent specific volume and apparent specific adiabatic compressibility of KCl was seen to have a continuous decrease from the highest to the lowest concentration used. This decrease was attributed to an increase of the electrostriction effect as the KCl ions were diluted. The electrostriction effect might have increased due to a decrease in ion-ion interactions that promoted greater dissociation of the K^+ and Cl^- ions and hence increased the number of water molecules located in the water shell around the ions which are exposed to the ions' charge. The partial specific volume calculated for KCl was 0.359 $cm^3\ g^{-1}$, which is within the range reported in the literature, 0.354 -0.360 $cm^3\ g^{-1}$ (Zen, 1957, Monnin, 1989).

The ultrasonic velocity and density were both seen to have linear relationships against concentration in the hemp seed peptide serial dilutions. The apparent specific volume and apparent specific adiabatic compressibility of the 1 kDa peptides was seen to be independent of the concentration in the higher concentration range, but starting at concentrations between 5 and 3.75 mg mL^{-1} the apparent specific volume was seen to continuously decrease as the concentration diminished. The decrease was attributed to the high amount of salts and sugars associated with the peptides (up to 35%) that might increase the electrostriction effect as the solution was diluted. A similar effect perhaps at play, as was commented on for the KCl serial dilutions, was that greater electrostriction occurred with the disassociation of salt from the peptide molecules as the solution was diluted. A contrary effect, an increase in the apparent specific volume and apparent specific adiabatic compressibility was seen for the 3 kDa hemp seed peptides as the solution was diluted. A mechanism for this effect was not completely clarified, but was associated with a similar concentration dependence that has been reported by Benhardt & Pauly (1975) and Bull & Bresse (1979) and which was attributed to changes in the hydration of the peptides and changes in the pH as the solution was diluted.

The three dairy bioactive peptides were seen to have strong linear relationships between ultrasonic velocity and concentration and between density and concentration. The apparent specific volume and the apparent specific adiabatic compressibility of the dairy peptides were seen to be independent of concentration in the high concentration range, but starting at 5 mg mL^{-1}, a continuous decrease was seen in both parameters as the concentration diminished. This effect was attributed to three factors: 1) the dissociation of the salts “bonded“ to the peptide structures, 2) disassociation of the salt molecules that have disassociated from the peptide-salt complexes, and 3) an increase in the number of water molecules interacting with the peptide. These three factors might cause an increase in the degree of electrostriction

as the solution was diluted. The difference in specific volume and compressibility between the three dairy peptides was attributed to differences in amino acid sequence; these differences were compatible with the variation in solubility observed for the three dairy peptides. Therefore, I am concluding that between the three dairy peptides, dairy peptide C should have the higher amount of polar amino acids and the dairy peptide D the higher amount of non-polar amino acids. The partial specific volume was determined, with values of 0.635 $cm^3\ g^{-1}$, 0.515 $cm^3\ g^{-1}$ and 0.636 $cm^3\ g^{-1}$ for dairy peptides A, C and D, respectively; these values were similar to the partial specific volume values of small peptides reported by different authors, 0.497 $cm^3\ g^{-1}$ and 0.630 $cm^3\ g^{-1}$ (Hedwig, 1994), 0.567 $cm^3\ g^{-1}$ and 0.689 $cm^3\ g^{-1}$ (Chalikian et al., 1998), 0.507 $cm^3\ g^{-1}$ and 0.547 $cm^3\ g^{-1}$ (Schwitzer & Hedwig, 2004), or in between the values of small peptides and globular proteins, 0.70 $cm^3\ g^{-1}$ to 0.75 $cm^3\ g^{-1}$ (Bernhardt & Pauly, 1975; Bull & Bresse, 1979; Durchschlag, 1982; Gekko & Hasegawa, 1986; Chalikian et al., 1996; Sitorkin et al., 2012). The partial specific adiabatic compressibility values obtained in this study for the dairy peptides A (-21.9 $x10^{-6}\ bar^{-1}$), C (-59.0 $x10^{-6}\ bar^{-1}$) and D (-20.7 $x10^{-6}\ bar^{-1}$) were below the values of small peptides, -1.7 $x10^{-6}\ bar^{-1}$ and -6.69 $x10^{-6}\ bar^{-1}$ (Chalikian et al., 1998) and the values of globular proteins, 1 $x10^{-6}\ bar^{-1}$ to 11 $x10^{-6}\ bar^{-1}$ (Gekko & Hasegawa, 1986; Kharakoz & Sarvazyan, 1993; Chalikian et al., 1996). However, the vales reported by Chalikian et al. (1998) for tri-peptides were assessed with synthesized peptides containing no salts or other impurities and at higher concentrations than was reported for the dairy peptides here.

The UV and MLV liposomes were both seen to have linear relationships between ultrasonic velocity and concentration and between density and concentration. The apparent specific volume and the apparent specific compressibility was seen to be independent of concentration at the higher concentration range, but between 5 and 2.5 mg mL^{-1}, a continuous

decrease in apparent specific volume was seen; this decrease was attributed to an increase in hydration of the liposomes as the solutions were diluted.

On the basis of the assessments of the volumetric properties of the KCl, various bioactive peptides and the liposomes, some conclusions on the structuring of water can be drawn. The partial specific volume and partial specific adiabatic compressibility coefficient obtained for the different materials are shown in Table 9.1. The differences in the partial specific adiabatic compressibility coefficient between the KCl, bioactive peptides and liposomes were compatible with the differences observed for the partial specific volume. KCl and the five bioactive peptides were seen to have a negative compressibility at infinite dilution, which indicates an incompressible interior and a reduction in the compressibility of the water molecules located in the hydration shell around the solutes compared with the water molecules of bulk water. The reduction of the size of the water molecules in the hydration shell was likely caused by contraction of the volume of these water molecules caused by the electrostriction effect produced by the charges on the solute surface that is in contact with the water. The most negative compressibility at infinite dilution was seen for the KCl, suggesting that the solvated K^+ and Cl^- ions in aqueous solution have an incompressible interior, and had a more negative value compared with the bioactive peptides because it had the higher amount of water molecules affected by the electrostriction caused by the solute-solvent interactions. The negative compressibility at infinite dilution of the five bioactive peptides was similar to the compressibility values reported for small peptides and amino acids in the literature (Hedwig, 1994; Chalikian et al., 1994; Chalikian et al., 1998; Pfeiffer et al., 2008). The content of salts of the bioactive peptides was seen to be directly related with the amount of electrostriction at infinite dilution and therefore with the magnitude of the negative compressibility of each bioactive peptide. However, dairy peptide D showed a rapid increase in compressibility as the

concentration increased, having a positive compressibility at 1.25 mg mL^{-1} and higher concentrations implying a hydrophobic association as concentration increased. Finally, the compressibility of both liposomes (UV and MLV) at infinite dilution was seen to be positive, which indicates that both have a compressible interior, and is also compatible with a larger partial specific volume of both liposomes compared with the KCl and the bioactive peptides. The partial compressibility coefficient of both liposomes was within the range reported for liposomes by Hianik et al. (1998).

Table 9.1. Partial specific volume and partial specific adiabatic compressibility of all materials analysed.

	$\overline{\beta}_S^0$ (x10^{-6} bar^{-1})	$\overline{V}^0$ (cm^3 g^{-1})
KCl	-164.0	0.359
1 kDa hemp seed peptide	-86.2	0.516
Dairy peptide C	-59.0	0.515
3 kDa hemp seed peptide	-22.0	0.712
Dairy peptide A	-21.9	0.635
Dairy peptide D	-20.7	0.636
Unilamellar vesicles (UV)	34.6	0.846
Multilamellar vesicles (MLV)	36.4	0.860

The ultrasonic velocity of the liposome-dairy peptides mixes over time did not show any clear change or tendency to change that was differentiated from the inherent variation of the measurements. The density of the liposome-dairy peptide mixes showed slight changes over time that could be caused by time-dependent changes in the volumetric properties due to interaction between liposomes and dairy peptides.

The comparison of the ultrasonic velocity experimental values with the calculated additive values, based on additive behavior of the volumetric properties (Hianik et al., 2011), showed that the experimental values of the liposomes-dairy peptides were lower than the additive values, except for the UV liposomes-dairy peptide D mixes. The lowered experimental ultrasonic velocity compared to the calculated additive value suggests that the decrease in the ultrasonic velocity is a consequence of the interaction between liposomes and the dairy peptides. A similar decrease of the ultrasonic velocity caused by interaction of liposomes with proteins and peptides has been reported by Hianik et al. (1997) and Krivanek et al. (2002). This result supports the value of the use of ultrasonic velocity measurements in order to assess interactions between liposomes and peptides. The comparison of the density experimental values of the liposomes-dairy peptides mixes with the density additive values did not show any differences associated with liposome-peptide interactions.

The use of ultrasonic velocity and density measurements was shown to be a useful method in order to analyse the interaction between bioactive peptides and liposomes used as model membranes. According to changes in ultrasonic velocity of the mixed suspension that are reported in this thesis it was possible to conclude that the liposomes and bioactive peptides interact. These changes are caused by changes in the amount of water molecules located in the hydration shell around the liposomes and bioactive peptides. The results suggest a potential use of ultrasonic and density measurements to determine the permeability of bioactive peptides through liposomes.

Ultrasonic velocity and density measurements can be then a useful tool for understanding the peptide-liposome interaction and liposomes permeability to bioactive peptides, with the advantage that is possible to use commercial products that contains some

impurities (e.g., salts, sugars) in order to understand how these impurities affect the interaction. Additionally, knowing the amino acid sequence of the peptides, ultrasonic velocity and density measurements could be also used to understand how the amino acid composition affects the interaction and permeability of bioactive peptides.

9.2. Recommendations

Analysis of the volumetric properties of the hemp seed peptides and dairy peptides showed the important influence of components like salts and sugars. Therefore, in future studies it would be useful to determine the exact nature of these components to have more information on the components of the system analysed. Also, knowledge about the sequence of the peptides might allow us to understand better the changes in the volumetric properties.

The use of the extrusion method to prepare unilamellar vesicles had good results, achieving a reduction in the size of the vesicles and allowing us to obtain a homogeneous size distribution. However the use of refined lecithin, which is a mixture of phosphatidylcholine with different acyl chains lengths, did not allow us to know the exact phase transition temperature. The use of more specialized phospholipids with a known phase transition temperature can help to improve the results of the extrusion method which is recommended to be conducted above the gel-liquid crystal transition temperature of the phospholipid (Hope et al., 1984).

Additionally the use of liposomes that also include cholesterol within the phospholipids bilayer, which is another component in cell membranes, can help us to understand the physical properties of liposomes with two components, as well as the effect of cholesterol on the interaction between bioactive peptides and liposomes.

References

Afanas'ev, V.N. (2011). Solvation of Electrolytes and Nonelectrolytes in Aqueous Solutions. Journal of Physical Chemistry B. 115, 6541-6563.

Afanas'ev, V.N., Tyunina, E.Y. (2001). Adiabatic Compressibility of Aqueous Potassium Chloride Solutions and Its Relation to Ion Hydration. Russian Journal of Inorganic Chemistry. 46, 1915-1920.

Afanas'ev, V.N., Ustinov, A.N. (2009). Adiabatic Compressibility of Hydrated Complexes of Electrolytes. Electrochimica Acta. 54, 6455-6463.

Agarwal, M., Kushwaha, H.R., Chakravarty, C. (2010). Local Order, Energy, and Mobility of Water Molecules in the Hydration Shell of Small Peptides. Journal of Physical Chemistry. 114, 651-659.

Almeida, P.F., Pokorny, A. (2010). Binding and Permeabilization of Model Membranes by Amphipathic Peptides. Antimicrobial Peptides, Methods in Molecular Biology. 618, 155-169.

Apenten, R.K.O., B. Buttner, B., Mignot, B., Pascal, D., Povey, M.J.W. (2000). Determination of the Adiabatic Compressibility of Bovine Serum Albumen in Concentrated Solution by a New Ultrasonic Method. Food Hydrocolloids 14, 83–91.

Avbelj, F. (2000). Amino Acid Conformational Preferences and Solvation of Polar Backbone Atoms in Peptides and Proteins. Journal of Molecular Biology. 300, 1335-1359.

Baginski, M., Cybulska, B., Gruszecki, W. (2006). Interaction of Polyene Macrolid Antibiotics with Lipid Model Membranes. Advances in Lipid Planar & Liposomes. 3, 271-313.

Bánó, M., Marek, J. (2006). How Thick is the Layer of Thermal Volume Surrounding the Protein?. Biophysical Chemistry. 120, 44-54.

Baum, S.J. (1987). Introduction to Organic and Biological Chemistry. New York. Macmillan Publishing Company.

Bechinger, B., Seelig, J. (1991). Conformational Changes of Phosphatidylcholine Headgroup due to Membrane Dehydration. A H-NMR Study. Chemistry and Physics of Lipids. 58, 1-5.

Bernhardt, J., Pauly, H. (1975). Partial Specific Volumes in Highly Concentrated Protein Solutions. I. Water-Bovine Serum Albumin and Water-Bovine Hemoglobin. The Journal of Physical Chemistry. 79, 584-590.

Bernhardt, J., Pauly, H. (1977). Partial Specific Volumes in Highly Concentrated Protein Solutions. 2. Mixtures of Water, Bovine Hernoglobin, and Sodium Chloride. The Journal of Physical Chemistry. 81, 1290-1295.

Bryant, C.M., McClements, D.J. (1999). Ultrasonic Spectroscopy Study of Relaxation and Scattering in Whey Protein Solutions. Journal of the Science of Food and Agriculture. 79,1754-1760.

Bull, H., Breese, K. (1979). Concentrations and Partial Volumes of Proteins. Archives, of Biochemistry & Biophysic. 197, 199-204.

Calandrini, V., Fioretto, D., Onori, G., Santucci, A. (2000). Role of Hydrophobic Interactions on the Stabilisation of Native State of Globular Proteins. Chemical Physics Letters. 324, 344–348.

Chabance, B., Marteau, P., Rambaud, J.C., Migliore-Samour, D., Boynard, M., Perrotin, P., Guillet, R., Jolles, P. Fiat, A.M. (1998). Casein Peptide Release and Passage to the Blood in Humans during Digestion of Milk or Yogurt. Biochimie. 80, 155,-165.

Chalikian, T.V. (2003). Volumetric Properties of Proteins. Annual Review of Biophysics & Biomolecular Structure. 32, 207-235.

Chalikian, T.V., Breslauer, K.J. (1996). Compressibility as a Means to Detect and Characterize Globular Protein States. Proceedings of the National Academy of Sciences of United States of America. 93, 1012-1014.

Chalikian, T.V., Breslauer, K.J. (1998). Thermodynamic Analysis of Biomolecules: a Volumetric Approach. Current Opinion in Structural Biology. 8, 657-664.

Chalikian, T.V., Filfil, R. (2003). How Large are the Volume Changes Accompanying Protein Transitions and Binding?. Biophysical Chemistry. 104, 489-499.

Chalikian, T.V. Macgregor, R.B. (2009). Origins of Pressure-Induced Protein Transitions. Journal of Molecular Biology. 394, 834-842.

Chalikian, T.V., Kharakoz, D.P., Sarvazyan, A.P., Cain, C.A., McGough, R.J., Pogosova, V., Gareginian, T.N. (1992). Ultrasonic Study of Proton-Transfer Reactions in Aqueous Solutions of Amino Acids. Journal of Physical Chemistry. 96, 876-883.

Chalikian, T.V., Sarvazyan, A.P., Breslauer, K.J. (1994). Hydration and Partial Compressibility of Biological Compounds. Biophysical Chemistry. 51, 89-109.

Chalikian, T.V., Sarvazyan, A.P., Funck, T., Cain, C.A., Breslauer, K.J. (1994). Partial Molar Characteristics of Glycine and Alanine in Aqueous Solutions at High Pressures Calculated from Ultrasonic Velocity Data. Journal of Physical Chemistry. 98, 321-328.

Chalikian, T.V., Gindikin, V.S., Breslauer, K.J. (1995). Volumetric Characterizations of the Native, Molten Globule and Unfolded States of Cytochrome c at Acidic pH. Journal of Molecular Biology. 250, 291-306.

Chalikian, T.V., Totrov, M., Abagyan, R., Breslauer, K.J. (1996). The Hydration of Globular Proteins as Derived from Volume and Compressibility Measurements: Cross Correlating Thermodynamic and Structural Data. Journal of Molecular Biology. 260, 588-603.

Chalikian, T.V., Gindikin, V.S., Breslauer, K.J. (1998). Hydration of Diglycyl Tripeptides with Non-Polar Side Chains: a Volumetric Study. Biophysical Chemistry. 75, 57-71.

Chatterjee, S.N., Agarwal, S. (1988). Liposomes as Membrane Model for Study of Lipid Peroxidation. Free Radical Biology & Medicine. 4, 51-72.

Chen, X., Weber, I., Harrison , R.W. (2008). Hydration Water and Bulk Water in Proteins Have Distinct Properties in Radial Distributions Calculated from 105 Atomic Resolution Crystal Structures. Journal of Physical Chemistry B. 112, 12073-12080.

Chug, T.D.Y., Raymond, K.N. (1993). Lactoferrin: The Role of Conformational Changes in Its Iron Binding and Release. Journal of the American Chemical Society. 115, 6765-6768.

Colotto, A., Kharakoz, D.P., Lohner, K., Laggner, P. (1993). Ultrasonic Study of Melittin Effects on Phospholipid Model Membranes. Biophysical Journal. 65, 2360-2367.

Corredig, M., Verespej, E., Dalgleish, D. (2004). Heat-Induced Changes in the Ultrasonic Properties of Whey Proteins. Journal of Agricultural & Food Chemistry. 52, 4465-4471.

Creamer, L.K., MacGibbon, A.K.H. (1996). Some Recent Advances in the Basic Chemistry of Milk Proteins and Lipids. International Dairy Journal. 6, 539-568.

Creighton, T.E. (1993). Proteins. Structures and Molecular Properties, 2nd edition. New York: W.H. Freeman and Company.

Creuzenet, C., Durand, C., Haertlé, T. (1997). Interaction of α_{s2}- and β-Casein Signal Peptides with DMPC and DMPG Liposomes. Peptides. 18, 463-472.

Dalgleish, D.G., Verespej, E., Alexander, M., Corredig, M. (2005). The Ultrasonic Properties of Skim Milk Related to the Release of Calcium from Casein Micelles during Acidification. International Dairy Journal. 15, 1105-1112.

Daniel, H. (2004). Protein Digestion and Absorption of Amino Acids and Peptides. Encyclopedia of Gastroenterology. USA. Elsevier.

Desnoyers, J.E., Verrall, R.E., Conway, B.E. (1965). Electrostriction in Aqueous Solutions of Electrolytes. Journal of Chemical Physics. 43, 243-250.

Durchschalg, H., Jaenicke, R. (1982). Partial Specific Volume Changes of Proteins. Densimetric Studies. Biochemical & Biophysical Research Communications. 108, 1074-1079.

Eggers, F., Funck, Th. (1973). Ultrasonic Measurements with Milliliter Liquid Samples in the 0.5-100 MHz Range. Review of Scientific Instruments. 44, 969-977.

Erdmann, K., Cheung, B.W.Y., Schröder, H. (2008). The Possible Roles of Food-Derived Bioactive Peptides in Reducing the Risk of Cardiovascular Disease. Journal of Nutritional Biochemistry. 19, 643-654.

Fahy, E., Subramaniam, S., Brown, H.A., Glass, C.K., Merril, A.H., Murphy, R.C., Raetz, C.R.H., Russell, D.W., Semaya, Y., Shaw, W., Shimizu, T., Spener, F., van Meer, G., VanNiewenhze, M.S., White, S.H., Witztum, J., Dennis, E.A. (2005). A Comprehensive Classification System for Lipid. Journal of Lipid Research. 46, 839-861.

Gekko K. (2002). Compressibility Gives New Insight into Protein Dynamics and Enzyme Function. Biochimica et Biophysica Acta. 1595, 382-386.

Gekko, K., Hasegawa, Y. (1986). Compressibility-Structure Relationship of Globular Proteins. Biochemistry. 25, 6563-6571.

Gekko, K., Obu, N., Li, J., Lee, J.C. (2004). A Linear Correlation between the Energetics of Allosteric Communication and Protein Flexibility in the *Escherichia coli* Cyclic AMP

Receptor Protein Revealed by Mutation-Induced Changes in Compressibility and Amide Hydrogen-Deuterium Exchange. Biochemistry. 43, 3844-3852.

Gill, I., Lopez-Fandiño, R., Jorba, X., Vulfson, E.N. (1996). Biologically Active Peptides Enzymatic Approaches to Their Production. Enzyme and Microbial Technology. 18, 162-183.

Girgih, A.T., Udenigwe, C.C., Aluko, R.E. (2011). In Vitro Antioxidant Properties of Hemp Seed (Cannabis sativa L.) Protein Hydrolysate Fractions. Journal of the American Oil Chemists Society. 88, 381-389.

Guang, C., Phillips, R.D. (2009). Plant Food-Derived Angiotensin I Converting Enzyme Inhibitory Peptides. Journal of Agricultural & Food Chemistry. 57, 5113-5120.

Guilloteau, P., Romé, V., Delaby, L., Mendy, F., Roger, L., Chayvialle, J.A. (2009). A New Role of Phosphopeptides as Bioactive Peptides Released during Milk Casein Digestion in the Young Mammal: Regulation of Gastric Secretion. Peptides. 30, 2221-2227.

Hackel, M., Hinz, H.J., Hedwig, G.R. (1999). Partial Molar Volumes of Proteins: Amino Acid Side-Chain Contributions Derived from the Partial Molar Volumes of some Tripeptides over the Temperature Range 10-90°C. Biophysical Chemistry. 82, 35-50.

Harpaz, Y., Gerstein, M., Chothia, C. (1994). Volume Changes on Protein Folding. Structure. 2, 641-649.

Hartmann, R., Meisel, H. (2007). Food-Derived Peptides with Biological Activity: from Research to Food Applications. Current Opinion in Biotechnology. 18, 163-169.

Hedwig, G.R. (1994). Partial Molar Heat Capacities Volumes and Compressibilities of Aqueous Solutions of Some Peptides that Model Side-Chains of Proteins. Pure & Applied Chemistry. 66, 387-392.

Heremans, K., Smeller, L. (1998). Protein Structure and Dynamics at High Pressure. Biochimica et Biophysica Acta. 1386, 353-370.

Hianik, T. (2006). Structure and Physical Properties of Biomembranes and Model Membranes. Acta Physica Slovaca. 56, 687-805.

Hianik, T. (2011). Mechanical Properties of Bilayer Lipid Membranes and Protein-Lipid Interactions. Advances in Planar Lipid Bilayers and Liposomes. 13, 33-72.

Hianik, T., Kaatze, U., Sargent, D.F., Krivanek, R., Halstenberg, S., Pieper, W., Gaburjaková, J., Gaburjaková, M., Pooga, M., Langel, U. (1997). A Study of the Interaction of Some Neuropeptides and Their Analogs with Bilayer Lipid Membranes and Liposomes. Bioelectrochemistry and Bioenergetics. 42, 123-132.

Hianik, T., Haburcák, M., Lohner, K., Prenner, E., Paltauf, F., Hermetter, A. (1998). Compressibility and Density of Lipid Bilayers Composed of Polyunsaturated Phospholipids and Cholesterol. Colloids Surfaces A: Physicochemical & Engineering Aspects. 139, 189-197.

Hianik, T., Kupcu, S., Sleytr, U.B., Rybar, P., Krivanek, R., Kaatze, U. (1999). Interaction of Crystalline Bacterial Cell Surface Proteins with Lipid Bilayers in Liposomes. A Sound Velocity Study. Colloids and Surfaces A: Physicochemical & Engineering Aspects. 147, 331-339.

Hianik, T., Rybár, P., Krivanek, R., Petríková, M., Roudna, M., Apell, H.J. (2011). Specific Volume and Compressibility of Bilayer Lipid Membranes with Incorporated Na,K ATPase. First Publ. In: General Physiology and Biophysics. 30, 145-153.

Hohlweg, W., Kosol, S., Zangger, K. (2012). Determining the Orientation and Localization of Membrane-Bound Peptides. Current Protein and Peptide Science. 13, 267-279.

Hope, M.J., Bally, M.B., Webb, G., Cullis, P.R. (1985). Production of Large Unilamellar Vesicles by a Rapid Extrusion Procedure. Characterization of Size Distribution, Trapped Volume and Ability to Maintain a Membrane Potential. Biochimica et Biophysica Acta. 812, 55-65.

Huang, C., Charlton J.P. (1971). Studies on Phosphatidylcholine Vesicles. The Journal of Biological Chemistry. 246, 2555-2560.

Hunter, H.N., Jing, W., Schibli, D.J., Trinh, T., Park, I.Y., Kim, S.C., Vogel, H.J. (2005). The Interactions of Antimicrobial Peptides Derived from Lysozyme with Model Membrane Systems. Biochimica et Biophysica Acta. 1668, 175-189.

Jain, M. K. 1988. Introduction to Biological Membranes. A Wiley-Interscience Plubication, John Wiley & Sons.

Jäkälä, P., Jauhiainen, T., Korpela, R., Vapaatalo, H. (2009). Milk Protein-derived Bioactive Tripeptides Ile-Pro-Pro and Val-Pro-Pro Protect Endothelial Function in Vitro in Hypertensive Rats. Journal of Functional Foods. 1, 266-273.

Joanne, P., Galanth, C., Goasdoué, N., Nicolas, P., Sagan, S., Lavielle, S., Chassaing, G., El Amri, C., Alves, I.D., (2009). Lipid Reorganization Induced by Membrane-active Peptides Probed Using Differential Scanning Calorimetry. Biochimica et Biophysica Acta. 1788, 1772-1781.

Kaatze, U., Eggers, F., Lautscham, K. (2008). Ultrasonic Velocity Measurements in Liquids with High Resolution-Techniques, Select Applications and Perspectives. Measurement Science & Technology. 19, 1-21.

Kamau, S.M., Cheison, S.C., Chen, W., Liu, X., Lu, R. (2010). Alpha-Lactalbumin: Its Production Technologies and Bioactive Peptides. Comprehensive Reviews in Food Science and Food Safety. 9, 197-212.

Kentish, S., Ashokkumar, M. (2011). The Physiscal and Chemical Effects of Ultrasound. pp. 1-12 in Ultrasound Technologies for Food and Bioprocesing. Barbosa-Cánovas, G.V. New York. Springer.

Khandelia, H., Ipsen, J.H., Mouritsen, O.G. (2008). The Impact of Peptides on Lipid membranes. Biochimica et Biophysica Acta. 1778, 1528–1536.

Kharakoz, D.P. (1991). Volumetric Properties of Proteins and Their Analogues in Diluted Water Solutions. 2. Partial Adiabatic Compressibilities of Amino Acids at 15-70 °C. Journal of Physical Chemistry. 95, 5634-5642.

Kharakoz, D.P. (1997). Partial Volumes and Compressibilities of Extended Polypeptide Chains in Aqueous Solution: Additivity Scheme and Implication of Protein Unfolding at Normal and High Pressure. Biochemistry. 36, 10276-10285.

Kharakoz D.P., Sarvazyan, A.P. (1993). Hydrational and Intrinsic Compressibilities of Globular Proteins. Biopolymers. 33, 11-26.

Koenig, B.W., Gawrisch, K. (2005). Specific Volumes of Unsaturated Phosphatidylcholines in the Liquid Crystalline Lamellar Phase. Biochimica et Biophysica Acta. 1715, 65-70.

König, G., Boresch, S. (2009). Hydration Free Energies of Amino Acids: Why Side Chain Analog Data are not Enough. Journal of Physical Chemistry. 113, 8967-8974.

Korhonen, H. (2009). Milk-derived Bioactive Peptides: From Science to Applications. Journal of Functional Foods. 1, 177-187.

Korhonen, H., Pihlanto, A. (2006). Bioactive Peptides: Production and Functionality. International Dairy Journal. 16, 945-960.

Krivanek, R., Rybar, P., Prenner, E.J., McElhaney, R.N., Hianik, T. (2001). Interaction of the Antimicrobial Peptide Gramicidin S with Dimyristoyl-phosphatidylcholine Bilayer Membranes: a Densitometry and Sound Velocimetry Study. Biochimica et Biophysica Acta. 1510, 452-463.

Krivanek, R., Rybar, P., Kupcu, S., Sleytr, U.B., Hianik, T. (2002). Affinity Interactions on a Liposome Surface Detected by Ultrasound Velocimetry. Bioelectrochemistry. 55, 57-59.

Kupke, D.W. (1973). Density and Volume Change Measurements. pp. 1-71 in Physical Principles and Techniques of Protein Chemistry, Part C. Leach, J. New York. Academic Press.

Kylara, A., Panyam, D. (2003). Peptides from Milk Proteins and Their Properties. Critical Reviews in Food Science and Nutrition. 43, 607-633.

Kyte, J. 1995. Structure in Protein Chemistry. New York & London: Garland Publishing, Inc.

Langel, U., Cravatt, B.F., Graslund, A., von Heijne, G., Land, T., Niessen, S., Zorko, M. (2010). Introduction to Peptides and Proteins. Boca Raton, FL. CRC Press Taylor & Francis Group.

Lee, S., Tikhomirova, A., Shalvardjian, N., Chalikian, T.V. (2008). Partial molar volumes and adiabatic compressibilities of unfolded protein states. Biophysical Chemistry. 134, 185–199.

Lee, S., Heerklotz, H., Chalikian, T.V. (2010). Effects of Buffer Ionization in Protein Transition Volumes. Biophysical Chemistry. 148, 144-147.

Lewis, R.N.A.H, Mannock, D.A., McElhaney, R.N. (1989). Effect of Fatty Acyl Chain Length and Structure on the Lamellar Gel to Liquid-Crystalline and Lamellar to Reversed Hexagonal Phase Transitions of Aqueous Phosphatidylethanolamine Dispersions. Biochemistry. 28, 541-548.

Lico, C., Santi, L., Twyman, R.M., Pezzotti, M., Avesani, L. (2012). The Use of Plants for the Production of Therapeutic Human Peptides. Plant Cell Reports. 31, 439-451.

Lipka, E., Crison, J., Amidon, G.L. (1996). Transmembrane Transport of Peptide Type Compounds: Prospects for Oral Delivery. Journal of Controlled Release. 39, 121-129.

López-Expósito, I., Recio, I. (2008). Protective Effect of Milk Peptides: Antibacterial and Antitumor Properties. Bioactive Components of Milk. Advances in Experimental Medicine & Biology. 606, 271-293.

Luckey, M. 2008. Membrane Structural Biology. Cambridge. Cambridge University Press.

MacDonald, R.C., MacDonald, R.I., Bert Ph.M. Menco, B.Ph.M., Takeshita, K., Subbarao, N.K., Hu, L. (1991). Small-Volume Extrusion Apparatus for Preparation of Large, Unilamellar Vesicles. Biochimica et Biophysica Acta. 1061, 297-303.

Madureira, A.R., Tavares, T., Gomes, A.M.P., Pintado, M.P., Malcata, F.X. (2010). Invited Review: Physiological Properties of Bioactive Peptides Obtained from Whey Proteins. Journal of Dairy Science. 93, 437-455.

Marcus, Y. (2005). Electrostriction, Ion Solvation, and Solvent Release on Ion Pairing. Journal of Physical Chemistry B. 109, 18541-18549.

Marcus, Y. (2006). Ionic Volumes in Solution. Biophysical Chemistry. 124, 200-207.

Marcus, Y. (2011). Electrostriction in Electrolyte Solutions. Chemical Reviews. 111, 2761-2783.

Mason, T.J., Sáez Bernal, V. (2012). An Introduction to Sonoelectrochemistry. pp.21-44 in Power Ultrasound in Electrochemistry, from versatile tool to eingeriering solution. Pollet, B.G. Chichester, West Sussex. Wiley.

Matos, P., Gonalvez, S., Santos, N. (2008). Interaction of Peptides with Biomembranes Assessed by Potential-sensitive Fluorescent Probes. Journal of Peptide Science. 14, 407-415.

McClements, D.J. (1991). Ultrasonic Characterisation of Emulsions and Suspensions. Advances in Colloid and Interface Science. 37, 33-72.

Meisel, H. (1997). Biochemical Properties of Bioactive Peptides Derived from Milk Proteins: Potential Nutraceuticals for Food and Pharmaceutical Applications. Livestock Production Science. 50, 125-138.

Meisel, H. (1998). Overview on Milk Protein-derived Peptides. Int. Dairy Journal. 8, 363-373.

Möller, N.P., Scholz-Ahrens, K.E., Roos, N., Schrezenmeir, J. (2008). Bioactive Peptides and Proteins from Foods: Indication for Health Effects. European Journal of Nutrition. 47, 171-182.

Monnin, C. (1989). An Ion Interaction Model for the Volumetric Properties of Natural Water: Density of the Solution and Partial Molal Volumes of Electrolytes to High Concentrations at 25°C. Geochimica et Cosmochimica Acta. 53, 1177-1188.

Mougin, P., Wilkinson, D., Roberts, K.J., Jack, R., Kippax, P. (2003). Sensitivity of Particle Sizing by Ultrasonic Attenuation Spectroscopy to Material Properties. Powder Technology. 134, 243-248.

Mozsolits, H., Lee, T., Wirth, H., Perlmutter, P., Aguilar, M. (1999). The Interaction of Bioactive Peptides with an Immobilized Phosphatidylcholine Monolayer. Biophysical Journal. 77, 1428-1444.

Murphy, L.R., Matubayasi, N., Payne, V.A., Levy, R.M. (1998). Protein Hydration and Unfolding — Insights from Experimental Partial Specific Volumes and Unfolded Protein Models. Folding & Design. 3, 105-118.

Nagle, J.F., Wilkinson, D.A. (1978). Lecithin Bilayers. Density Measurements and Molecular Interactions. Biophysical Journal. 23, 159-175.

Nelson, P.V., Povey, M.J.W., Wang, Y. (2001). An Ultrasound Velocity and Attenuation Scanner for Viewing the Temporal Evolution of a Dispersed Phase in Fluids. Review of Scientific Instruments. 72, 4234-4241.

Nölting, B. (1995). Relation between Adiabatic and Pseudoadiabatic Compressibility in Ultrasonic Velocimetry. Journal of Theoretical Biology. 175, 191-196.

Orioni, B., Bocchinfuso, G., Kim, J.Y., Palleschi, A., Grande, G., Bobone, S., Park, Y., Kim, J.I., Hahm, K., Stella, L. (2009). Membrane Perturbation by the Antimicrobial Peptide PMAP-23: A Fluorescence and Molecular Dynamics Study. Biochimica et Biophysica Acta. 1788, 1523-1533.

Orlovskaya, T.V., Sultanova, E.M., Oshchepkova, Y.I., Arzanova, I.A., Kuznetsova, N.N., Veshkurova, O.N., Salikhov, S.I. (2010). Bioactive Peptides from Several plant Species. Chemistry of Natural Compounds. 46, 331-332.

Ostro, M.J. (1983). Liposomes. New York. Marcel Dekker Inc.

Owusu-Apenten, R.K. (2010). Bioactive Peptides: Applications for Improving Nutrition and Health. CRC Press.

Pavlovskaya, G., McClements, D.J., Povey, M.J.W. (1992). Ultrasonic Investigation of Aqueous Solutions of a Globular Protein. Food Hydrocolloids. 6, 253-262.

Peetla, C., Stine, A., Labhasetwar, V. (2009). Biophysical Interactions with Model Lipid Membranes: Applications in Drug Discovery and Drug Delivery. Molecular Pharmaceutics. 6, 1264–1276.

Perven, S. (2012). Effect of Hofmeister Salts on the Compressibility of the Protein of Bovine Serum Albumin, Unexamined Thesis. The University of Manitoba.

Pfeiffer, H., Heremans, K., Wevers, M. (2008). The Influence of Correlated Protein–water Volume Fluctuations on the Apparent Compressibility of Proteins Determined by Ultrasonic Velocimetry. Biochimica et Biophysica Acta.1784, 1546–1551.

Phelan, M., Aherne, A., FitzGerald, R.J., O'Brien, N.M. (2009). Casein-derived Bioactive Peptides: Biological Effects, Industrial Uses, Safety Aspects and Regulatory Status. International Dairy Journal. 19, 643-654.

Picariello, G., Ferranti, P., Fierro, O., Mamone, G., Caira, S., Luccia, A., Monica, S., Addeo, F. (2010). Peptides Surviving the Simulated Gastrointestinal Digestion of Milk Proteins: Biological and Toxicological Implications. Journal of Chromatography B. 878, 295-308.

Picker, P., Tremblay, E., Jolicoeu, C. (1974). A high-precision digital readout flow densimeter for liquids. Journal of Solution Chemistry. 3, 377-384.

Poliwoda, A., Ilczuk, N., Wieczorek, P.P. (2007). Transport Mechanism of Peptides Through Supported Liquid Membranes. Separation and Purification Technology. 57, 444-449.

Povey, M.J.W., Moore, J.D., Braybrook, J., Simons, H., Belchamber, R., Raganathan, M., Pinfield, V. (2011). Investigation of Bovine Serum Albumin Denaturation Using Ultrasonic Spectroscopy. Food Hydrocolloids. 25, 1233-1241.

Quirós, A., Contreras, M., Ramos, M., Amigo, L., Recio, I. (2009). Stability to Gastrointestinal Enzymes and Structure–activity Relationship of β-casein-peptides with Antihypertensive Properties. Peptides. 30, 1848-1853.

Ramaswami, V., Haaseth, R.C., Matsunaga, T.O., Hruby, V.J., O'Brien, D.F. (1992). Opioid Peptide Interactions with Lipid Bilayer Membranes. Biochimica et Biophysica Acta. 1109, 195-202.

Raschke, T.M. (2006). Water Structure and Interactions with Protein Surfaces. Current Opinion in Structural Biology. 16, 152-159.

Rasper J, Kauzmann W. (1962). Volume Changes in Protein Reactions. I. Ionization Reactions of Proteins. Journal of American Chemical Society. 84, 1771–77.

Retsina, T., Richardson, S.M., Wakeham, W.A. (1986). The Theory of Vibrating-rod Densimeter. Applied Scientific Research. 43, 127-158.

Romanowski, M., Zhu, X.Y., Kim, K., Hruby, V.J, O'Brien, D.F. (2002). Interaction of Enkephalin Peptides with Anionic Model Membranes. Biochimica et Biophysica Acta. 1558, 45-53.

Roy, F., Boye, J.I., Simpson, B.K. (2010). Bioactive Proteins and Peptides in Pulse Crops: Pea, Chickpea and Lentil. Food Research International. 43, 432-442.

Rybar, P., Krivanek, R., Samuely, T., Lewis, R.N.A.H., McElhaney, R.N., Hianik, T. (2007). Study of the Interaction of α-Helical Transmembrane Peptide with Phosphatidylcholine Bilayer Memebrane by Means of Densimetry and Ultrasound Velocimetry. Biochimica et Biophysica Acta. 1768, 1466-1478.

Sabaté, R., Gallardo, M., Estelrich, J. (2005). Spontaneous Incorporation of β-amyloid Peptide into Neutral Liposomes. Colloids and Surfaces A: Physicochemical Engineering Aspects. 270-271, 13-17.

Saito, T. (2008). Antihypertensive Peptides Derived from Bovine Casein and Whey Proteins. Advances in Experimental Medicine & Biology. 606, 295-317.

Sarvazyan, A.P. (1991). Ultrasonic Velocimetry of Biological Compounds. Annual Reviews Biophysics & Biophysical Chemistry. 20, 321-42.

Sarvazyan, A.P., Kharakoz, D.P., Hemmes, P. (1979). Ultrasonic Investigation of the pH-Dependent Solute-Solvent Interactions in Aqueous Solutions of Amino Acids and Proteins. The Journal of Physical Chemistry. 83, 1796-1799.

Scattering in Whey Protein Solutions. Journal of the Science of Food & Agriculture. 79, 1754-1760.

Schwitzer, M.A., Hedwig, G.A. (2005). Thermodynamic Properties of Peptide Solutions. Part 17. Partial Molar Volumes and Heat Capacities of the Tripeptides GlyAspGly and GlyGluGly, and Their Salts K[GlyAspGly] and Na[GlyGluGly] in Aqueous Solution at 25 °C. Journal of Solution Chemistry. 34, 801-821.

Seelig, J. (2004). Thermodynamics of Lipid–Peptide Interactions. Biochimica et Biophysica Acta. 1666, 40-50.

Sewald, N., Jakubke, H.D. (2002). Peptides: Chemistry and Biology (pp. 2-54, 61-129). Wiley-VCH, Germany.

Silva-Sanchez, C., Barba de la Rosa, A.P., Leon-Galvan, M.F., De Lumen, B.O., De Leon.Rodriguez, A., Gonzalez de Mejia, E. (2008). Bioactive Peptides in Amaranth (Amaranthus hypochondriacus) Seed. Journal of Agricultural & Food Chemistry. 56, 1233-1240.

Sirotkin, V.A., Komissarov, I.A., Khadiullina, A.V. (2012). Hydration of Proteins: Excess Partial Volumes of Water and Proteins. Journal of Physical Chemistry B. 116, 4098-4105.

Strom-Jensen, P.R., Magin, R.L., Dunn, F. (1984). Ultrasonic Evidence for Structural Relaxation in Large Unilamellar Liposomes. Biochimica et Biophysica Acta. 769, 179-186.

Tang, C., Wang, X., Yang, X. (2009). Enzymatic Hydrolysis of Hemp (Cannabis sativa L.) Protein Isolate by Various Proteases and Antioxidant Properties of the Resulting Hydrolysates. Food Chemistry. 114, 1484-1490.

Tang, Y.C., Deber, C.M. (2004). Aqueous Solubility and Membrane Interactions of Hydrophobic Peptides with Peptides Tags. Biopolymers (Peptide Science). 76, 110-118.

Taulier, N., Chalikian, T.V. (2001). Characterization of pH-induced Transitions of β-Lactoglobulin: Ultrasonic, Densimetric, and Spectroscopic Studies. Journal of Molecular Biology. 314, 873-889.

Taulier, N., Chalikian, T.V. (2002). Compressibility of protein transitions. Biochimica et Biophysica Acta. 1595, 48-70.

Tidona, F., Criscione, A., Guastella, A.M., Zuccaro, A., Bordonaro, S., Marletta, D. (2009). Bioactive Peptides in Dairy Products. Italian Journal of Animal Science. 8, 315-340.

Tiengo, A., Faria, M., Netto, F.M. (2009). Characterization and ACE-Inhibitory Activity of Amaranth Proteins. Journal of Food Science. 74, 121-126.

Thompson, A., Boland, M., Singh, H. (2009). Milk Proteins From Expression to Food, Elsevier Inc, San Diego, CA, USA.

Trevino, S.R., Scholtz, J.M., Pace, N. (2007). Amino Acid Contribution to Protein Solubility: Asp, Glu, and Ser Contribute more favorably than the other Hydrophilic Amino Acids in RNase Sa. Journal of Molecular Biology. 366, 449-460.

Uhríková, D., Rybar, P., Hianik, T., Balgavý, P. (2007). Component Volumes of Unsaturated Phosphatidylcholines in Fluid Bilayers: a Densitometric Study. Chemistry and Physics of Lipids. 145, 97-105.

Valdez, D., Le Huerou, J.Y., Gindre, M., Urbach, W., Waks, M. (2001). Hydration and Protein Folding in Water and in Reverse Micelles: Compressibility and Volume Changes. Biophysical Journal. 80, 2751-2760.

Vegarud, G.E., Langsrud, T., Svenning, C. (2000). Mineral-binding Milk Proteins and Peptides; Occurrence, Biochemical and Technological Characteristics. British Journal of Nutrition. 84, S91-S98.

Vila, J.A., Ripoll, D.R., Villegas, M. E., Vorobjev, Y.N., Scheraga, H.A. (1998). Role of Hydrophobicity and Solvent-Mediated Charge-Charge Interactions in Stabilizing α-Helices. Biophysical Journal. 75, 2637-2646.

Vogl, T. Hinz, H.J., Hedwig, G.R. (1995). Partial Molar Heat Capacities and Volumes of Gly-X-Gly Tripeptides in Aqueous Solution: Model Studies for the Rationalization of Thermodynamics Parameters of Proteins. Biophysical Chemistry. 54, 261-269.

Walter, E., Kissel, T., Amidon, G.L. (1996). The Intestinal Peptide Carrier: A Potential Transport System for Small Peptide Derived Drugs. Advanced Drug Delivery Reviews. 20, 33-58.

White, S.H., Jacobs, R.E., King, G.I. (1987). Partial Specific Volumes of Lipid and Water in Mixtures of Egg Lecithin and Water. Biophysical Journal. 52, 663-665.

with DMPC and DMPG Liposomes. Peptides. 18, 463-472.

Wrobel, B.M., Time, R.W. (2012). Ultrasonic Measurement and Characterization of a Low Concentration System of Solid Particles in Liquid, in High Shear Flow. Applied Acoustics. 73, 117-131.

Yoshie-Stark, Y., Wada, Y., Wäsche, A. (2008). Chemical Composition, Functional Properties, and Bioactivities of Rapeseed Protein Isolates. Food Chemistry. 107, 32-39.

Yu, L., Guo, L., Ding, J.L., Ho, B., Feng, S., Popplewell, J., Swann, M., Wohland, T. (2009). Interaction of an Artificial Antimicrobial Peptide with Lipid Membranes. Biochimica et Biophysica Acta. 1788, 333-344.

Yuno-Ohta, N., Corredig, M. (2007). Characterization of α-Lactoglobulin A Gelation in the Presence of Sodium Caprate by Ultrasound Spectroscopy and Electron Microscopy. Biomacromolecules. 8, 2542-2548.

Zamyatnin, A.A. (1984). Amino Acid, Peptide, and Protein Volume in Solution. Annual Reviews of Biophysics & Bioengineering. 13, 145-165.

Zen, E. (1957). Partial Molar Volumes of some Salts in Aqueous Solutions. Geochimica et Cosmochimica Acta. 12, 103-122.

Zorko, M., Langel, U. (2005). Cell-penetrating Peptides: Mechanism and Kinetics of Cargo Delivery. Advanced Drug Delivery Reviews. 57, 529-545.