APPLICATION OF ULTRA HIGH HYDROSTATIC PRESSURE FOR

INVESTIGATING THE BINDING OF FLAVOR COMPOUNDS

TO β-LACTOGLOBULIN VIA HEADSPACE SOLID PHASE

MICROEXTRACTION-GAS CHROMATOGRAPHY

By

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To the Faculty of Washington State University:

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Abstract

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Fluorescence spectroscopy and headspace-solid phase microextraction (HS-SPME) gas chromatography analysis were used to evaluate the effects of ultra high hydrostatic pressure (UHP) (600 MPa) and pH (3.0-9.0) on β-lactoglobulin (BLG) surface hydrophobicity and binding of selected flavor compounds. An increase in tryptophan intrinsic fluorescence intensity of BLG was observed after UHP of 16 min, which suggested that the tryptophan residues were exposed during the unfolding of BLG. A 2 nm red-shift in tryptophan emission wavelength was observed after UHP come-up time, indicating changes in the polarity of tryptophan residues from a less polar to a more polar microenvironment. After UHP treatment come-up time of BLG at pH 9.0, there was an increase in BLG surface hydrophobicity, suggesting a flexible molecular structure due to surface denaturation of BLG at alkaline pH. UHP treatment of BLG solutions at pH 3.0, 5.0, and 9.0 resulted in decreases in the number of binding sites for the nonpolar fluorescence probe 6-propionyl-2-(dimethylamino)-naphthalene (PRODAN). UHP

treatment did not show significant influences in the apparent dissociation constant of PRODAN.

An extraction time of 10 min was used for HS-SPME of δ-decalactone, 2-methylbutyraldehyde, ethyl lactate, and diacetyl. Although the CAR/PDMS fiber was able to detect diacetyl, ethyl lactate, and 2-methylbutyraldehyde, the PDMS/DVB SPME fiber was selected for the extraction of selected flavor compounds due to reproducible and linear ($R^2 > 0.954$) calibration plots, and its semi-polar nature to extract δ-decalactone.

As observed by fluorescence quenching, there is no linear relationship between UHP treatment of BLG at 600 MPa and the number of binding sites for diacetyl, 2-methylbutyraldehyde, δ-decalactone, and ethyl lactate. BLG has low binding affinity for the selected flavor compounds with polar groups, and UHP treatment of BLG did not influence binding of 2-methylbutyraldehyde as observed by fluorescence quenching experiments. Headspace analysis of UHP–treated BLG resulted in significant increases ($p < 0.05$) in flavor retention over native BLG. In addition, a short UHP treatment time (come-up time) may be adequate for flavor retention.

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CHAPTER ONE

INTRODUCTION

In the United States, health is a powerful driver for the food industry, especially when nearly 60% of grocery shoppers are overweight and 12.5% of children are diagnosed with two or more risk factors for heart disease (Sloan 2006). In Western societies, excessive consumption of dietary fat is linked to obesity (Bray and Popkin 1999) and cardiovascular diseases (Law 2000). High intake of saturated fats, trans fats, and cholesterol increase the risk of coronary heart disease (Law 2000). Currently, dietary fat comprises nearly 36% of the energy content of the American diet. . The recommended total fat intake is between 20 and 35 percent of calories for adults, and for Americans, it is important to decrease their intake of saturated fats to less than 10 percent based on population studies of American diets (Thompson and Veneman 2005).

One way to decrease energy from fat in the diet is to substitute low-fat foods for high-fat foods. However, it may be difficult for some people to limit their dietary choices to the low-fat foods. The National Dairy Council reports that foods high in energy density are more palatable because of a high fat content, which may lead to overeating and consequent weight gain (McBean 2000). Fat modifies the overall perception of flavor of many foods through their effect on mouthfeel (e.g. creaminess of ice cream, richness of whole milk) and on the volatility and threshold value of flavor compounds present (Nawar 1996). Most flavor compounds are nonpolar, and fat is a flavor carrier for these hydrophobic compounds (Guyot and others 1996). Therefore, when a considerable amount of fat is removed, the flavor will be lost resulting in food that is often bland and monotonous.

Advancements in food technology may offer ways of reducing fat and energy consumption while satisfying consumer preference for high fat foods. High-fat foods appeal to consumers because the foods are often flavorful and rich. Fat replacers or fat substitutes are used to describe ingredients that replace fat or provide the physical and sensory properties of fat to reduce fat in food applications, and are derived from several chemical compounds that include carbohydrates, proteins, lipids, and synthetic compounds (Lindsay 1996). Among the sensory qualities of reduced-fat foods, flavor is one of the most important attributes consumers use as an index of acceptability, and within the last 30 years, there is continued interest in improving protein functionality through the binding of flavor compounds to proteins to improve flavor release and perception of foods (Gremli 1974; Damodaran and Kinsella 1980; Dumont and Land 1986; Lubbers and others 1998; Guichard and Langourieux 2000; Burova and others 2003).

Basic properties of β-lactoglobulin

The Food and Drug Administration defines whey as "the liquid substance obtained by separating the coagulum from milk, cream, or skim milk in cheese making" (FDA 2005). For every 1 pound of cheese produced, approximately 9 pounds of liquid whey result, and according to USDA statistics in 1999, total cheese production in the US was 7.94 billion pounds, which equates to almost 72 billion pounds of liquid whey (USDA 1999). Although not every dairy facility has the capability to process whey into a usable product and often rely on disposing the whey by-product, whey can be recovered and processed into animal feed, human food products, and pharmaceuticals.

β-Lactoglobulin (BLG) is a major whey protein in mammalian species, except for rodents, lagomorphs and humans. In bovine whey, BLG is the predominant protein, at a concentration of 2-4 g/L compared to α-lactalbumin (1-1.5 g/L), serum albumin (0.1-0.4 g/L), and immunoglobulin (0.6-1.0 g/L) (Swaisgood 1996). The characterization of BLG by amino acid sequencing and isoelectric focusing has identified seven genetic polymorphisms, A, B, C, D, E, F, and G (Phillips and others 1994). The most prevalent are the A and B forms (Croguennec and others 2004) that differ by two amino acids (substitutions Asp64 (A) → Gly (B); Val118 (A) → Ala (B)) (Brignon and Dumas 1973), and is commercially available in pure form of either variant or as a mixture of both. BLG is a polypeptide with a molecular weight of 18,350 Da (Brownlow and others 1997), consisting of 162 amino acid residues. In milk, it exists as a dimer with a molecular weight of 32,400 Da. BLG is a globular protein containing thiol and disulfide groups.

The orthorhombic crystal structure of BLG at pH 7.6 contains a nine-stranded, flattened β-barrel or calyx and a flanking three-turn α-helix (Phillips and others 1994). One curved face of the calyx is formed from a twisted antiparallel β-sheet containing the N-terminal half of strand A, together with strands B-D (Figure 1) (Brownlow and others 1997). The other curved face is a similarly twisted antiparallel β-sheet containing strands E-H and the C-terminal half of strand A. Strand A (residues 17-26) has a 90° bend at its midpoint, Leu22, reflecting its participation in both sheets. A ninth strand (I) extends the EFGHA sheet. The extended A-B loop (i.e. the loop connecting strand A to strand B) is involved in hydrogen bonding interactions that form part of the dimer interface along with strand I. The α-helix lies in the sequence between strands H and I. A disulfide bond (Cys66-Cys160) connects the C-D loop to the carboxyl-terminal region and is on the

outside of the molecule, close to the mouth of the hydrophobic pocket (Brownlow and others 1997). The second disulfide bond (Cys106-Cys119) links strands G and H. Cys121 is buried at the sheet-helix interface (Phillips and others 1994), and does not normally participate in a disulfide linkage in BLG (Uhrinova and others 2000).

The structure of BLG is pH dependent. At acidic pH less than 3.5, the dimer reversibly dissociates due to strong electrostatic repulsive interactions, but no conformation changes occur (Molinari and others 1996). At pH 2.6, an overall positive charge of BLG may cause a disruption of tertiary as well as quaternary structure (Uhrinova and others 2000). At pH 2 to 3, at which the protein has a net charge of ~+20, BLG is essentially monomeric under salt-free conditions (Timasheff and Townend 1961; Baldini and others 1999; Sakurai and others 2001). In the pH range of 3.7 to 5.2, BLG reversibly forms a larger oligomer. This self-association process has a maximum around pH 4.6, just below the isoelectric point, and is more pronounced for BLG-A than for BLG-B (Townend and Timasheff 1960; Timasheff and Townend 1961; McKenzie and others 1967). Light scattering studies indicated that intermediate oligomers (tetramers and hexamers) are present in significant amounts at 8 °C and 15 °C and at BLG concentrations of less than 1 mM (Kumosinski and Timasheff 1966). At neutral pH and BLG concentration greater than 50μM, BLG is predominantly dimeric. Six percent of the monomer surface area is buried at the dimer interface where 12 intermolecular hydrogen bonds and 2 ion pairs are involved (Brownlow and others 1997; Qin and others 1998). In the pH range of 6-8, a reversible refolding of BLG polypeptide chains is called the Tanford transition (Tanford and others 1959). At pH greater than 8, the open end of the BLG calyx provides an access route to a cavity at the center of BLG, which is a binding

site for small hydrophobic molecules; this may be considered as an "open" conformation, as opposed to a "closed" conformation occurring at acidic pH (2.6) (Uhrinova and others 2000).

Most lipocalins bind small hydrophobic molecules within the calyx. It has been suggested that BLG is involved in the transport of retinol and/or fatty acids (Cogan and others 1976; Sawyer and others 1998). A proposed retinol binding site, or hydrophobic pocket, is located in the cavity surrounded by β strands BCDEFG where residue side chains that line the cavity are all hydrophobic (Sawyer and others 1998). There is a concentration of hydrophobic residues lining the cleft formed between the α-helix and the β-sheet with charged or polar groups at one end of the cleft, thus forming a possible site for fatty acid binding (Figure 1).

Intrinsic Protein Fluorescence

Ultraviolet spectrophotometry is a fast, accurate quantitative and nondestructive technique used in studies of protein structure and function of membranes, polymers, and biological macromolecules (Demchenko 1992). Fluorescence is the emission of photons which results from the transition of paired electrons from a higher-energy orbital to the lower orbital (Lakowicz 1983). Following light absorption, a fluorophore is excited to a higher vibrational level. The electronic transition down to the lowest electronic level results in an excited vibrational state. The absorption spectrum of the molecule reflects the vibrational levels of the electronically excited states, and the emission spectrum reflects the vibrational levels of the ground electronic state. As a result, the vibrational structures seen in the absorption and the emission spectra are similar (Lakowicz 1983).

There are several intrinsic fluorophores in proteins (mainly aromatic amino acids: tryptophan, tyrosine, and phenylalanine) that have been used to explore protein structure-function relationships or molecule interactions in association with other physical parameters such as temperature and pressure (Lakowicz 1983). Among these fluorophores, the tryptophan residue (Trp) is most often used. The characteristics of its fluorescence emission spectra depend on its environment. The emission maximum wavelength (λ_{max}) of free Trp in aqueous solution is at about 352 nm and becomes shorter (blue shift) when Trp is in an nonpolar solvent or in a hydrophobic environment such as inside the protein core. The more nonpolar the environment, the larger the Trp λ_{max} blue shift. When the protein is gradually unfolded, the Trp residue becomes more and more exposed to the bulk aqueous solution. Then the λ_{max} shifts to the red and finally reaches 352 nm (free Trp in water) when Trp is totally solvated, i.e. when the protein is fully denatured. Therefore protein Trp fluorescence can be used as a probe related to the medium polarity allowing for detection and following protein conformational changes, from which information about protein structure-function, unfolding and molecule interaction, can be obtained. In comparison with Trp, tyrosine and phenylalanine residues in proteins have not been often used for such studies, mainly because their fluorescence quantum yields are lower and less sensitive to the environment (Lakowicz 1983), i.e. less sensitive to conformational changes. To measure the Trp fluorescence emission coming only from Trp residues, without disturbance from the excitation of tyrosine and phenylalanine residues, the protein is often excited at 295 nm or at longer wavelength, where tyrosine and phenylalanine do not absorb (Lakowicz 1983).

Extrinsic Fluorescence

Some extrinsic fluorescence probes including 1-anilinonaphthalene-8-sulfonic acid (ANS) and *cis*-parinaric acid (CPA) are widely used in BLG structure-function studies, especially for determining protein hydrophobicity. Upon binding of the probes to accessible hydrophobic regions of proteins, an increase in fluorescence is observed, which is used as a measure of protein surface hydrophobicity. However, due to the possible contribution of both electrostatic and hydrophobic interactions to the binding of these anionic probes, the interpretation based on these probes has not been easy.

PRODAN (6-propionyl-2-dimethylaminonaphthalene), introduced by Weber and Farris (1979), is a fluorescent probe that is very sensitive to the polarity of the environment. The binding of PRODAN to protein results in a fluorescence enhancement and a blue shift that provides for easy separation of the fluorescence contribution from the free and bound probe (Moreno and others 1999). PRODAN is suitable for studies of polarity of many protein cavities by spectroscopic techniques (McGregor and Weber 1986). Other uses for PRODAN were described for the study of pressure effects on ligand-protein complexes (Chong and Weber 1983) and on the dielectric constant in phosphatidylcholine lipid bilayers (Chong 1988).

Using an uncharged probe (PRODAN) for measuring protein surface hydrophobicity, especially under conditions of varying pH, is advantageous over using anionic probes ANS and CPA. Alizadeh-Pasdar and Li-Chan (2000) reported low surface hydrophobicity of BLG when using ANS or CPA at pH 3.0 as opposed to relatively high surface hydrophobicity when using PRODAN for unheated and heated (80°C) BLG. The presence or absence of a permanent charge results in electrostatic interactions that may overestimate protein hydrophobicity determined under selected pH conditions (Alizadeh-

Pazdar and Li-Chan 2000). Haskard and Li-Chan (1998) reported that increasing the ionic strength of buffers to 1.0 M NaCl had a negative effect on surface hydrophobicity determination of bovine serum albumin when using ANS and no significant effect when using PRODAN.

Flavor-Protein Interactions

Flavor is considered one of the most important attributes of food in the determination of acceptance by a consumer. The mixture of odorous compounds that is present in food is often very complex. Individual components may be present in extremely small concentrations (μg to sub-pg/Kg) and may be unstable or volatile. As a consequence, it is difficult to concentrate flavor compounds from foods representative of the original flavor and free of contaminants and artifacts.The quantities of flavor compounds perceived by the human nose are determined by the release of flavor compounds from the food matrix. The rate of release is based on the affinity of the flavor compounds for biopolymers that make up the food matrix.

The predominant influence of proteins on flavor release and perception is caused by interactions of flavor components with the protein. The interactions that occur between proteins and flavor compounds are either reversible binding, including hydrogen bonds, hydrophobic interactions, and ionic bonds, or irreversible binding via covalent linkages and the condensation of aldehydes with amino (NH_2) and sulfhydryl (SH) groups (Izzo and Ho 1993; Fischer and Widder 1997; Lubbers and others 1998; Adams and others 2001).

One of the most widely used proteins in flavor binding studies is BLG, which is known to interact with many flavor compounds, such as ketones, ionones, aldehydes, and

esters (van Ruth and Villeneuve 2002). Increases in percentage of retention of BLG with

increasing chain length for a series of alkanones and ethyl esters suggest hydrophobic

interactions (Andriot and others 2000). Most of the binding that occurs between the

flavor compound and BLG is reversible through hydrophobic affinity and hydrogen

binding (Tromelin and Guichard 2003; Guichard 2006). Any factor affecting

hydrophobic affinity or surface hydrophobicity of BLG influences flavor binding.

Salting-in-type salts, such as $BaCl_2$, $CaCl_2$, $MgCl_2$ and $MnCl_2$, destabilizes hydrophobic

interactions thereby decreasing flavor binding, whereas salting-out type salts, such as

$MgSO_4$ and Na_2SO_4, increase flavor binding through preferential hydration of proteins

(Arakawa and Timasheff 1984). Two opposing factors, the surface tension effect

contributing to an unfavorable free energy change and the salt binding to peptide bonds

and some side chains as major sources of favorable free energy, are responsible for

protein preferential interactions with salts (Arakawa and Timasheff 1984). The binding of

salting-out type salts to proteins is greater than the binding of salting-in type salts to

proteins is due to the surface tension effect that leads to significant preferential hydration

of proteins.

BLG is a possible carrier for flavor compounds and may be effective in delivering

or delaying release of flavor compounds. BLG can be altered to bind to a wide range of

volatile flavor compounds during food manufacturing or to release these flavor

compounds in a more or less controlled way upon modifications through chemical or

thermal means (Kuhn and others 2006).

Ultra High Hydrostatic Pressure of Foods

Introduced in 1899 by Bert H. Hite for the preservation of milk, meats, and juices by high pressure processing (Johnston 1995), ultra-high pressure (UHP), or high hydrostatic pressure (HHP), treatment of foods and other biological tissues affects the structural stability of chemical constituents, including proteins, vitamins, lipids, saccharides and pigments in such a way that may improve their intrinsic functional properties in terms of color, flavor, and texture retention. UHP treatment involves subjecting food materials to a high pressure (up to 87,000 pounds per square inch or 600 MPa) and holding (isobarically) the food under constant pressure for a specific period of time (less than 30 min) before pressure release. UHP is sufficient in bringing about the necessary level of treatment in terms of protein conformational change, microbial deactivation, and extended shelf-life (Tedford and others 1999). There is a growing body of literature concerning the effects of UHP on selected food ingredients. Reversible effects such as dissociation of polymeric structures or partial unfolding are observed below 200 MPa (Iametti and others 1997). Structural changes are induced in proteins when pressures greater than 200 MPa are employed. Pressures greater than 500 MPa result in irreversible unfolding of monomeric proteins, aggregation of monomers stabilized by thiol/disulfide interchange reactions (Futenberger and others 1997), and formation of gel structures of stable polymers made of denatured monomers, yet only occurs in 10% of the total protein as observed by circular dichroism (Iametti and others 1997). However, temperature induces irreversible denaturation of the whole protein through the hydrolysis of covalent bonds and/or aggregation of denatured proteins (Tedford and others 1999).

Effects on protein structure

The effects of pressure on proteins can be understood in terms of the Le Chatelier principle which states that any reaction, conformational change, or phase transition that is accompanied by a decrease in volume will be enhanced when pressure increases, while reactions involving an increase in volume will be inhibited (Johnston 1995; Ledward 2000). For pressure-induced protein unfolding, the equilibrium constant at atmospheric pressure (K_{atm}) is a function of the equilibrium constant at a given pressure (K_p) by the relationship:

$$Kp = K_{atm}exp(-p\Delta V_{unf}/RT) \text{ ,}$$

where p is pressure, ΔV_{unf} is the standard volume change of unfolding, R is the gas constant and T is the absolute temperature. As the equilibrium constant at a given pressure is directly related to the degree of unfolding ($Kp = \alpha /1- \alpha$), where α is the degree of denaturation, it follows that (Botelho and others 2000):

$$\ln(\alpha/1- \alpha) = \ln K_{atm} - p\Delta V_{unf}/RT$$

The Gibbs energy that determines thermodynamic equilibrium among conformers (stereoisomers) of a protein in solution is driven by pressure according to the following relation (Lasalle and others 2003),

$$\Delta V^0(p-p_0) - \frac{1}{2} \Delta\kappa(p-p_0)^2 + \Delta G^0 = \Delta G_p$$

where p_0 is the atmospheric pressure (1 bar), ΔG^0 and ΔV^0 are differences in the Gibbs energy and partial volume at 1 bar, respectively, and $\Delta\kappa$ denotes the difference in compressibility. Pressure changes the conformational equilibrium by acting on volumetric properties, while denaturants such as urea directly perturb the interaction energy and entropy embedded in ΔG^0 (Wu and Wang 1999). That is, pressure drives the

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equilibrium to increase the population of the lower volume conformer to the higher volume conformer (Weber and Drickamer 1983).

Proteins in solution may adopt a variety of conformations between the native state and the fully denatured state. The molten globule state is one of the conformations observed in globular proteins that is thermodynamically stable (Ptitsyn 1995) and is a compact denatured state under mild denaturing conditions with native-like secondary structure, with loss of tight native tertiary structure (Yang and others 2001). While in the molten globule state, the amino acid residues exhibit increased mobility compared to the native state (Ptitsyn 1995). Molten globules have increased exposure and hydration of hydrophobic residues compared with the native state (Dagget and Levitt 1992), as evidenced by increased binding of hydrophobic probes, especially ANS (Yang and others 2001), decreased solubility in water, increased susceptibility to aggregation, and increased heat capacity (Ptitsyn 1987).

The molten globule state can be induced for many proteins using chemical and physical methods such as acid denaturation, chemical denaturants (urea or guanidine hydrochloride), heating, high pressure treatment, or ethanol (Kumar and others 2004) (Engelhard and Evans 1995; Yang and others 2001; Mazon and others 2004; Paci and others 2005).

Pressure may affect the secondary, tertiary, and quaternary structure of proteins. Moderate pressure does not disrupt secondary structures because hydrogen bonds that stabilize the secondary structure are not affected by the little effect of pressure (Masson and Clery 1996). Belloque and others (2000) showed that at pressure treatments of 300 and 400 MPa, the BLG structure exhibits a high degree of flexibility through the

exposure of a thiol group near β-strands FGH, and exists in a completely unfolded state. However, the conformation of the BLG core after pressure release is identical to the native structure. The preservation of the core implies that most of the β-barrel is structured. Belloque and others (2000) suggested that after pressure treatment of 400 MPa, complete refolding of BLG may occur when BLG is returned to ambient pressure or during pressurization BLG did not unfold completely. Iametti and others (1997) used circular dichroism and fluorometry and observed that only 10% of the BLG structure was lost at both 600 and 900 MPa. Moller and others (1998) reported that the exposure of the thiol group after pressure treatment of 150 MPa decreased after 2 days, indicating the refolding of BLG. Hydrophobic amino acids are found in discrete patches on the surface of proteins. Iametti and Bonomi (1993) reported that physical denaturing agents can modify the number and properties of these surface hydrophobic patches. After pressure treatment of 900 MPa, a decrease in the number of surface hydrophobic sites was observed with a concomitant decrease of the affinity of BLG for hydrophobic probes (Iametti and others 1997).

Effects on flavor-protein binding

UHP treatment changes the structure of BLG, thus affecting the protein-flavor interactions. Liu and others (2005) have reported effects of UHP treatment on whey protein-flavor binding using fluorescence spectroscopy and headspace analysis. Depending on the structure of the flavor compounds, benzaldehyde and methyl ketones, the flavor concentration, and UHP treatment times, the number of binding sites and apparent dissociation constants were either unaffected or increased upon UHP treatment (Liu and others 2005).

Solid Phase Microextraction

Sample preparation techniques based on adsorption have been applied for the extraction of volatiles for instrumental analysis (Yang and Peppard 1994). Solid phase microextraction (SPME), developed by Pawliszyn and co-workers (Arthur and Pawliszyn 1990; Zhang and others 1994), involves the extraction of compounds onto a chemically modified fused silica optical fiber. It is a convenient and solvent-free method that is suitable for the analysis of food, beverages, oils (Page and Lacroix 1993; Yang and Peppard 1994; Steffen and Pawliszyn 1996; Ai 1997; Kataoka and others 2000), and many dairy products, including the detection of flavor compounds in headspace of β-lactoglobulin (Jung and Ebeler 2003). SPME is used in combination with gas chromatography (GC) (Rocha and others 2001), GC-mass spectrometry (GC-MS) (Jelen and others 2003), and GC-olfactometry (GC-O) (Rega and others 2003), and introduced for direct coupling with HPLC and LC-MS for the analysis of weak volatile or thermally labile compounds not amenable to GC or GC-MS (Pawliszyn 1999).

SPME is a multiphase equilibrium process. Most extraction systems are complex consisting of an aqueous phase with suspended particles having various adsorption interactions with analytes, and a gaseous headspace. During extraction, analytes migrate between all three phases until equilibrium is reached. A number of factors must be considered to ensure good accuracy and precision in the development of a SPME method. The SPME fiber coating is determined by the chemical nature of the target analyte, and its polarity and volatility characteristics. Polydimethylsiloxane (PDMS) is often considered first for its rugged liquid coating to withstand high injector temperatures, up to about 300 °C. PDMS is a nonpolar phase that extracts nonpolar analytes very well.

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However, it can be successfully applied to extract more polar analytes, particularly after optimizing extraction conditions. It is important to consider the use of mixed phase coatings for extraction of volatile compounds, and perform experiments with different fibers to identify the optimal coating type for a broad range of compound characteristics (Pawliszyn 1997).

The extraction mode is selected by considering the sample matrix, analyte volatility, and its affinity to the matrix. Headspace extraction is preferred due to faster equilibration times and for medium to high volatile analytes. For very polar analytes, such as acids and bases, which can have high affinity toward the matrix, changing the pH of the matrix can improve extraction.

Efficient desorption and rapid transfer of the analytes from the injector of the GC to the column require high linear flow rates of the carrier gas around the coating, and can be accomplished by using a narrow bore injector insert. Temperature also plays an important role in accelerating the desorption process. When the maximum allowable coating temperature is used as the injector temperature, adjustment of the desorption time facilitates quantitative desorption in a single injection.

The equilibration time is defined as the time after which the amount of analyte extracted remains constant and corresponds within the limits of experimental error to the amount extracted at infinite extraction time (Pawliszyn 1999). When equilibration times are excessively long, shorter extraction times can be used. Care must be taken when determining the equilibration time in HS-SPME determinations, especially when a rapid rise of the equilibration curve is followed by a very slow increase that is related to the mass transfer of the analyte from water through the headspace to the fiber. At

equilibrium, small variations in the extraction time do not affect the amount of the analyte extracted by the fiber. However, at the steep part of the curve, even small variations in the extraction time may result in significant variations in the amount extracted.

Gas Chromatography

Gas chromatography (GC) with inactive glass capillary columns is the most suitable method for the separation of flavor compounds. Gas chromatography is used extensively to analyze fatty acids, sterols, aldehydes, ketones, carbonyls, alcohols, and other volatiles and derivatized flavor compounds. Some of the purposes of using GC are to separate mixtures of compounds; determine the quantity of individual compounds in a mixture; and analyze aroma components, solvents, pesticides, or additives.

GC is a technique for separating chemical compounds in which a compound is carried by a mobile phase streaming through a column where separation is being affected by interaction of individual compounds with the stationary phase of the column. The mobile phase may be a gas and the stationary phase may be a liquid film on the surface of an inert support material or a solid surface. Affinity of the solute for the stationary phase results in retardation of its movement through the chromatography system. The distribution ratio (K) defines the distribution of compounds between the mobile phase and the stationary phase at equilibrium, and is described as

$$K = \frac{[C_S]}{[C_M]}$$

where C_S is the concentration of a compound in the stationary phase per unit volume, and C_M is the concentration of a compound in the mobile phase per unit volume. Each

compound separated will ideally have a different K, reflecting relative affinities for the stationary phase.

The factors that influence the distribution and retention of volatile compounds are composition and properties of the mobile phase and stationary phase; the intermolecular forces among component(s), stationary and mobile phases; and temperature. The intermolecular forces that influence retardation of components are based on Coulomb's Law,

$$F = k\frac{q_a q_b}{r^2}$$

where F is the force between charged particles, q_a and q_b. The separation between the particles is r, and k is a constant ($8.99 \times 10^9 \; Nm^2/c^2$). Coulomb's Law is the interaction between charged molecules, such that when F is negative the force between the molecules is attractive (Pratt and Pumplin; Braithwaite and Smith 1999). The two main types of electrostatic interactions are polar van der Waal's retention forces from interaction between molecules with surface charge and nonpolar dispersion forces between neutral molecules or functional groups.

An ideal chromatogram is judged by the resolution between peaks where adjacent peaks are resolved sufficiently so that accurate determination of the peak areas can be obtained (Braithwaite and Smith 1999). Therefore, there should be a baseline separation between the peaks and no overlap of the tail of one peak with the leading edge of the next peak. Resolution is a function of retention characteristics of the components (k), column efficiency (N) and the selectivity or separating capabilities (α) of the column:

$$N = 5.54\left(\frac{t_R}{w_k}\right)^2$$

where t_R is the retention time, w_h is the width at half height of the peak, and N is column efficiency. For a given column length, optimum column efficiency is obtained when the equilibrium step or plate height is at a minimum, described by the van Deemter equation:

$$H = A + \frac{B}{\bar{u}} + C_S \bar{u} + C_M \bar{u}$$

where the A term describes the eddy diffusion of component molecules and the variable pathways the mobile phase may follow through the stationary phase packing in the capillary column; the B term describes the random motion of molecules dispersed in the mobile phase along the longitudinal axis of the column, or longitudinal diffusion; and the C term describes molecules' constant movement between the mobile and stationary phases to establish equilibrium defined by the distribution ratio, K.

Many studies on the binding between flavor compounds and proteins focused on methods to study interactions between protein and flavor compounds (Jung and others 2002; Jung and Ebeler 2003), modeling flavor release (Castellano and Snow 2001), reduction of off-flavors of soy products (Zhou and Cadwallader 2006), developing new soy products (Zhou and others 2006), adding flavor to emulsions with proteins as emulsifiers (Pelletier and others 1998), and adding sulfur-containing compounds for roasted and savory flavors to meat products (Adams and others 2001). Considine and others (2005) reported that nonflavor ligands added prior to high pressure treatment inhibited the formation of intermediate, non-native protein species. Therefore, further investigation on the binding of flavor compounds associated with high-fat foods with proteins for use in reduced-fat foods, and the addition of flavor ligands to protein prior to UHP treatment is warranted.

There is an increasing demand for healthier low-fat foods. In reduced-fat foods, carbohydrates and proteins are dominant components that interact differently with flavor compounds compared with fat, and thus change the perceived flavor. Therefore, a better understanding of the science behind protein-flavor interactions is required for the development of improved food flavor, particularly that of low-fat foods.

Investigation of UHP on the flavor binding capabilities of BLG will assist in the design of proteins as a flavor carrier system in reduced-fat foods.

The objectives of this research are to:

1) evaluate the combined effects of UHP and holding time at selected pH values on the hydrophobic properties of BLG using intrinsic tryptophan and an extrinsic fluorescence probe;

2) develop a method to extract and quantitate selected flavor compounds using headspace-solid phase microextraction gas chromatography from a buffer solution; and

3) investigate the binding properties of selected flavor compounds with both untreated and UHP treated BLG through the use of intrinsic fluorescence quenching and static headspace analysis.

REFERENCES

Adams RL, Mottram DS, Parker JK, Brown HM. 2001. Flavor-protein binding: disulfide interchange reactions between ovalbumin and volatile disulfides. J. Agric. Food Chem. 49: 4333-4336.

Ai J. 1997. Headspace solid phase microextraction. Dynamics and quantitative analysis before reaching a partition equilibrium. Anal. Chem. 69: 3260-3266.

Alizadeh-Pazdar N, Li-Chan ECY. 2000. Comparison of protein surface hydrophobicity measured at various pH values using three different fluorescent probes. J. Agric. Food Chem. 48: 328-334.

Andriot I, Harrison M, Fournier N, Guichard E. 2000. Interaction between methyl ketones and β-lactoglobulin: sensory analysis, headspace analysis, and mathematical modeling. J. Agric. Food Chem. 48: 4246-4251.

Arakawa T, Timasheff SN. 1984. Mechanism of protein salting in and salting out by divalent cation salts; balance between hydration and salt binding. Biochem. 23: 5912-5923.

Arthur CL, Pawliszyn J. 1990. Solid phase microextraction with thermal desorption using fused silica optical fibers. Anal. Chem. 62: 2145-2148.

Baldini G, Beretta S, Chirica G, Franz H, Maccioni E, Mariani P, Spinozzi F. 1999. Salt-induced association of b-lactoglobulin by light and X-ray scattering. Macromolecules 32: 6128-6138.

Botelho MM, Valente-Mesquita VL, Oliveira KMG, Polikarpov I, Ferreira ST. 2000. Pressure denaturation of β-lactoglobulin. Eur. J. Biochem 267: 2235-2241.

Braithwaite A, Smith FJ. 1999. Chromatographic methods. Dordrecht: Kluwer Academic Publishers. 559p.

Bray GA, Popkin BM. 1999. Dietary fat intake does affect obesity. Amer. J. Clin. Nutr. 68(6): 1157-1173.

Brignon G, Dumas BR. 1973. Localisation dans la chaine peptidique de la b lactoglobuline bovine de la substitution Glu/Gln differenciant les variants genetiques B et D. FEBS Lett. 33: 73-76.

Brownlow S, Cabral JHM, Cooper R, Flower DR, Yewdall SJ, Polikarpov I, North ACT, Sawyer L. 1997. Bovine β-lactoglobulin at 1.8A resolution - still an enigmatic lipocalin. Structure 5: 481-495.

Burova TV, Grinberg NV, Grinberg VY, Tolstoguzov VB. 2003. Binding of odorants to individual proteins and their mixtures. Effects of protein denaturation and association. A plasticized globule state. Colloids Surfaces. A. Physico. Eng. Aspects 213: 235-244.

Castellano J, Snow NH. 2001. Modeling flavor release using inverse gas chromatography-mass spectrometry. J. Agric. Food Chem. 49: 4296-4299.

Chong PL. 1988. Effects of hydrostatic pressure on the location of PRODAN in lipid bilayers and cellular membranes. Biochem. 27: 399-404.

Chong PL, Weber G. 1983. Pressure dependence of 1,6-diphenyl-1,3,5-hexatrine fluorescence in single-component phosphatidylcholine liposomes. Biochem. 22: 5544-5551.

Cogan U, Kopelman M, Mokady S, Shinitzky M. 1976. Binding affinities of retinol and related compounds to retinol bindin proteins. Eur. J. Biochem. 65: 71-78.

Considine T, Singh H, Patel HA, Creamer LK. 2005. Influence of binding of sodium dodecyl sulfate, all-trans-retinol, and 8-anilino-1-naphthalenesulfonate on the high-pressure-induced unfolding and aggregation of b-lactoglobulin B. J. Agric. Food Chem. 53: 8010-8018.

Croguennec T, O'Kennedy BT, Mehra R. 2004. Heat-induced denaturation/aggregation of β-lactoglobulin A and B: kinetics of the first intermediates formed. Int. Dairy J. 14: 399-409.

Dagget V, Levitt M. 1992. A model of the molten globule state from molecular dynamics simulations. Proc. Natl. Acad. Sci. 89: 5142-5146.

Damodaran S, Kinsella JE. 1980. Flavor protein interactions. Binding of carbonyls to bovine serum albumin: thermodynamic and conformation effects. J. Agric. Food Chem. 28: 567-571.

Demchenko AP. 1992. Fluorescence and dynamics in proteins. In: Lakowicz JR. Topics in fluorescence spectroscopy. New York: Plenum Press. p 65-111.

Dumont JP, Land DG. 1986. Binding of diacetyl by pea proteins. J. Agric. Food Chem. 34: 1041-1045.

Engelhard M, Evans PA. 1995. Kinetics of interaction of partially folded proteins with a hydrophobic dye: evidence that molten globule character is maximal in early folding intermediates. Prot. Sci. 4: 1553-1562.

Food and Drug Administration. 2005. Direct food substances affirmed as generally recognized as safe. FDA.

Fischer N, Widder S. 1997. How proteins influence food flavor. Food Technol. 51(1): 68-70.

Futenberger S, Dumay E, Cheftel JC. 1997. High-pressure promotes β-lactoglobulin aggregation through SH/S-S interchange reactions. J. Agric. Food Chem. 45: 912-921.

Gremli HA. 1974. Interaction of flavor compounds with soy protein. J. Am. Oil Chem. Soc. 51: A95-A97.

Guichard E. 2006. Flavour retention and release from protein solutions. Biotech. Adv. 24: 226-229.

Guichard E, Langourieux S. 2000. Interactions between β-lactoglobulin and flavor compounds. Food Chem. 71: 301-308.

Guyot C, Bonnafont C, Lesschaeve I, Issanchou S, Voilley A, Spinnler HE. 1996. Effect of fat content on odor intensity of three aroma compounds in model emulsions: D-decalactone, diacetyl, and butyric acid. J. Agric. Food Chem. 44(8): 2341-2348.

Iametti S, Transidico P, Bonomi F, Vecchio G, Pittia P, Rovere P, Dall'Aglio G. 1997. Molecular modifications of β-lactoglobulin upon exposure to high pressure. J. Agric. Food Chem. 45: 23-29.

Izzo HV, Ho CT. 1993. Effect of residual amide content on aroma generation and browning in heated gluten-glucose model systems. J. Agric. Food Chem. 41: 2364-2367.

Jelen HH, Majcher M, Zawirska-Wojtasiak R, Wiewiorowska M, Wasowicz E. 2003. Determination of geosmin, 2-methylisoborneol, and a musty-earthy odor in wheat grain by SPME-GC-MS, profiling volatiles, and sensory analysis. J. Agric. Food Chem. 51: 7079-7085.

Johnston DE. 1995. High pressure effects on milk and meat. In: Ledward DA, Johnston DE, Earnshaw RG and Hasting APM. High pressure processing of foods. Loughborough: Nottingham University Press. p 99-121.

Jung D, Ebeler SE. 2003. Headspace solid-phase microextraction method for the study of the volatility of selected flavor compounds. J. Agric. Food Chem. 51: 200-205.

Jung D, Ebeler SE. 2003. Investigation of binding behavior of α- and β-ionones to β-lactoglobulin at different pH values using a diffusion-based NOE pumping technique. J. Agric. Food Chem. 51: 1988-1993.

Jung D-M, de Ropp JS, Ebeler SE. 2002. Application of pulsed field gradient NMR techniques for investigating binding of flavor compounds to macromolecules. J. Agric. Food Chem. 50: 4262-4269.

Kataoka H, Lord HL, Pawliszyn J. 2000. Applications of solid-phase microextraction in food analysis. J. Chrom. A 880: 35-62.

Kuhn J, Considine T, Singh H. 2006. Interactions of milk proteins and volatile flavor compounds: Implications in the development of protein foods. J. Food Sci. 71: R72-R82.

Kumar Y, Tayyab S, Muzammil S. 2004. Molten-globule like partially folded states of human serum albumin induced by fluoro and alkyl alcohols at low pH. Arch. Biochem. Biophys. 426: 3-10.

Kumosinski TF, Timasheff SN. 1966. Molecular interactions in b-lactoglobulin, X: The stoichiometry of the β-lactoglobulin mixed tertramerization. J. Am. Chem. Soc. 88(5635-5642).

Lakowicz JR. 1983. Principles of fluorescence spectroscopy. New York: Plenum Press. 496p.

Lasalle MW, Li H, Yamada H, Akasaka H, Redfield C. 2003. Pressure-induced unfolding of the molten globule of all-Ala α-lactalbumin. Prot. Sci. 12: 66-72.

Law M. 2000. Dietary fat and adult diseases and the implications for childhood nutrition: an epidemic approach. Am. J. Clin. Nutr. 72: 1291S-1296S.

Ledward DA. 2000. Effects of pressure on protein structure. High Pressure Res. 19: 1-10.

Lindsay RC. 1996. Food additives. In: Fennema OR. Food Chemistry. Third Edition. New York: Marcel Dekker, Inc. p 767-821.

Liu X, Powers JR, Swanson BG, Hill HH, Clark S. 2005. Modification of whey protein concentrate hydrophobicity by high hydrostatic pressure. Innov. Food Sci. Emerg. Technol. 6(3): 310-317.

Lubbers S, Landy P, Voilley A. 1998. Retention and release of aroma compounds in foods containing proteins. Food Technol. 52: 68-74, 208-214.

Masson P, Clery C. 1996. Pressure-induced molten globule states of proteins. In: Hayashi R. High pressure bioscience and biotechnology. London: Elsevier Applied Science Publishers. p 117-126.

Mazon H, Marcillat O, Forest E, Smith DL, Vial C. 2004. Conformational dynamics of the GdmHCl-induced molten globule state of creatine kinase monitored by hydrogen exchange and mass spectrometry. Biochem. 43: 5045-5054.

McBean LD. 2000. Insights into weight management. Dietary determinants of obesity. http://www.nationaldairycouncil.org/NationalDairyCouncil/Health/Digest/dcd71-2Page3.htmT. 05-02-06

McGregor RB, Weber G. 1986. Estimation of the polarity of the protein interior by optical spectroscopy. Nature 319: 70-73.

McKenzie HA, Sawyer WH, Smith MB. 1967. Optical rotatory dispersion and sedimentation in the study of association-dissociation: bovine β-lactglobulins near pH 5. Biochim. Biophys. Acta 147: 73-92.

Molinari H, Ragona L, Varani L, Musco G, Consonni R, Zetta L, Monaco HL. 1996. Partially folded structure of monomeric bovine β-lactoglobulin. FEBS Letters 381: 237-243.

Moller RE, Stapelfeldt H, Skibsted LH. 1998. Thiol reactivity in pressure-unfolded β-lactoglobulin. Antioxidative properties and thermal refolding. J. Agric. Food Chem. 46: 425-430.

Moreno F, Cortijo M, Gonzalez-Jimenez J. 1999. The fluorescent probe Prodan characterizes the warfarin binding site on human serum albumin. Photochem. and Photobiol. 69(1): 8-15.

Nawar WW. 1996. Lipids. In: Fennema OR. Food chemistry. Third Edition. New York: Marcel Dekker, Inc. p 225-319.

Paci E, Greene LH, Jones RM, Smith LJ. 2005. Characterization of the molten globule state of retinol-binding protein using a molecular dynamics simulation approach. FEBS J. 272: 4826-4838.

Page B, Lacroix G. 1993. Application of solidphase microextraction to the headspace gas chromatographic analysis of halogenated volatiles in selected foods. J. Chrom. 48: 199-211.

Pawliszyn J. 1997. Solid phase microextraction: theory and practice. New York: Wiley-VCH, Inc. 247p.

Pawliszyn J. 1999. Applications of solid phase microextraction. Cambridge: The Royal Society of Chemistry. 655.

Pelletier E, Sostmann K, Guichard E. 1998. Measurement of interactions between b-lactoglobulin and flavor compounds (esters, acids, and pyrazines) by affinity and exclusion size chromatography. J. Agric. Food Chem. 46(4): 1506-1509.

Phillips LG, Whitehead DM, Kinsella JE. 1994. Structural and chemical properties of β-lactoglobulin. In: Structure-function properties of food proteins. New York: Academic Press. p 75-106.

Pratt S, Pumplin J. Coulomb's law. http://www.pa.msu.edu/courses/1997spring/PHY232/lectures/coulombslaw/. July 15, 2006

Ptitsyn OB. 1987. Protein folding: hypotheses and experiments. J. Prot. Chem. 6: 273-293.

Ptitsyn OB. 1995. Molten globule and protein folding. Adv. Prot. Chem. 47: 83-229.

Qin BY, Bewley MC, Creamer LK, Baker HM, Baker EN, Jameson GB. 1998. Structural basis of the tanford transition of bovine β-lactoglobulin. Biochem. 37: 14014-14023.

Rega B, Fournier N, Guichard E. 2003. Solid phase microextraction (SPME) of oragne juice flavor: odor representativeness by direct gas chromatography olfactometry (D-GC-O). J. Agric. Food Chem. 51: 7092-7099.

Rocha S, Ramalheira V, Barros A, Delgadillo I, Coimbra MA. 2001. Headspace solid phase microextraction (SPME) analysis of flavor compounds in wine. Effect of the matrix volatile composition in the relative response factors in a wine model. J. Agric. Food Chem. 49: 5142-5151.

Sakurai K, Oobatake M, Goto Y. 2001. Salt-dependent monomer-dimer equilibrium of bovine β-lactoglobulin at pH 3. Prot. Sci. 10: 2325-2335.

Sawyer L, Brownlow S, Polikarpov I, Wu SY. 1998. β-Lactoglobulin: structural studies, biological clues. Int. Dairy J. 8: 65-72.

Sloan AE. 2006. Top 10 functional food trends. Food Technol. 60(4): 22-39.

Steffen A, Pawliszyn J. 1996. Analysis of flavor volatiles using headspace solid-phase microextraction. J. Agric. Food Chem. 44: 2187-2193.

Swaisgood HE. 1996. Characteristics of milk. In: Fennema OR. Food chemistry. New York: Marcel Dekker, Inc. p 841-878.

Tanford C, Bunville LG, Nozaki Y. 1959. The reversible transformation of β-lactoglobulin at pH 7.5. J. Am. Chem. Soc. 81: 4032-4035.

Tedford L-A, Smith D, Schaschke CJ. 1999. High pressure processing effect on the molecular structure of ovalbumin, lysozyme and β-lactoglobulin. Food Res. Int. 32: 101-106.

Thompson TG, Veneman AM. 2005. Dietary guidelines for Americans 2005. www.health.gov/dietaryguidelines/dga2005/document/html/chapter6.htm. 10-6-06

Timasheff SN, Townend R. 1961. Molecular interactions in β-lactoglobulin, V: The association of the genetic species of β-lactoglobulin below the isoelectric point. J. Am. Chem. Soc. 83: 464-469.

Timasheff SN, Townend R. 1961. Molecular interactions in β-lactoglobulin. VI. The dissociation of the genetic species of β-lactoglobulin at acid pH's. J. Am. Chem. Soc. 83: 470-473.

Townend R, Timasheff SN. 1960. Molecular interactions in β-lactoglobulin. III. Light scattering investigation of the stoichiometry of the association between pH 3.7 and 5.2. J. Am. Chem. Soc. 82: 3168-3174.

Tromelin A, Guichard E. 2003. Use of catlyst in a 3D-QSAR study of the interactions between flavor compounds and β-lactoglobulin. J. Agric. Food Chem. 51: 1977-1983.

Uhrinova S, Smith MH, Jameson GB, Uhrin D, L. S, Barlow PN. 2000. Structure changes accompanying pH-induced dissociation of the β-lactoglobulin dimer. Biochem. 39: 3565-3574.

USDA. 1999. Dairy products 1999 summary. http://usda.mannlib.cornell.edu/reports/nassr/dairy/pdp-bban/daryan00.txt.

van Ruth SM, Villeneuve E. 2002. Influence of β-lactoglobulin, pH and presence of other aroma compounds on the air/liquid partition coefficients of 20 aroma compounds varying in functional group and chain length. Food Chem. 79: 157-164.

Weber G, Drickamer HG. 1983. The effect of high pressure upon proteins and other biomolecules. Q. Rev. Biophys. 16: 89-112.

Wu JW, Wang ZX. 1999. New evidence for the denaturant binding model. Prot. Sci. 8: 2090-2097.

Yang J, Dunker AK, Powers JR, Clark S, Swanson BG. 2001. β-Lactoglobulin molten globule induced by high pressure. J. Agric. Food Chem. 49: 3236-3243.

Yang X, Peppard T. 1994. Solid-phase microextraction for flavor analysis. J. Agric. Food Chem. 42: 1925-1930.

Zhang Z, Yang MJ, Pawliszyn J. 1994. Solid phase micro-extraction. J. Anal. Chem. 66: 844A-853A.

Zhou Q, Cadwallader KR. 2006. Effect of flavor compound chemical structure and environmental relative humidity on the binding of volatile flavor compounds to dehydrated soy protein isolates. J. Agric. Food Chem. 54: 1838-1843.

Zhou Q, Lee S-Y, Cadwallader KR. 2006. Inverse gas chromatographic evaluation of the influence of soy protein on the binding of selected butter flavor compounds in a wheat soda cracker system. J. Agric. Food Chem. 54: 5516-5520.
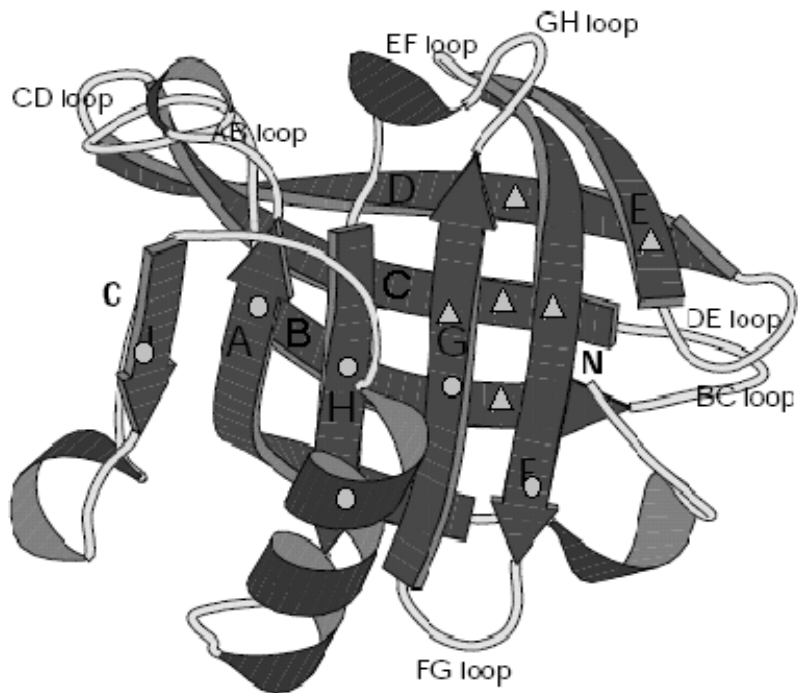
△ Hydrophobic Pocket
◎ Hydrophobic Surface Pocket

Figure 1. Ribbon diagram of a single subunit of β-lactoglobulin. The diagram was reproduced from (Brownlow and others 1997). The triangle and circle dots indicate positions for the hydrophobic pocket or hydrophobic surface pocket, respectively, proposed by Sawyer and others (1998).

CHAPTER TWO

Surface Hydrophobicity of Ultra High Pressure Treated β-Lactoglobulin – PRODAN Fluorescent Probe

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ABSTRACT

The effects of ultra-high hydrostatic pressure (UHP) treatment at 600 MPa with treatment times of 0 to 32 min and pH values of 3.0 to 9.0 on intrinsic tryptophan fluorescence of β-lactoglobulin (BLG) and the binding properties of 6-propionyl-2-(dimethylamino)-naphthalene (PRODAN) extrinsic probe to BLG were studied. UHP treatment of BLG at selected pH values resulted in increases in tryptophan fluorescence peak intensities with unfolding of BLG structure. A red shift in the emission peak wavelength after UHP come-up time at pH 7.0 indicated an increase in the polarity of the microenvironment of tryptophan. Significant increases in surface hydrophobicity of UHP-treated BLG were observed with the greatest value at pH 9.0 after come-up time, probably resulting from irreversible unfolding and exposure of hydrophobic sites of BLG to PRODAN. Significant changes in the number of binding sites and increases in dissociation constants of BLG for PRODAN were observed as a function of pH and UHP treatment time. A linear relationship was not observed between UHP treatment and number of binding sites. However, at pH 7.0, BLG had relatively low dissociation constants, with more binding sites than BLG of other pH values, suggesting that UHP-treated BLG can be used as an ingredient for improvement of flavor in most reduced fat foods close to neutral pH.

INTRODUCTION

Recent developments and advances in ultra high pressure (UHP) processing technology are now at a stage where certain foodstuffs can be commercially processed isobarically at pressures on the order of hundreds of mega-pascals (MPa). In contrast to thermal processing, UHP processing affects the structural stability of food constituents such as proteins in such a way that can improve their intrinsic functional properties for new ingredients.

UHP involves subjecting a food material to pressures up to 600 MPa and holding the food isobarically for up to 30 min before pressure release (Ramaswamy and others). UHP treatment is sufficient in bringing about necessary molecular change, microbial deactivation, pasteurization, and extended shelf-life.

High pressures act by altering the balance of intramolecular and solvent-protein interactions. Pressure-induced denaturation results from the disruption of both hydrophobic interactions and salt bridges. The extent of the changes in proteins depends on factors such as temperature, pH, solvent, and ionic strength, as well as on the nature of the native protein structure and pressure applied to the proteins (Iametti and others 1997). Pressure-induced protein structure unfolding is accompanied by a reduction in volume and hydration of nonpolar amino acid residues (Damodaran 1996). The unfolding mechanism of pressure-induced protein denaturation is not yet fully understood, although UHP treatment results in changes in the structure of the protein molecules due to the cleavage of weak hydrogen bonds and van der Waals forces while covalent bonds are unaffected (Tedford and others 1999).

Fluorescence spectroscopy is a useful technique to study the structure and dynamics of protein molecules, providing the primary protein structure harbors intrinsic chromophores. The sensitivity and noninvasiveness of fluorometry is a promising and widely used technique in medicine, biology, biochemistry, and molecular biophysics (Royer 1995). The intrinsic fluorescence of tryptophan residues in proteins is particularly sensitive to protein microenvironments and provides a sensitive method to study conformational changes of proteins (Busti and others 2002).

Extrinsic fluorescent probes can bind specifically to proteins, influencing both the intrinsic fluorescence of the protein and the fluorescence of the probe. PRODAN (6-propionyl-2-(dimethylamino)-naphthalene) exhibits sensitivity to the polarity of the environment in biological materials (Alizadeh-Pazdar and Li-Chan 2000). Although PRODAN exhibits low quantum yield in aqueous solutions, PRODAN becomes highly fluorescent in nonpolar solvents or when bound to hydrophobic sites on proteins or membranes (Vazquez and others 2005). Without a charge on PRODAN, electrostatic interactions are eliminated during the determination of surface hydrophobicity. Spectral shifts of PRODAN fluorescence that reflect changes in polarity of the environment and absence of ionic interactions due to lack of a permanent charge are supported by studies on spectral properties of PRODAN (Weber and Farris 1979), and binding with spectrin, egg and milk proteins (Alizadeh-Pazdar and others 2004), bovine serum albumin, ovalbumin (Haskard and Li-Chan 1998), thionin (Huang and others 1997), and lipid bilayers (Kusube and others 2005).

Recently the PRODAN probe was used by Alizadeh-Pasdar and Li-Chan (2000) for the quantitation of protein surface hydrophobicity at pH 3.0, 5.0, 7.0, and 9.0. In that

study (Alizadeh-Pazdar and Li-Chan 2000), a method was developed with PRODAN to

quantitate protein surface hydrophobicity comparing the binding of PRODAN versus

aromatic 1-anilinonaphthalene-8-sulfonic acid (ANS) and anionic aliphatic *cis*-parinaric

acid (CPA) fluorescent probes to whey protein isolate (WPI), β-lactoglobulin (BLG), and

bovine serum albumin (BSA) during thermal treatment at 80°C. Alizadeh-Pazdar and Li-

Chan (2000) reported lower hydrophobicity at pH 3.0 than other pH values for BSA,

BLG, and WPI determined using PRODAN, whereas hydrophobicity determined using

ANS was high at pH 3.0 for the proteins compared to other pH values. They also reported

that heating (80 °C for 30 min) of BSA, BLG and WPI at pH 3.0, 5.0, 7.0, or 9.0 either

had no effect or significantly decreased ($p \leq 0.05$) surface hydrophobicity determined

using PRODAN.

Some authors have reported using ANS and CPA to determine hydrophobicity of

UHP treated BLG or whey protein concentrate (Yang and others 2001; Liu and others

2005) and others have reported using PRODAN for characterizing binding sites of human

serum albumin (Moreno and others 1999). It is the aim of this research to use PRODAN

to observe changes in hydrophobicity of BLG as a function of UHP treatment and pH.

MATERIALS & METHODS

β-Lactoglobulin (BLG) and PRODAN were purchased from Sigma Chemical Co.

(St. Louis, MO, no. L-0130) and Molecular Probes (Eugene, OR), respectively.

Ultra-high Hydrostatic Pressure (UHP) Treatment

BLG solutions, at selected protein concentrations of 0.05 to 0.50 mg/mL in 0.05

mg/mL increments were prepared with sodium phosphate buffer (0.01 M) and adjusted to

selected pH values of 3.0, 5.0, 7.0, or 9.0. The concentrations of BLG at pH 7.0 were

spectrophotometrically determined using a molar extinction coefficient of $\varepsilon_{280} = 17600$ $M^{-1} cm^{-1}$ at 280 nm. BLG solutions, at an initial temperature of 4°C, were treated with an isostatic pressing system (Engineered Pressure Systems, Inc., Andover, MA) composed of a cylindrical pressure chamber (height = 0.25 m, diameter = 0.10 m) at 600 MPa for 0 (come-up time), 4, 8, 16, or 32 min. The come-up time (5.25 min) is the compression time required to reach a pressure of 600 MPa. After exposure to high pressure, the BLG solutions were assayed immediately or stored at 4 °C.

Intrinsic and Extrinsic Fluorescence Spectra

Conformational changes of BLG solutions were monitored by observing intrinsic tryptophan and extrinsic fluorescence spectra. Intrinsic fluorescence was assayed using an excitation wavelength of 295 nm to avoid excitation of tyrosine (Bhattacharjee and Das 2000; Muresan and others 2001) and observing fluorescence intensity at the emission wavelength of 338 nm. Extrinsic PRODAN fluorescence of BLG solutions was assayed using an excitation wavelength of 365 nm and observing the fluorescence intensity at the emission spectra at wavelengths from 375 to 600 nm. Ten microliters of PRODAN (0.153 mM in methanol) solution was added to 4 mL of untreated or UHP-treated BLG solutions (0.05-0.5 mg/mL) for the extrinsic PRODAN assay. Intrinsic and extrinsic fluorescence data were collected with a Fluoro Max-3 fluorometer (Jobin Yvon Horiba Spex, Edison, NJ) and data-conversion software (DATAMAX and GRAMS/32™, Jobin Yvon Horiba Spex, Edison, NJ).

Surface Hydrophobicity

The extrinsic aromatic uncharged hydrophobic PRODAN fluorescent probe is unlike other probes used to determine hydrophobicity of proteins. Unlike PRODAN,

fluorescent probes such as ANS or CPA are anionic and may contribute charged interactions on the determination of surface hydrophobicity at various pH values (Alizadeh-Pazdar and Li-Chan 2000). PRODAN, on the other hand, has no charge, eliminating possible electrostatic contributions, and hence the PRODAN probe was used to assay the hydrophobic nature of BLG.

Surface hydrophobicity of BLG was determined using the PRODAN probe. A stock solution of 1.53×10^{-4} M PRODAN was prepared in methanol, sealed with Parafilm to prevent evaporation of methanol, covered with aluminum foil to avoid exposure to light, and stirred for 5 hr. The PRODAN stock solution was stored in the freezer (-11°C) until the day of the assay, and held on ice throughout the experiment. The concentration of PRODAN in the stock solution was spectrophotometrically determined using a molar absorption coefficient of $\varepsilon_{360} = 1.8 \times 104$ M^{-1} cm^{-1} (Chakrabarti 1996) before performing the assay.

Intrinsic tryptophan fluorescence determinations were performed using excitation/emission wavelengths of 295 nm/338 nm and slits were set at 5 nm/5 nm to avoid excitation of tyrosine. Even though tyrosine has a high quantum yield, the tyrosine emission of most proteins is small and undetectable due to quenching by the presence of nearby charged or uncharged amino groups (Lakowicz 1983). The fluorescence emission of tryptophan is often studied for its solvent sensitivity and characterization of the protein structure during denaturation as evidenced by its blue or red shifts (Lakowicz 1983). Extrinsic fluorescence determination of PRODAN assay hydrophobicity of BLG was performed with excitation/emission wavelengths of 365 nm/465 nm and slits were set at 5 nm/5 nm. A 10 µL aliquot of the PRODAN stock solution was added to 4 mL of diluted

native and UHP treated proteins, and mixed well by vortexing. After 20 min equilibration in the dark, the relative fluorescence intensity (RFI) of each solution was determined in replicate, starting with a buffer blank (buffer plus probe) and then the smallest to the greatest protein concentration. The fluorometer quartz cell (Starna Cells, Inc, Atascadero, CA, no.3-Q-10) was rinsed between fluorescence assays with a small volume of the future solution being assayed. The RFI of each protein dilution blank (no probe) was subtracted from the RFI of each corresponding protein solution with PRODAN to obtain net RFI. Line fitting analysis (Microsoft Excel 2002 for Windows XP) was used to determine the linearity of the plot of net RFI versus protein concentration, and the resulting slope was observed as an index of BLG surface hydrophobicity according to Alizadeh-Pazdar and Li-Chan (2000).

Determination of the Apparent Dissociation Constants

PRODAN binding properties were calculated with the Cogan method (Cogan and others 1976). The number of accessible binding sites and apparent dissociation constants of PRODAN with BLG were calculated with equation (1)

$$P_0 \alpha = \left(\frac{1}{n}\right)\left(\frac{R_0 \alpha}{1-\alpha}\right) - \left(\frac{K_d'}{n}\right) \tag{1}$$

where P_0 is the total protein concentration, α is defined as the fraction of free binding sites on the protein molecules, n is the number of independent binding sites for PRODAN, R_0 is total PRODAN concentration, and $K'd$ is the apparent dissociation constant of a single site. The value of α was calculated with equation (2)

$$\alpha = \frac{F_{max} - F}{F_{max} - F_0} \tag{2}$$

where F represents the fluorescence intensity of PRODAN at selected R_0, F_{max} represents the fluorescence intensity of PRODAN upon saturation of protein molecules, and F_0 is the initial fluorescence intensity of PRODAN in the absence of BLG.

Statistical Analysis

Data collected from the binding studies of PRODAN to BLG were analyzed using an analysis of variance (ANOVA) procedure using the General Linear Model, with further analysis using Tukey's pairwise comparison test to determine differences (p < 0.05) between UHP and pH treatment means (SAS for Windows, version 9.1 TS Level 1M2, SAS Institute Inc., Cary, NC, USA.).

RESULTS & DISCUSSION

Conformational Change - Intrinsic Fluorescence

The tryptophan fluorescence spectra of native BLG treated at selected pH values are presented in Figure 1. A difference in peak intensity of the emission spectra was observed between native BLG at pH 3.0 and pH 7.0, and can be accounted for the stability of native BLG through dimerization at the higher pH as observed by Busti and others (2002). A high fluorescence peak intensity observed for native BLG at pH 5.0 results from the displacement of water through protein-protein interactions of monomers near its isoelectric point of 5.2 (Phillips and others 1994). In Figure 1, the emission wavelengths occurring at peak intensity for native BLG at pH 7.0 and pH 9.0 were observed at 339 and 345 nm. The change in emission wavelength, or red shift, with an increase in pH indicates that the microenvironment surrounding Trp19 and Trp 61 is becoming more polar (Lakowicz 1983).

The tryptophan fluorescence spectra of BLG treated at 600 MPa for selected times and pH values are presented in Figures 2-5. At pH 3.0 (Figure 2), BLG exhibited a two-stage increase in tryptophan emission peak maximum with the first increase observed after pressure was applied (come-up time) and the second increase of fluorescence emission peak maximum after 16 min of pressurization. The increases in tryptophan fluorescence maxima of BLG upon unfolding by UHP are consistent with the results of Yang and others (2001) and Liu and others (2005). The increase in tryptophan fluorescence maxima of BLG during UHP treatment suggests the dissociation of the BLG dimer. Small increases in tryptophan fluorescence intensities observed after UHP treatment indicates that unfolding of the compact structure of BLG at acid pH may or may not be complete (Phillips and others 1994).

After exposure of BLG solutions of pH 5.0 to UHP, precipitation of BLG from the solution was observed visually. The tryptophan emission peak maximum of native BLG increased by 18% after UHP come-up time, and remained at this intensity after UHP of 32 min (Figure 3). After UHP treatment of 16 min, the peak emission wavelength increased by 2 nm, indicating that the tryptophan microenvironment became more polar. Although BLG was near its isoelectric point of 5.3, a precipitate was only noticeable visually after UHP treatment. Around pH 4.5, BLG is prone to form octameric structures, and Timasheff and others (1966) proposed that the transition between the native isoelectric form of BLG and the acid forms facilitates this octamer formation. The applied pressure to BLG at pH 5.0 may have unfolded the subunits exposing the hydrophobic interior of BLG. The exposed cysteine groups of each BLG molecule then can readily participate in sulfhydryl-disulfide interchange reactions with neighboring

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BLG molecules (Swaisgood 1996) to minimize unfavorable interactions of hydrophobic groups with water, thereby causing the precipitation of BLG from solution (Petsko and Ringe 2004).

Following UHP come-up time at pH 7.0, the tryptophan fluorescence emission peak maximum decreased slightly from 1.37×10^7 to 1.34×10^7 with 2 nm red shift in the tryptophan emission wavelength (Figure 4). By increasing pressure times to 16 min, the tryptophan fluorescence maximum increased to 1.39×10^7. The observed decrease in the tryptophan emission spectra can be accounted for by the Tanford transition that occurs in BLG between pH 6.0 and 8.0 (Taulier and Chalikian 2001). Taulier and Chalikian (2001) believed that the transition induced a change in local microenvironment of aromatic residues with loosening of the interior packing. This loosening may explain the increase in the tryptophan emission peak maximum after 16 or 32 min of UHP, and also results from the transition to the intermediate molten globule structure as reported by Yang and others (2001). The increase in the fluorescence intensity observed is attributed to the β-strand EF loop of BLG adopting an "open" conformation that promotes access to large internal hydrophobic pockets (Adams and others 2006).

The tryptophan emission peak maxima of BLG after UHP treatment at pH 9.0 exhibited a decrease from 1.36×10^7 to 1.25×10^7 after 4 min of UHP and an increase to 1.34×10^7 after UHP of 16 min (Figure 5). Although lower than the observed fluorescence emission maxima of BLG at pH 3.0 to 7.0, this value may be possibly due to the retention of BLG secondary structure (Casal and others 1988). At alkaline pH, BLG is susceptible to surface denaturation, or base-induced denaturation (Timasheff and others 1966), accompanied by a more open flexible molecular structure (Phillips and others

1994). The increases in tryptophan intrinsic fluorescence after 16 min of UHP treatment may indicate further unfolding of the BLG monomer with loosening of secondary structure as observed for effects of chaotropic agents, such as urea, greater than 1.5 M on BLG (Phillips and others 1994; Yang and others 2001).

Conformational Change - Extrinsic PRODAN Fluorescence

PRODAN fluorescence emission spectra were observed at 25°C as a function of UHP (600 MPa) for selected times and pH (Figures 6-9). The PRODAN fluorescence emission spectrum of untreated BLG exhibited two peaks: free PRODAN showed a green emission peak at 520 nm, and PRODAN bound to BLG showed a blue emission peak at 450 nm (Hiratsuki 1998). The binding of PRODAN, as observed by increases in extrinsic fluorescence intensity, did not indicate binding to specific binding sites of BLG, but may suggest similar binding properties of other nonpolar flavor compounds. It can be seen in Figure 6 that at pH 3.0 or 5.0, native BLG exhibited an emission wavelength 25 nm less than observed emission wavelengths of 450 nm for BLG at pH 7.0 or 9.0, suggesting that the binding areas for PRODAN in BLG is nonpolar at acidic pH. UHP treatment of BLG at pH 3.0 (Figure 7), after come-up time, resulted in a decrease in the PRODAN extrinsic fluorescence intensity. The compact structure of BLG at pH less than 4.0 may increase BLG resistance to pressure denaturation occluding PRODAN molecules from entering the hydrophobic interior (Phillips and others 1994). However, after 4 min UHP treatment of BLG, BLG may not resist pressure denaturation hence an observed increase in extrinsic fluorescence.

At pH 5.0 (Figure 8), the PRODAN fluorescence emission peak of native BLG observed at 450 nm increased with increasing UHP treatment time. After an 8 min UHP

treatment of BLG, a 152% increase in PRODAN fluorescence intensity was observed, indicating large numbers of PRODAN molecules bound to BLG. The binding of PRODAN molecules suggests that BLG is unfolding under UHP and exposing the hydrophobic interior, suggesting under these conditions, BLG is favorable for binding of hydrophobic flavor compounds.

The difference in fluorescence intensity maxima between native BLG at pH 5.0 and 7.0 (Figure 6) and continued increase in intensity after UHP treatment (Figure 9) indicate that the conformation of a loop containing β-strands, β-E and β-F, changes as a function of pH, and the EF loop is folded back to reveal the interior of the calyx in an open conformation (Qin and others 1998).

Unlike the fluorescence intensities as observed of BLG at pH 7.0, the changes in intensities observed of BLG at pH 9.0 (Figure 10) is the result of base-induced denaturation disrupting the native dimeric structure into unfolded monomers (Taulier and Chalikian 2001). The small decreases in PRODAN fluorescence intensities observed after UHP treatment of BLG may account for hydration of BLG monomers, decreasing access of PRODAN molecules to the hydrophobic interior.

In summary, BLG may resist pressure-induced denaturation at acid pH, however, as pH increases towards the isoelectric point (pH 5.3), precipitation of BLG allows PRODAN molecules to bind BLG. At neutral pH, the molten globule state of BLG occurs after pressurization, and hydrophobic binding of PRODAN is enhanced at pH 9.0 through base-induced denaturation.

Surface Hydrophobicity

Linear plots that were generated from net RFI versus protein concentration gave a slope as an index of BLG surface hydrophobicity. The quantitation of protein hydrophobicity is important for predicting protein functionality. Surface or effective hydrophobicity is termed for a portion of nonpolar amino acid residues, positioned in the interior of protein molecules in solutions to avoid contact with the aqueous environment, which participate in polar and nonpolar interactions, and does not correlate with total hydrophobicity (Nakai 1983).

The pH significantly affected surface hydrophobicity (Figure 11) of untreated BLG ($p < 0.05$), with the smallest surface hydrophobicity observed at pH 3.0, which is consistent with results of Alizadeh-Pasdar and Li-Chan (2000). The findings also support the suggestion that the E-F loop (residues 86-89) is arranged so as to occlude the open end of the calyx, giving the closed conformation (Uhrinova and others 2000). UHP treatment of BLG after 4, 8, or 16 min at pH 3.0 significantly increased surface hydrophobicity ($p < 0.05$). UHP treatment come-up time or 4 min significantly decreased surface hydrophobicity ($p < 0.005$) at pH 5.0 through formation of protein aggregates. The formation of disulfide bonds following SH/S-S interchange (Futenberger and others 1995) decreased the accessibility of PRODAN to the surface hydrophobic site, especially at or near the isoelectric point (pH 5.3) of BLG. UHP treatment had little observed effect on surface hydrophobicity of BLG at pH 7.0. However, there was a decrease in surface hydrophobicity observed after UHP of 16 min, probably due to the interaction of partially denatured proteins resulting in burial of effective hydrophobic regions (Alizadeh-Pazdar and Li-Chan 2000). Surface hydrophobicity of BLG increased significantly ($p < 0.005$) after UHP come-up time at pH 9.0, which are consistent with results of thermal treatment

of BLG observed using PRODAN from Alizadeh-Pasdar and Li-Chan (2000).

Additionally, the E-F loop is in the open conformation, providing access to the

hydrophobic pocket.

The surface hydrophobic site of BLG at pH 3.0 has little accessibility to

PRODAN molecules. The observed increase in PRODAN extrinsic fluorescence at pH

7.0 may indicate that BLG has other hydrophobic sites to which PRODAN molecules can

bind. At alkaline pH, UHP treatment of BLG after come-up time may partially unfold

BLG at the cleft formed by β strands A, H, and I, thereby increasing BLG surface

hydrophobicity through exposure of other nonpolar amino acid residues.

PRODAN Binding to BLG

Titrations of BLG with PRODAN (Figures 12-35) were plotted according to the

Cogan method (1976). At pH 3.0, native BLG exhibited 2.26 binding sites for PRODAN

per molecule of protein with an apparent dissociation constant of 114.79 x 10^{-7} M (Table

1). At pH 5.0 and 7.0, native BLG exhibited 1 binding site for PRODAN and a K'$_d$ of

5.57 x 10^{-7} M and 6.14 x 10^{-7} M, respectively. Yang and others (2003) reported that

native BLG at pH 7.0 exhibited 0.41 binding sites for ANS with a dissociation constant

of 4.5 x 10^{-5} M. At pH 9.0, native BLG had 0.80 binding sites with K'$_d$ of 3.71 x 10^{-7} M.

The number of binding sites of BLG for PRODAN observed in the present research is

higher than the results Yang and others (2003) reported for BLG to ANS, which suggests

that anionic probes, such as ANS, may underestimate the number of binding sites through

ionic interactions between ANS and BLG, or uncharged probes, like PRODAN, may

overestimate the number of binding sites.

There were significant differences ($p < 0.05$) between dissociation constants of BLG for PRODAN as a function of pH and UHP treatment times. A 1.2-fold increase in the dissociation constant of BLG was observed after 32 min of UHP treatment (Table 1) at pH 3.0. The number of binding sites at pH 3.0 decreased with UHP treatment from 2.26 to 1.32 after UHP of 8 min. However after 16 min, the number of binding sites increased to 1.92 probably due to a loosening of the compact structure from an extended UHP hold time. At pH 5.0, untreated BLG exhibited the largest number of binding sites and UHP-treated BLG after 16 min exhibited a large dissociation constant of 7.16×10^{-7} M, which may indicate a decrease in the binding affinity of BLG for PRODAN. At pH 7.0, there was a slight increase in the number of binding sites after UHP of 4 min with highest K'd (8.42×10^{-7} M) after UHP of 32 min, which may suggest that during UHP treatment, changes in secondary structure around the loosely structured surface loops may decrease accessibility to the binding sites for PRODAN (Creamer 1995). At pH 9.0, the highest n was observed after come-up time and 32 min.

Yang and others (2003) reported that the surface hydrophobic site of UHP-induced BLG dimers were surrounded by hydrophobic amino acid residues, which resulted in an increase of hydrophobic affinity of BLG for CPA at the surface hydrophobic site. This is consistent with the current study in which increases in the binding affinity of BLG of pH 3.0 for PRODAN were observed after 4 min of UHP treatment. It can be seen from Table 1 that BLG of pH 3.0 was observed of having the most number of binding sites, regardless of UHP treatment times, and the greatest dissociation constants than BLG of other pH values. On the other hand, BLG of pH 7.0 exhibits lower values of dissociation constants compared to BLG of pH 3.0, and may be

appropriate for further binding experiments due to the number of binding sites of more than 0.8 under the different UHP treatment times tested. Most foods occur close to neutral pH, and binding studies of BLG with flavor compounds carried out at pH 7.0 may show improvement in flavor characteristics of reduced-fat foods.

CONCLUSIONS

Tryptophan emission spectra and PRODAN binding provide evidence that UHP treatment at 600 MPa and pH 7.0 induces BLG to form a molten globule. PRODAN may be used to determine the surface hydrophobicity of proteins over a wide range of pH values. In addition to pressure-induced denaturation, the base-induced denaturation of BLG at alkaline pH may prove useful in increasing the binding properties of BLG.

REFERENCES

Adams JJ, Anderson BF, Norris GE, Creamer LK, Jameson GB. 2006. Structure of bovine β-lactoglobulin (variant A) at very low ionic strength. J. Struct Biol. In Press.

Alizadeh-Pazdar N, Li-Chan ECY. 2000. Comparison of protein surface hydrophobicity measured at various pH values using three different fluorescent probes. J. Agric. Food Chem. 48: 328-334.

Alizadeh-Pazdar N, Li-Chan ECY, Nakai S. 2004. FT-raman spectroscopy, fluorescent probe, and solvent accessibility study of egg and milk proteins. J. Agric. Food Chem. 52: 5277-5283.

Bhattacharjee C, Das KP. 2000. Thermal unfolding and refolding of β-lactoglobulin: an intrinsic and extrinsic fluorescence strudy. Eur. J. Biochem. 267: 3957-3964.

Busti P, Scarpeci S, Gatti C, Delorenzi N. 2002. Use of flourescence methods to monitor unfolding transitions in β-lactoglobulin. Food Res. Int. 35: 871-877.

Casal HL, Kohler U, Mantsch HH. 1988. Structural and conformation changes of β-lactoglobulin B: an infrared spectroscopic study of the effect of pH and temperature. Biochim. Biophys. Acta 957: 11-20.

Chakrabarti A. 1996. Fluorescence of spectrin-bound Prodan. Biochem. Biophys. Res. Comm. 226: 495-497.

Cogan U, Kopelman M, Mokady S, Shinitzky M. 1976. Binding affinities of retinol and related compounds to retinol bindin proteins. Eur. J. Biochem. 65: 71-78.

Creamer LK. 1995. Effect of sodium dodecyl sulfate and palmitic acid on the equilibrium unfolding of bovine β-lactoglobulin. Biochem. 34: 7170-7176.

Damodaran S. 1996. Amino acids, peptides, and proteins. In: Fennema OR. Food chemistry. Third Edition. New York: Marcel Dekker, Inc. p 321-429.

Futenberger S, Dumay E, Cheftel JC. 1995. Pressure-induced aggregation of β-lactoglobulin in pH 7.0 buffers. Lebensm.-Wiss.-Technol. 28: 410-418.

Haskard CA, Li-Chan ECY. 1998. Hydrophobicity of bovine serum albumin and ovalbumin determined using uncharged (PRODAN) and anionic (ANS) flourescent probes. J. Agric. Food Chem. 46: 2671-2677.

Hiratsuki T. 1998. Prodan fluorescence reflects differences in nucleotide-induced conformational states in the myosin head and allows continuous visualization of the ATPase reactions. Biochem. 37: 7167-7176.

Huang W, Vernon LP, Hansen LD, Bell JD. 1997. Interactions of thionin from Pyrularia pubera with dipalmitoylphosphatidylglycerol large unilamellar vesicles. Biochem. 36(10): 2860-2866.

Iametti S, Transidico P, Bonomi F, Vecchio G, Pittia P, Rovere P, Dall'Aglio G. 1997. Molecular modifications of β-lactoglobulin upon exposure to high pressure. J. Agric. Food Chem. 45: 23-29.

Kusube M, Tamai N, Matsuki H, Kaneshina S. 2005. Pressure-induced phase transitions of lipid bilayers observed by fluorescent probes prodan and laurdan. Biophys. Chem. 117(3): 199-206.

Lakowicz JR. 1983. Principles of fluorescence spectroscopy. New York: Plenum Press. 496p.

Liu X, Powers JR, Swanson BG, Hill HH, Clark S. 2005. Modification of whey protein concentrate hydrophobicity by high hydrostatic pressure. Innov. Food Sci. Emerg. Technol. 6(3): 310-317.

Moreno F, Cortijo M, Gonzalez-Jimenez J. 1999. The fluorescent probe Prodan characterizes the warfarin binding site on human serum albumin. Photochem. and Photobiol. 69(1): 8-15.

Muresan S, van der Bent A, de Wolf FA. 2001. Interaction of β-lactoglobulin with small hydrophobic ligands as monitored by fluorometry and equilibrium dialysis: nonlinear quenching effects related to protein-protein association. J. Agric. Food Chem. 49: 2609-2618.

Nakai S. 1983. Structure-function relationships of food proteins with an emphasis on the importance of protein functionality. J. Agric. Food Chem. 31: 676-683.

Petsko GA, Ringe D. 2004. Protein structure and function. London: New Science Press Ltd. 195p.

Phillips LG, Whitehead DM, Kinsella JE. 1994. Structural and chemical properties of β-lactoglobulin. In: Structure-function properties of food proteins. New York: Academic Press. p 75-106.

Qin BY, Bewley MC, Creamer LK, Baker HM, Baker EN, Jameson GB. 1998. Structural basis of the tanford transition of bovine β-lactoglobulin. Biochem. 37: 14014-14023.

Ohio State University. High pressure processing: Fact sheet for food processors. Ramaswamy R, Balasubramaniam VM, Kaletunc G. Columbus. 1-3

Royer CA. 1995. Approaches to teaching fluorescence spectroscopy. Biophys. J. 68: 1191-1195.

Swaisgood HE. 1996. Characteristics of milk. In: Fennema OR. Food chemistry. New York: Marcel Dekker, Inc. p 841-878.

Taulier N, Chalikian TV. 2001. Characterization of pH-induced transitions of β-lactoglobulin: ultrasonic, densimetric, and spectroscopic studies. J. Mol. Biol. 314: 873-889.

Tedford L-A, Smith D, Schaschke CJ. 1999. High pressure processing effect on the molecular structure of ovalbumin, lysozyme and β-lactoglobulin. Food Res. Int. 32: 101-106.

Timasheff SN, Mescanti L, Basch JJ, Townend R. 1966. Conformational transitions of bovine β-lactoglobulin A, B, C. J. Biol. Chem. 241: 2496-2501.

Uhrinova S, Smith MH, Jameson GB, Uhrin D, L. S, Barlow PN. 2000. Structure changes accompanying pH-induced dissociation of the β-lactoglobulin dimer. Biochem. 39: 3565-3574.

Vazquez ME, Blanco JB, Imperiali B. 2005. Photophysics and biological applications of the environment-sensitive fluorophore 6-N,N-dimethylamino-2,3-naphthalimide. J. Am. Chem. Soc. 127(4): 1300-1306.

Weber G, Farris FJ. 1979. Synthesis and spectral properties of a hydrophobic fluorescent probe: 6-propionyl-2-(dimethylamino)-naphthalene. Biochem. 18: 3075-3078.

Yang J, Dunker AK, Powers JR, Clark S, Swanson BG. 2001. β-Lactoglobulin molten globule induced by high pressure. J. Agric. Food Chem. 49: 3236-3243.

Yang J, Dunker AK, Powers JR, Clark S, Swanson BG. 2003. Ligand and flavor binding functional properties of β-lactoglobulin in the molten globule state induced by high pressure. J. Food Sci. 68: 444-452.
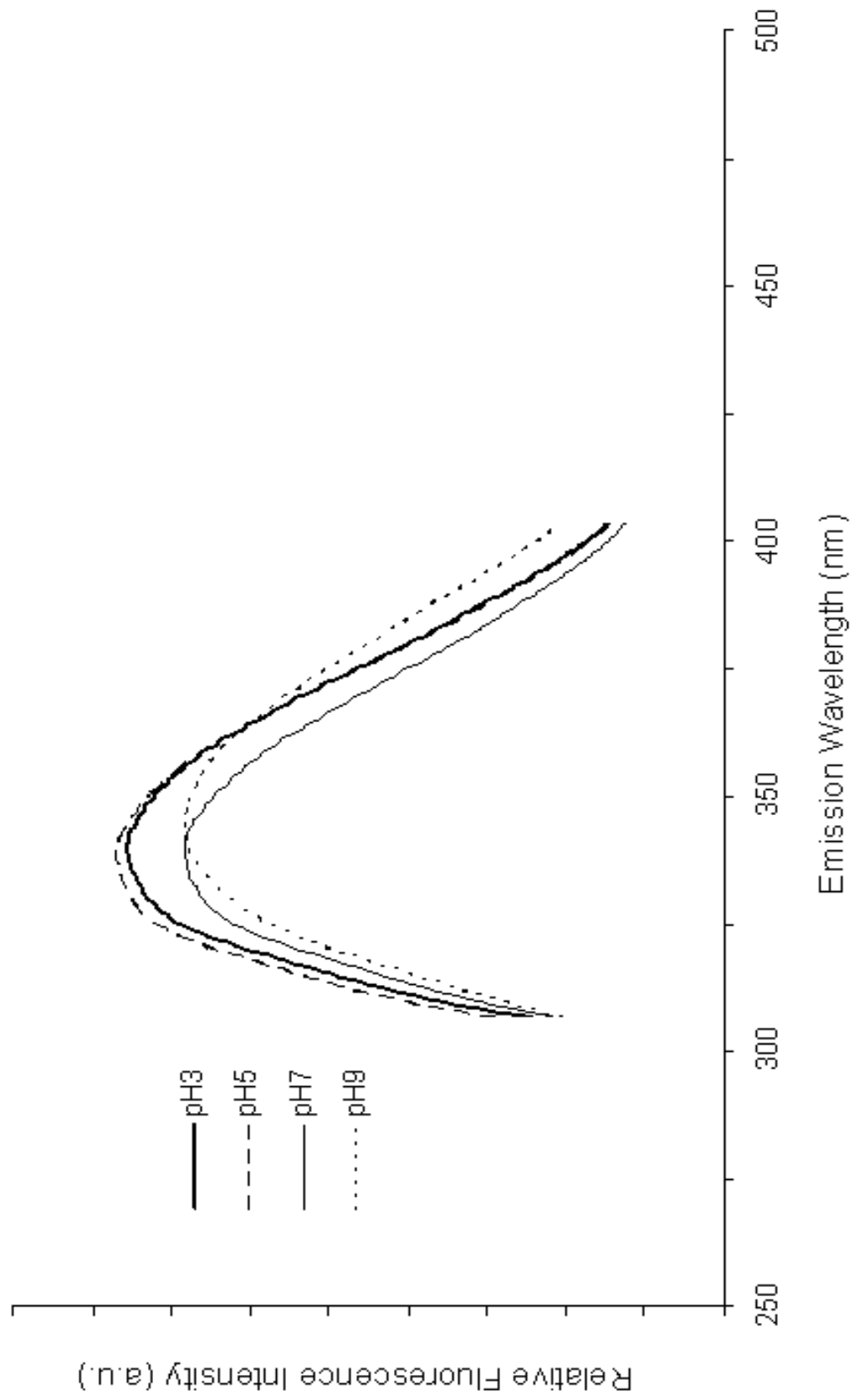
Figure 1. Intrinsic tryptophan emission spectra of native BLG solutions at pH 3.0, 5.0, 7.0, or 9.0.
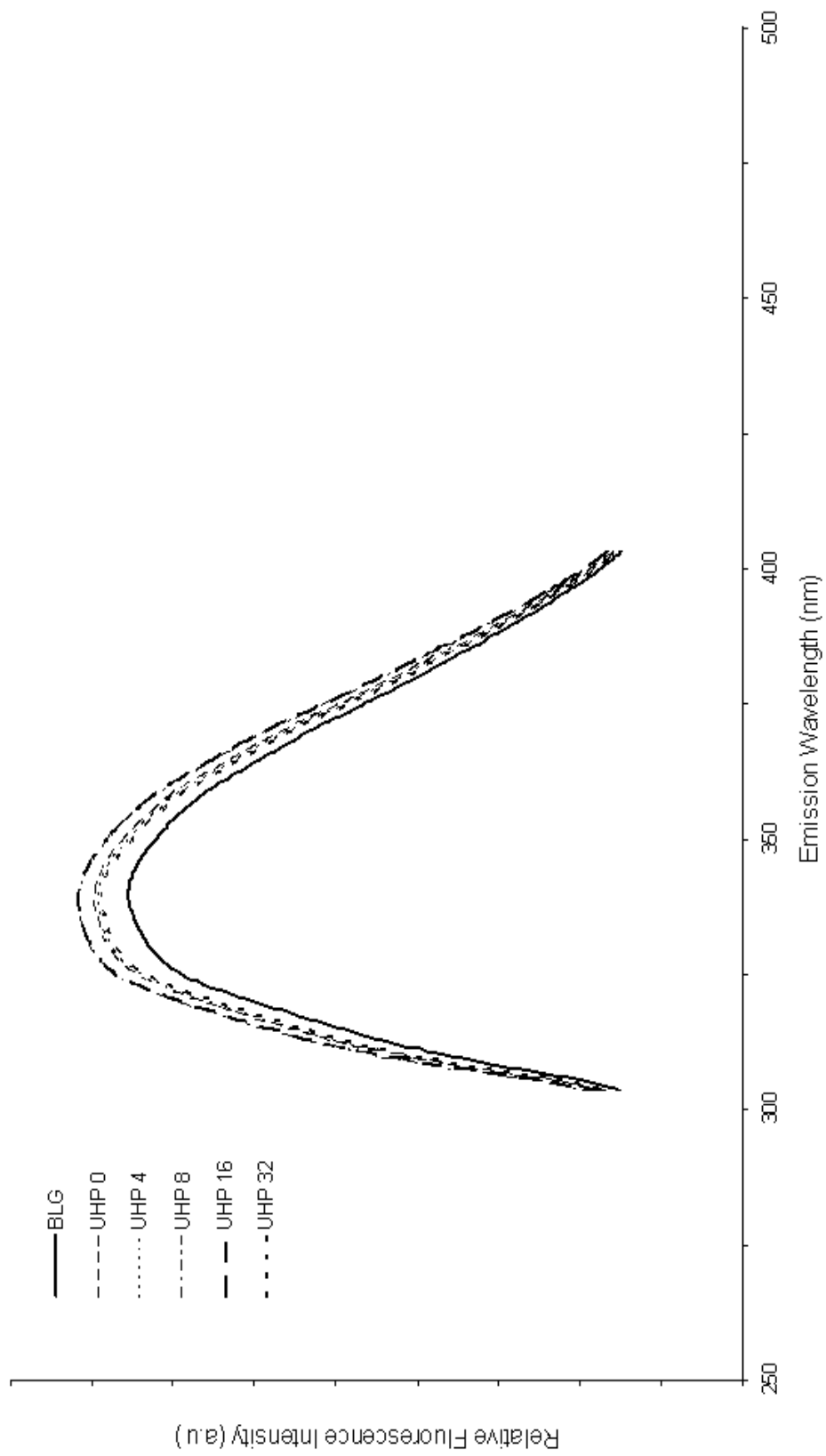
Figure 2. Intrinsic tryptophan emission spectra of BLG solutions at pH 3.0 treated with UHP at 600 MPa for selected holding times from 0 to 32 min (UHP0-UHP32).
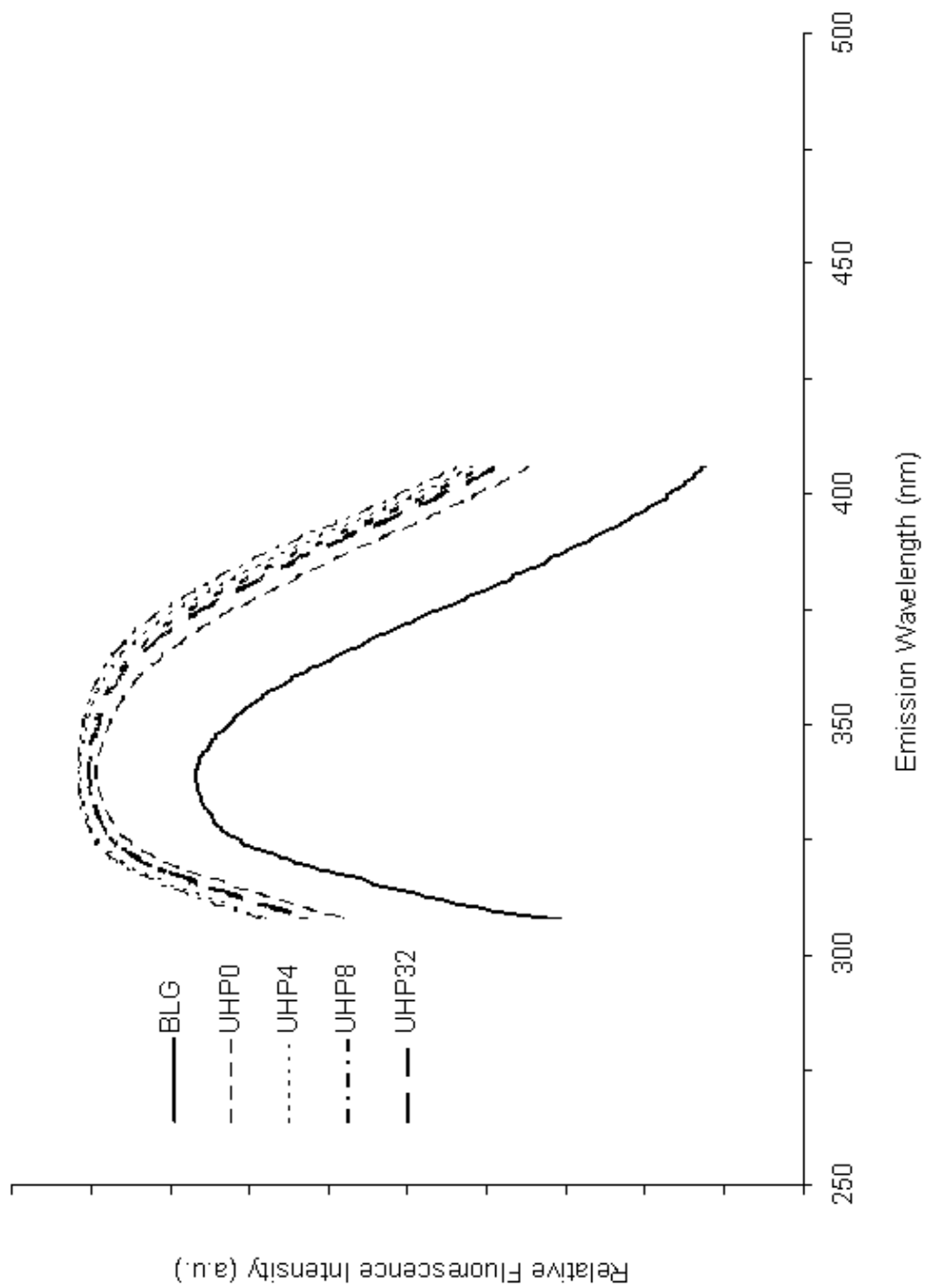
Figure 3. Intrinsic tryptophan emission spectra of BLG solutions at pH 5.0 treated with UHP at 600 MPa for selected holding times from 0 to 32 min (UHP0-UHP32).
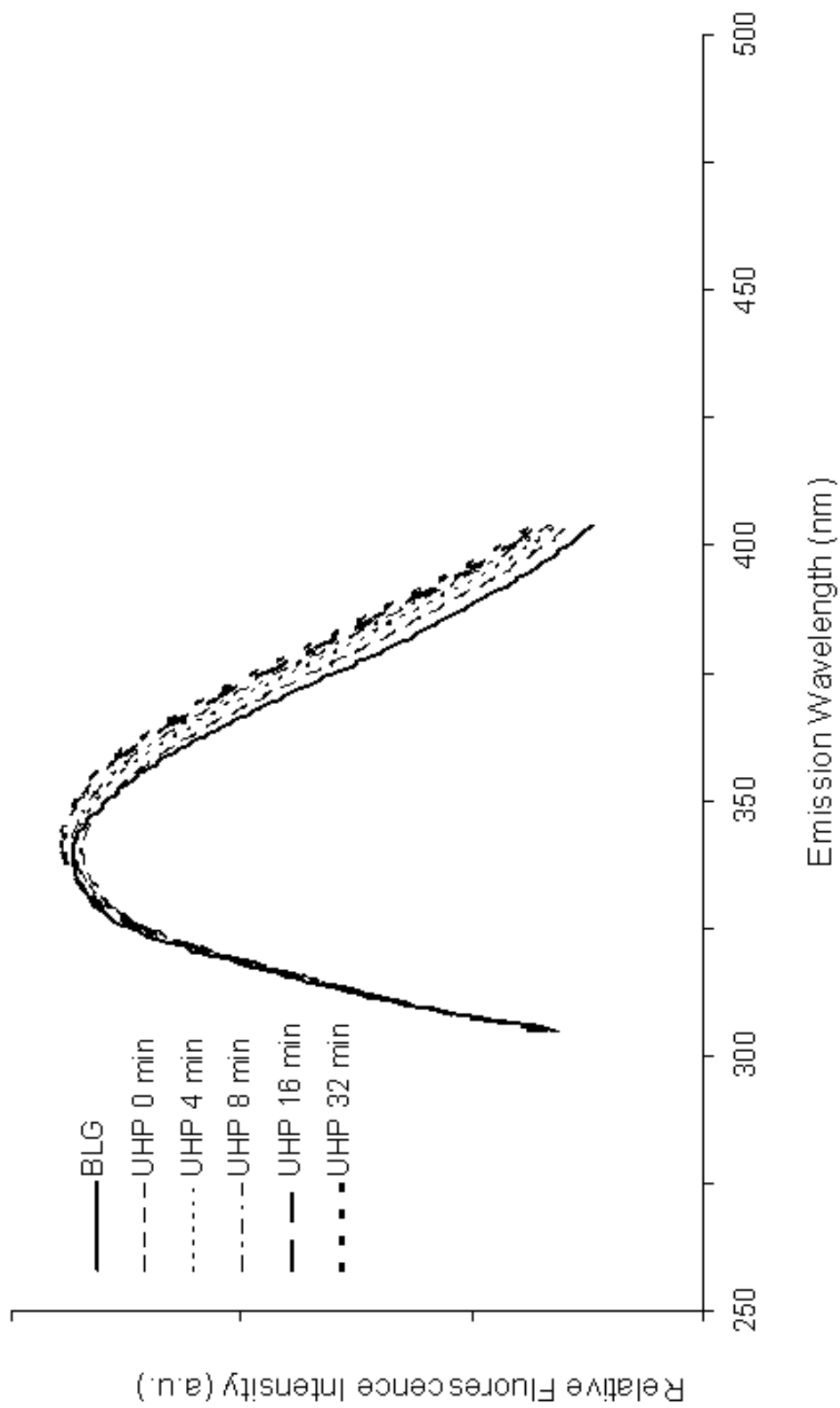
Figure 4. Intrinsic tryptophan emission spectra of BLG solutions at pH 7.0 treated with UHP at 600 MPa for selected holding times from 0 to 32 min (UHP0-UHP32).
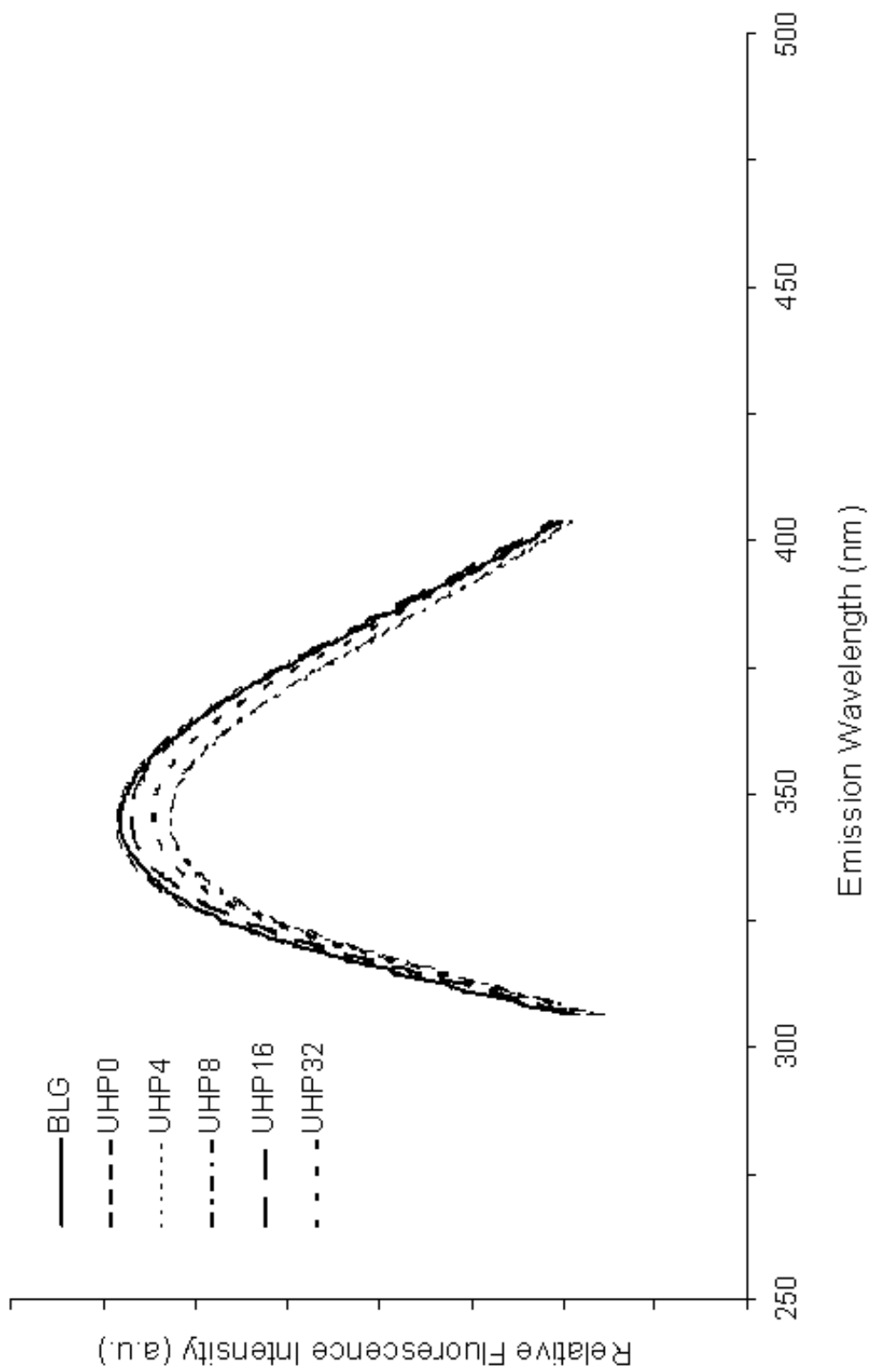
Figure 5. Intrinsic tryptophan emission spectra of BLG solutions at pH 9.0 treated with UHP at 600 MPa for selected holding times from 0 to 32 min (UHP0-UHP32).
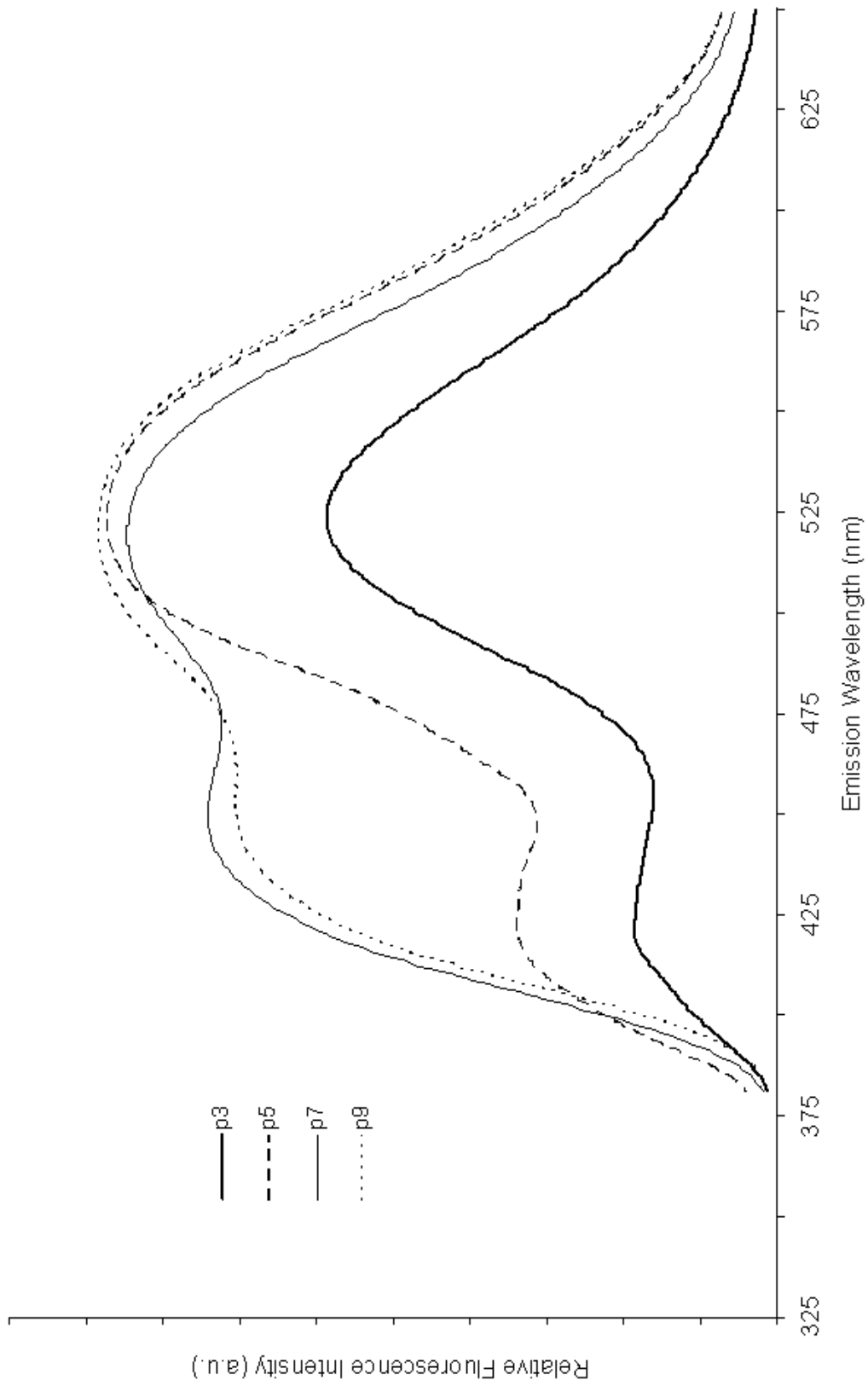
Figure 6. Extrinsic PRODAN emission spectra of native BLG solutions at pH 3.0-9.0.

x-axis: Emission Wavelength (nm)
y-axis: Relative Fluorescence Intensity (a.u.)

Legend:
p3
p5
p7
p9
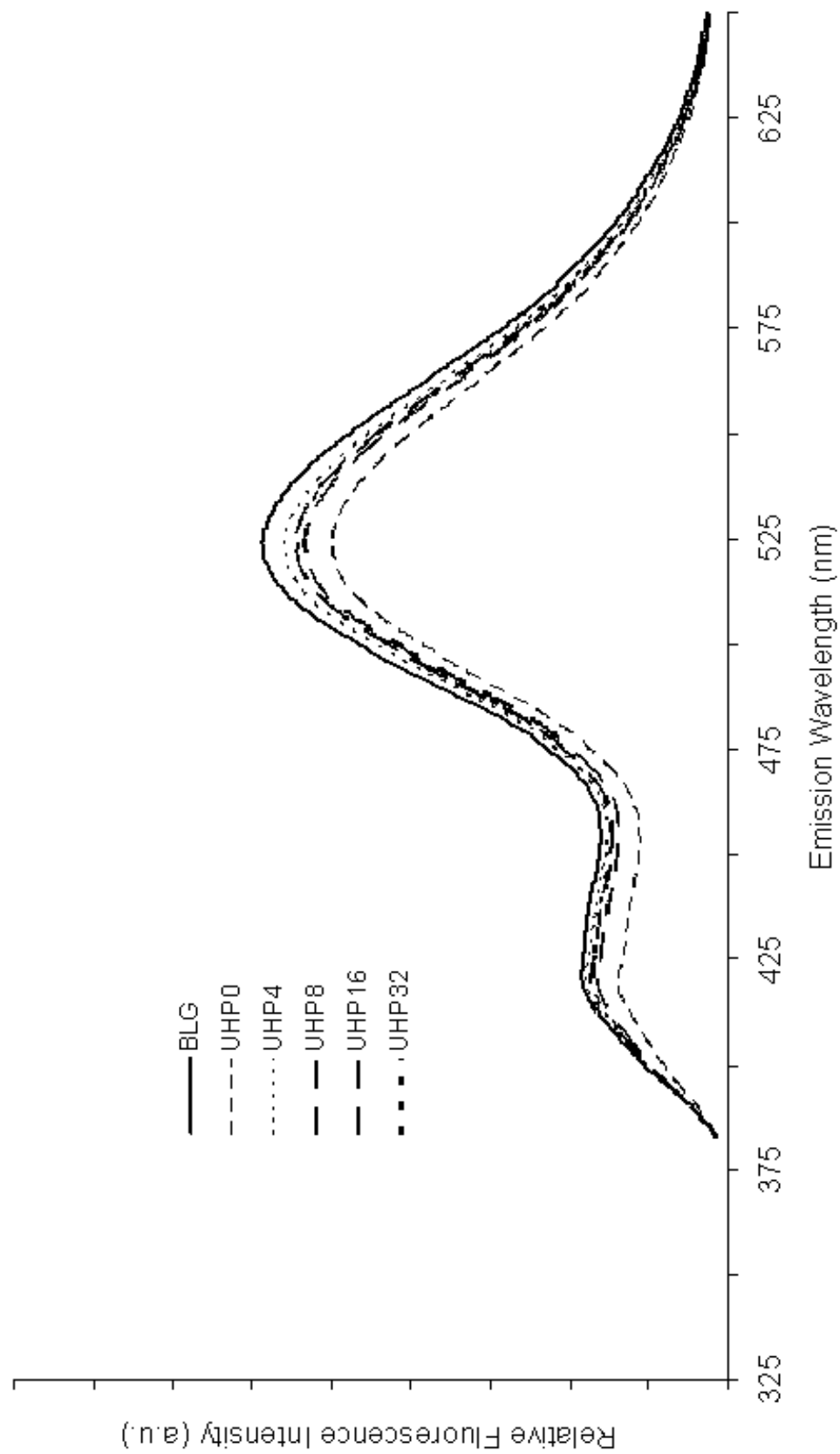
Figure 7. Extrinsic PRODAN emission spectra of BLG solutions at pH 3.0 treated with UHP at 600 MPa for selected holding times from 0 to 32 min (UHP0-UHP32).
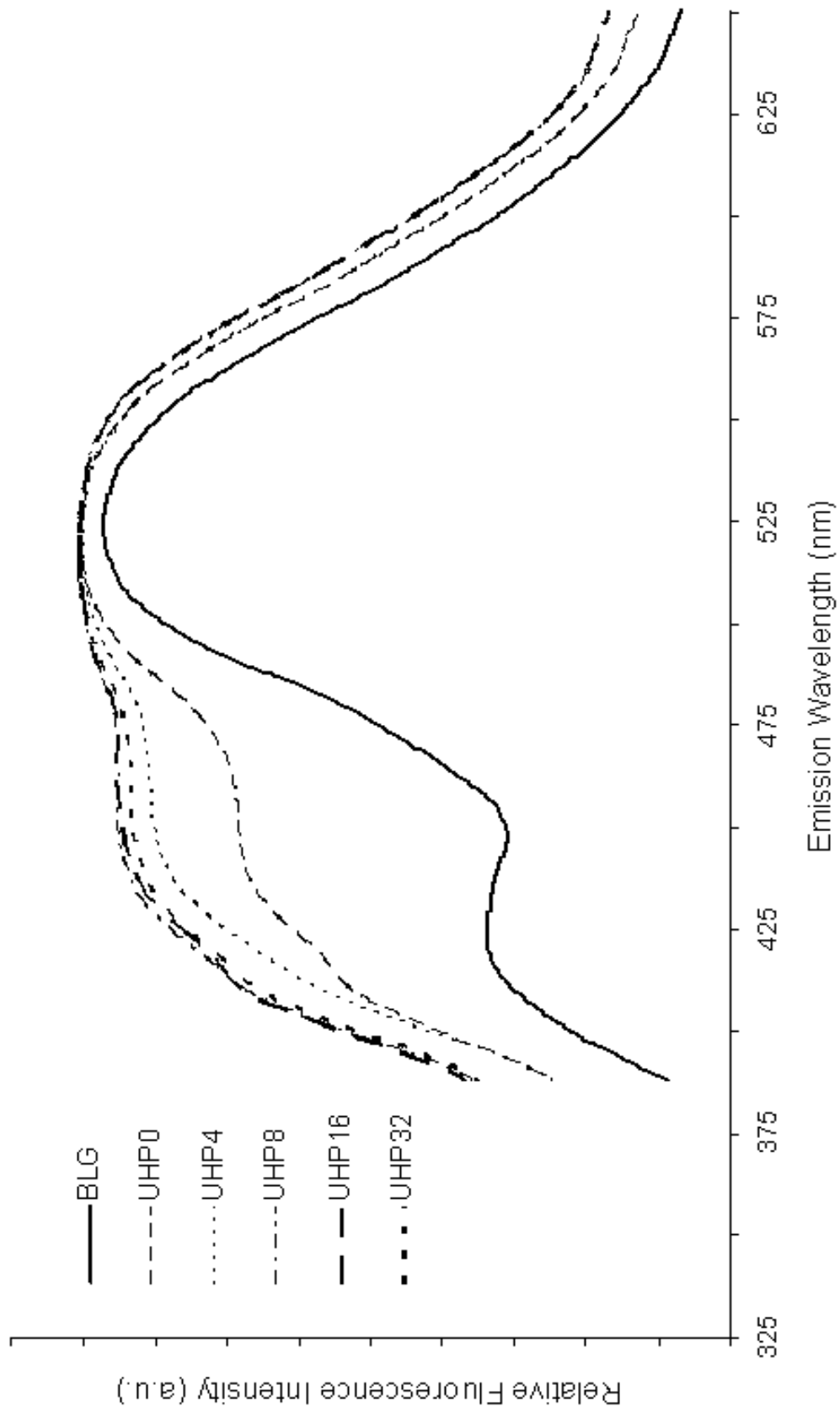
Figure 8. Extrinsic PRODAN emission spectra of BLG solutions at pH 5.0 treated with UHP at 600 MPa for selected holding times from 0 to 32 min (UHP0-UHP32).
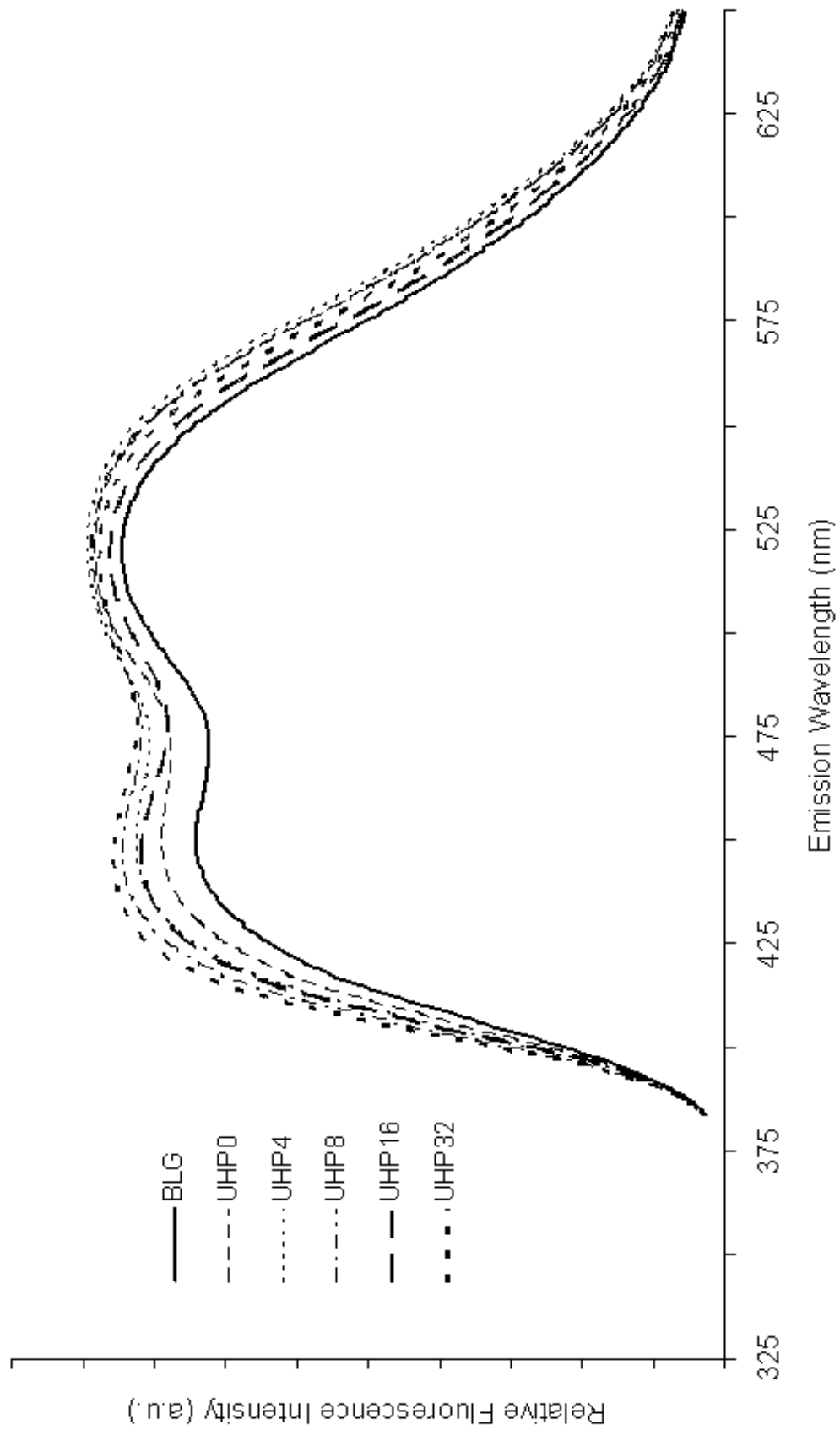
Figure 9. Extrinsic PRODAN emission spectra of BLG solutions at pH 7.0 treated with UHP at 600 MPa for selected holding times from 0 to 32 min (UHP0-UHP32).

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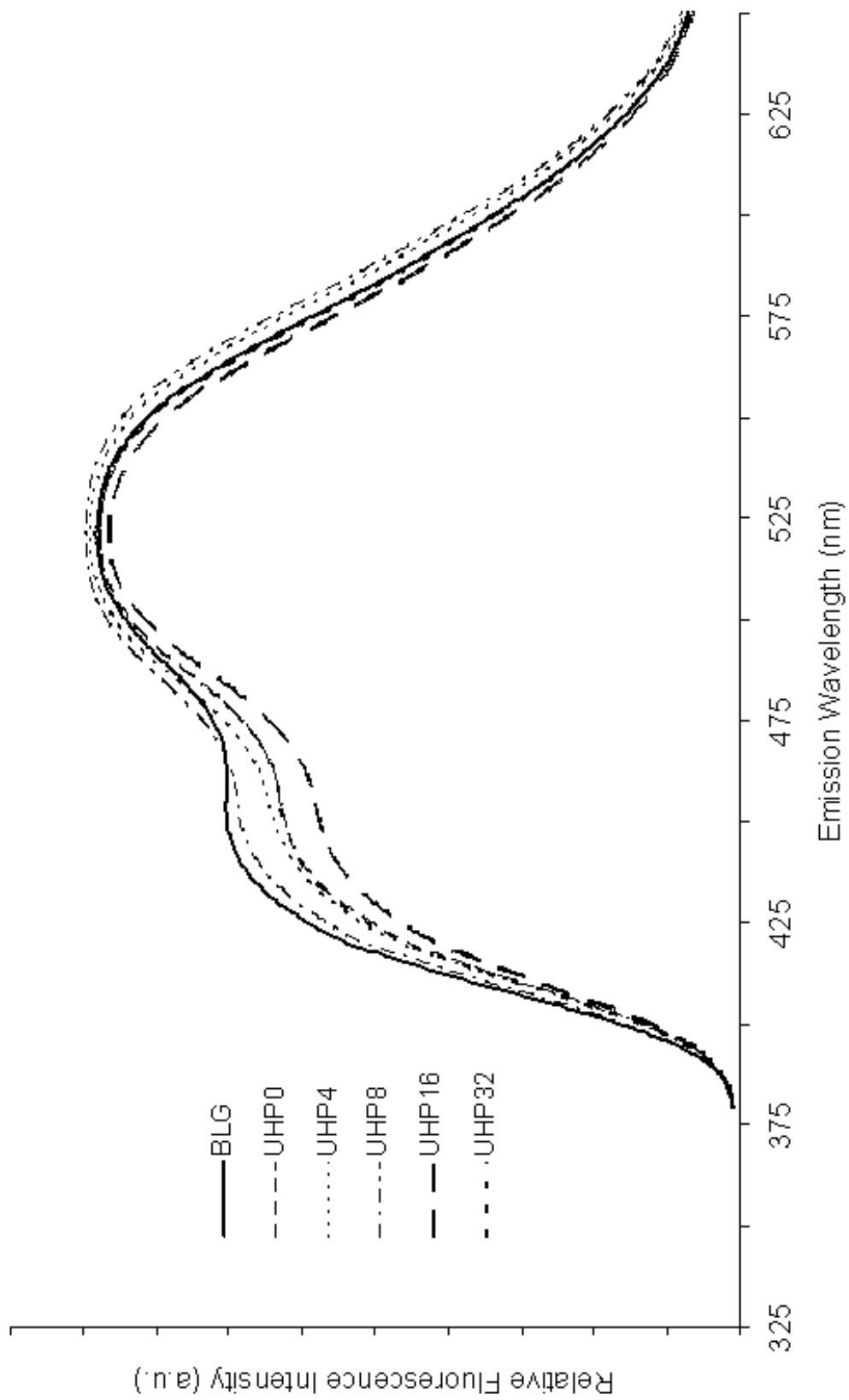
Figure 10. Extrinsic PRODAN emission spectra of BLG solutions at pH 9.0 treated with UHP at 600 MPa for selected holding times from 0 to 32 min (UHP0-UHP32).
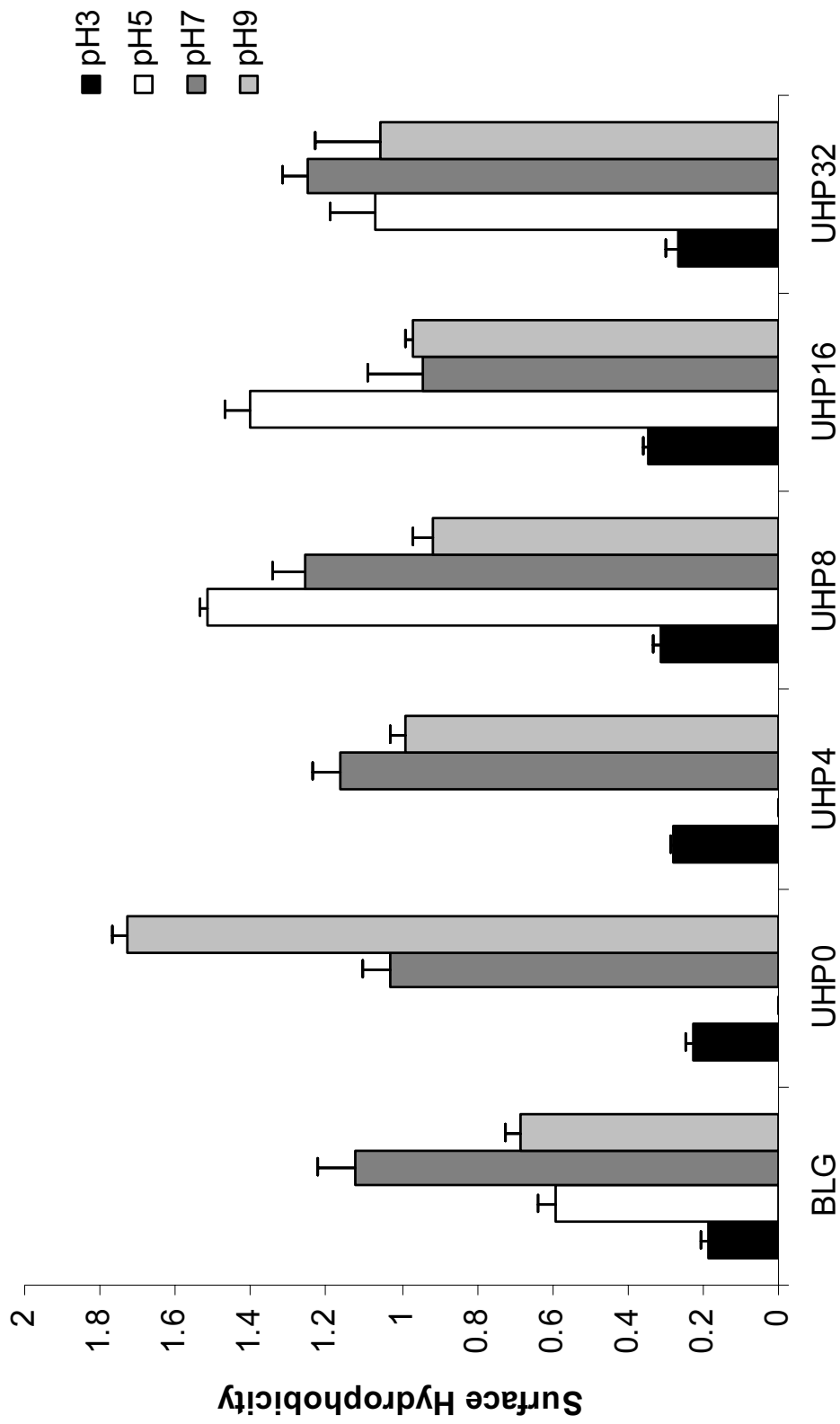
Figure 11. Surface hydrophobicity of BLG determined at pH 3.0-9.0 after ultra high pressure (UHP) treatment times 0, 4, 8, 16 or 32 min with PRODAN.
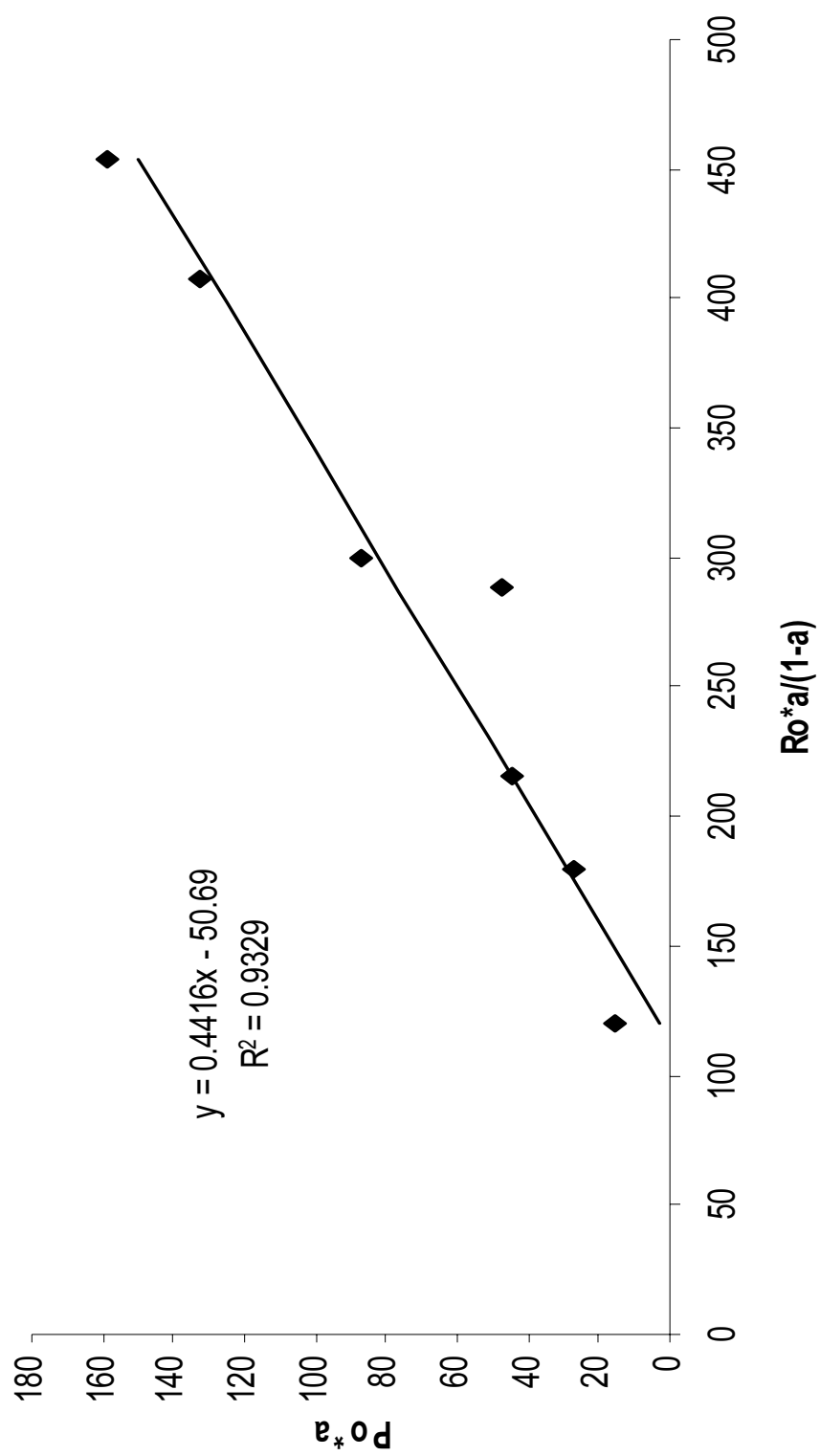
Figure 12. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for native BLG at pH 3.0.

The plot shows Po^*a on the y-axis versus $Ro^*a/(1-a)$ on the x-axis with the fitted line:

$$y = 0.4416x - 50.69$$
$$R^2 = 0.9329$$

Figure 13. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP treated (600 MPa) BLG with 0 min holding time at pH 3.0.

The chart shows:
- y-axis: $Po*a$, ranging from -20 to 160
- x-axis: $Ro*a/(1-a)$, ranging from 0 to 350
- $y = 0.5205x - 37.876$
- $R^2 = 0.8298$

Figure 14. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP treated (600 MPa) BLG with 4 min holding time at pH 3.0.

$y = 0.5455x - 29.945$

$R^2 = 0.9255$

Figure 15. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP-treated (600 MPa) BLG with 8min holding time at pH 3.0.

$y = 0.7572x - 83.523$

$R^2 = 0.9063$

Figure 16. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP-treated (600 MPa) BLG with 16 min holding time at pH 3.0.

$$y = 0.5196x - 35.653$$
$$R^2 = 0.9446$$

Figure 17. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP-treated (600 MPa) BLG with 32 min holding time at pH 3.0.

$y = 0.997x - 5.5514$
$R^2 = 0.9967$
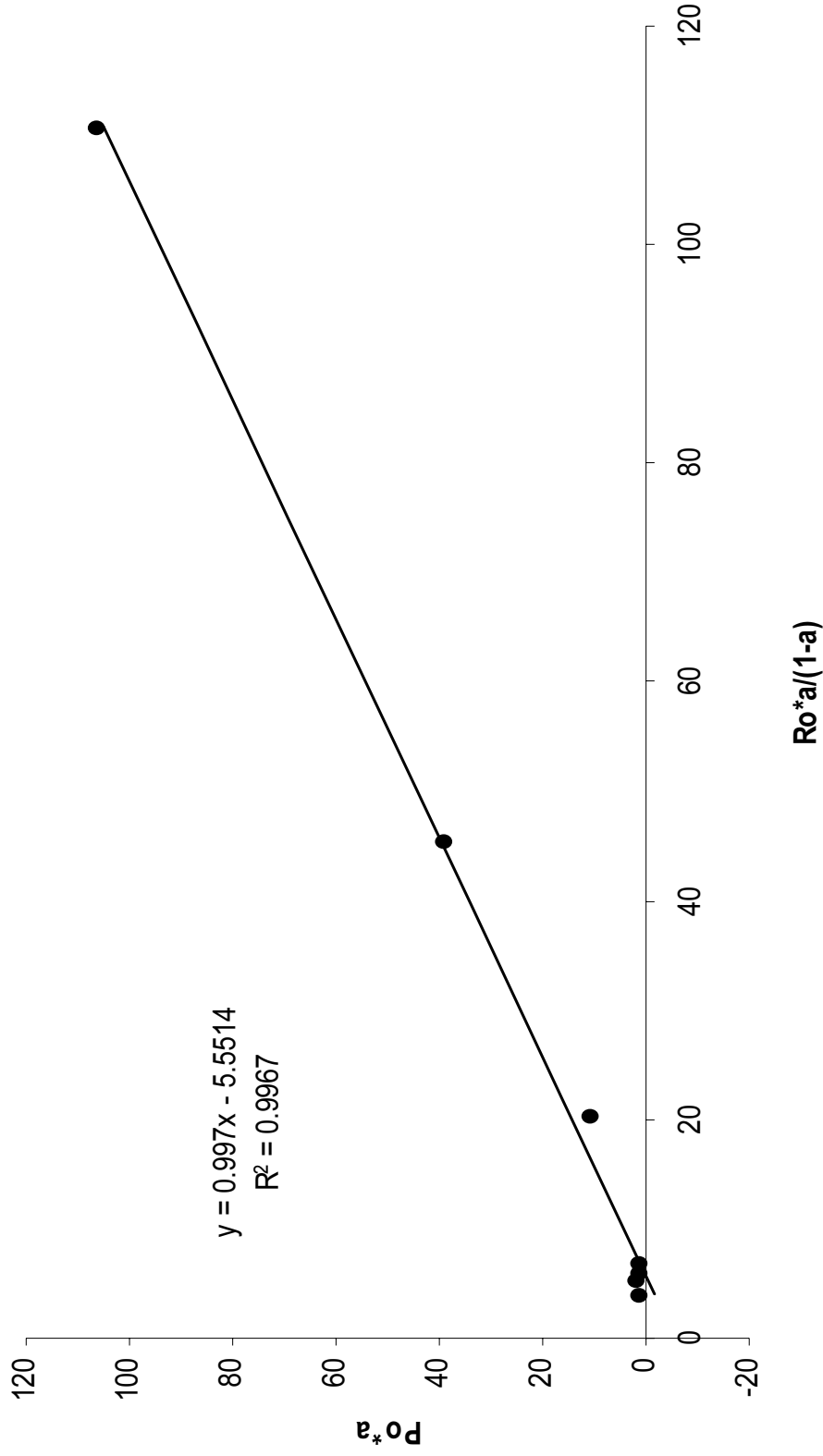
Figure 18. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for native BLG at pH 5.0.
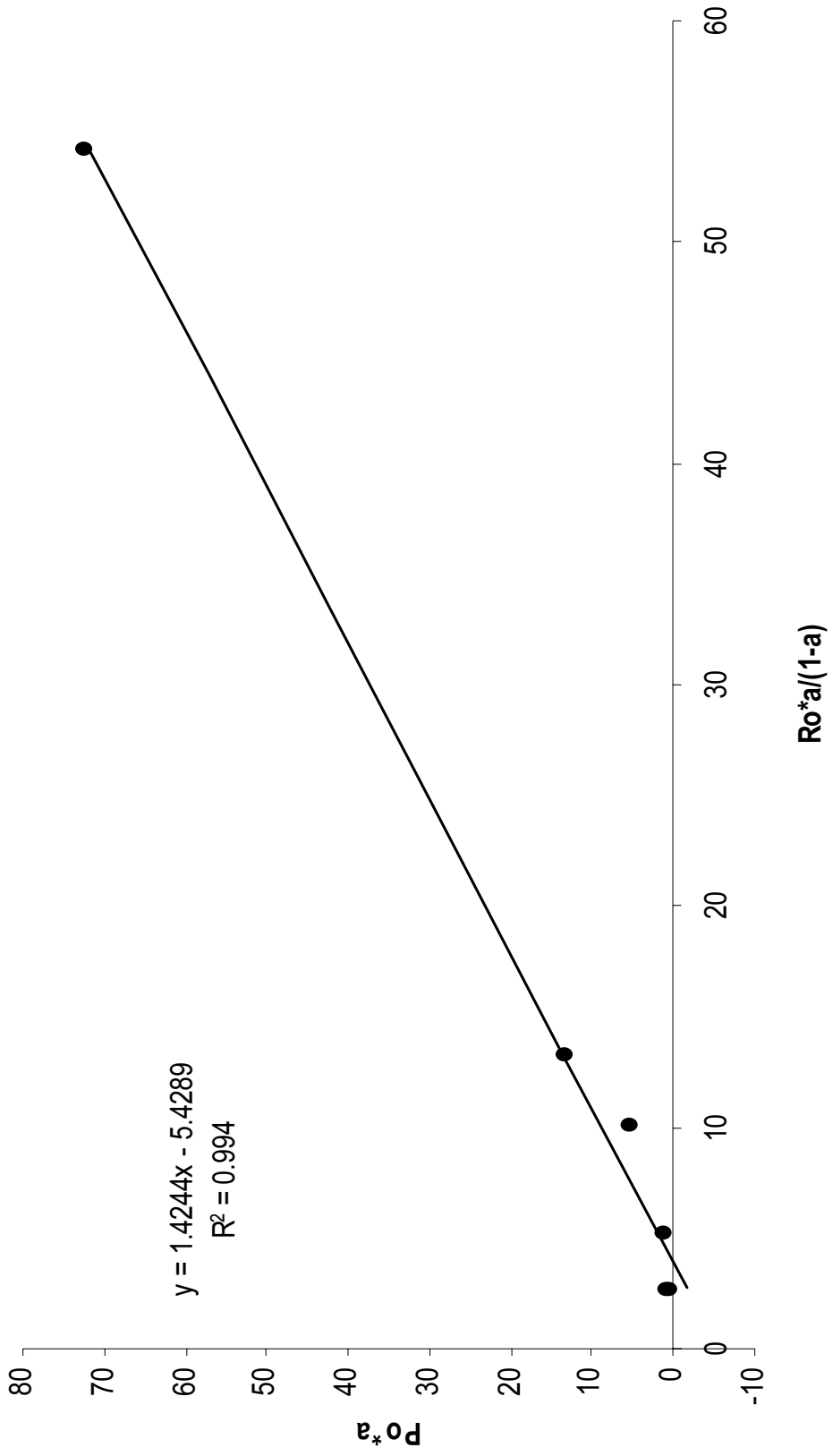
Figure 19. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for untreated BLG and UHP-treated (600 MPa) BLG with 0 min holding time at pH 5.0.
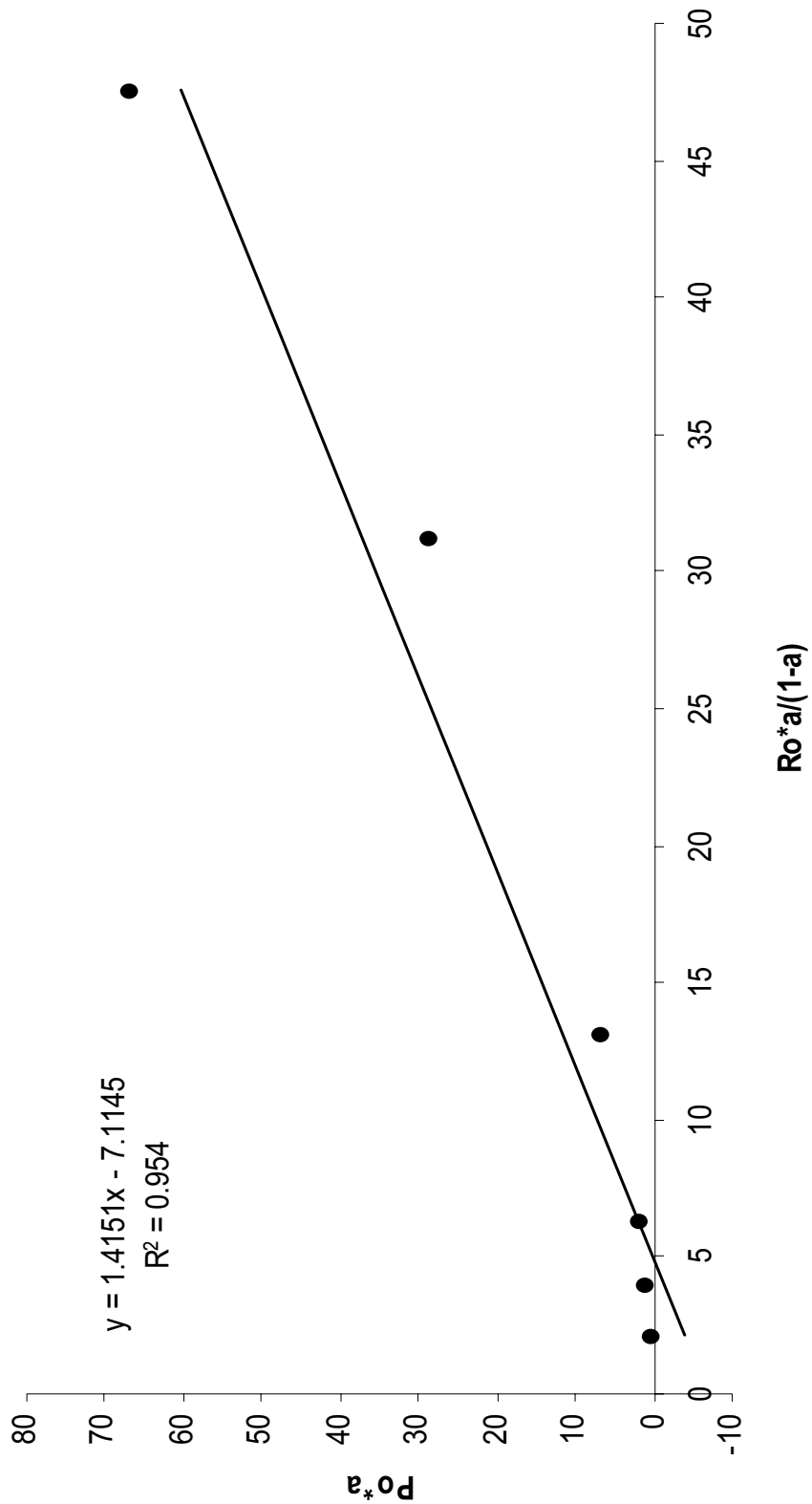
Figure 20. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for untreated BLG and UHP-treated (600 MPa) BLG with 4 min holding time at pH 5.0.

The plot shows a linear regression with equation $y = 1.4151x - 7.1145$ and $R^2 = 0.954$, where the x-axis is labeled $Ro*a/(1-a)$ and the y-axis is labeled $Po*a$.

Figure 21. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP treated (600 MPa) BLG with 8 min holding time at pH 5.0.

$y = 1.4706x - 5.3086$
$R^2 = 0.9816$
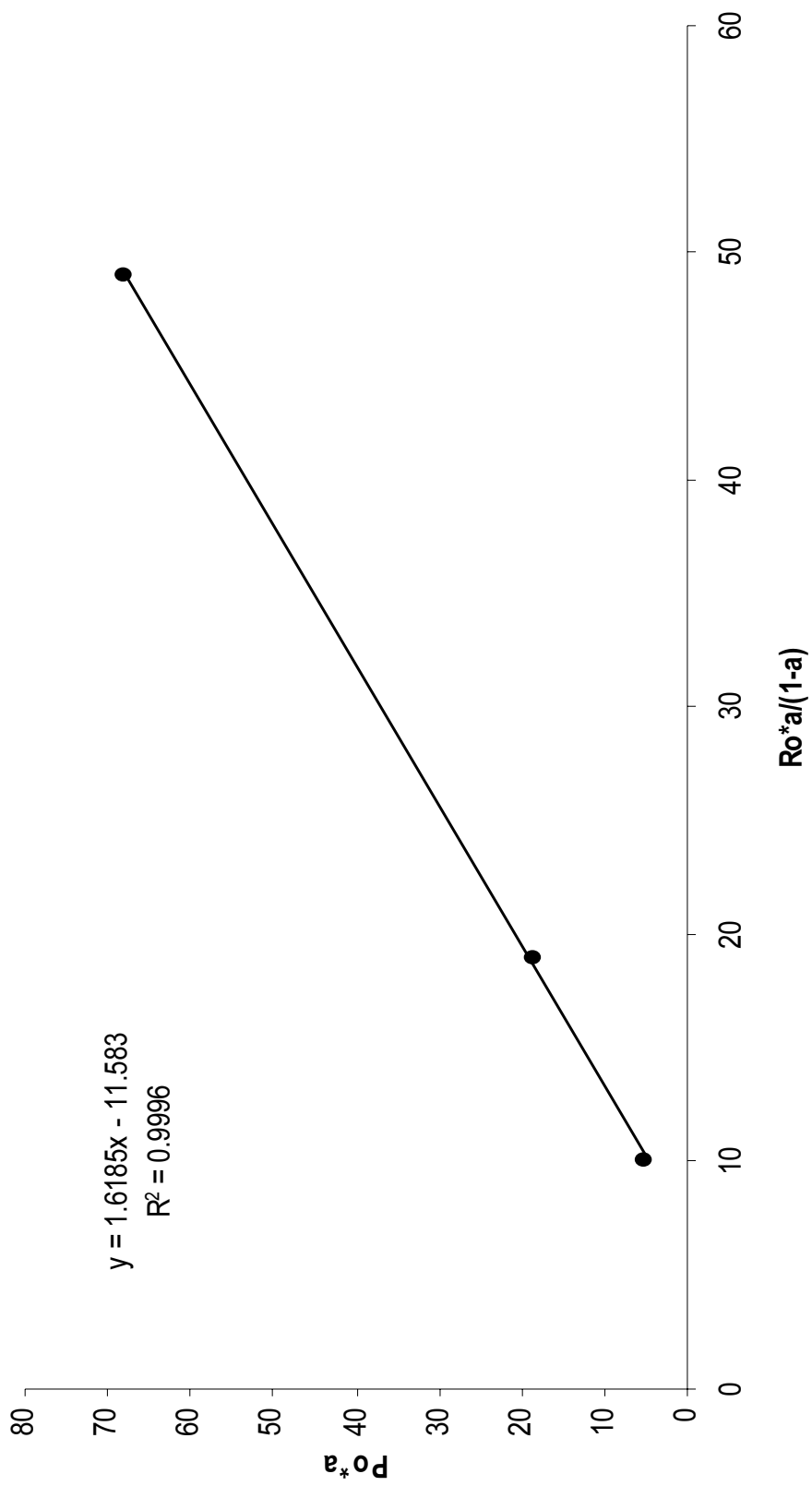
Figure 22. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP treated (600 MPa) BLG with 16 min holding time at pH 5.0.

$y = 1.6185x - 11.583$
$R^2 = 0.9996$
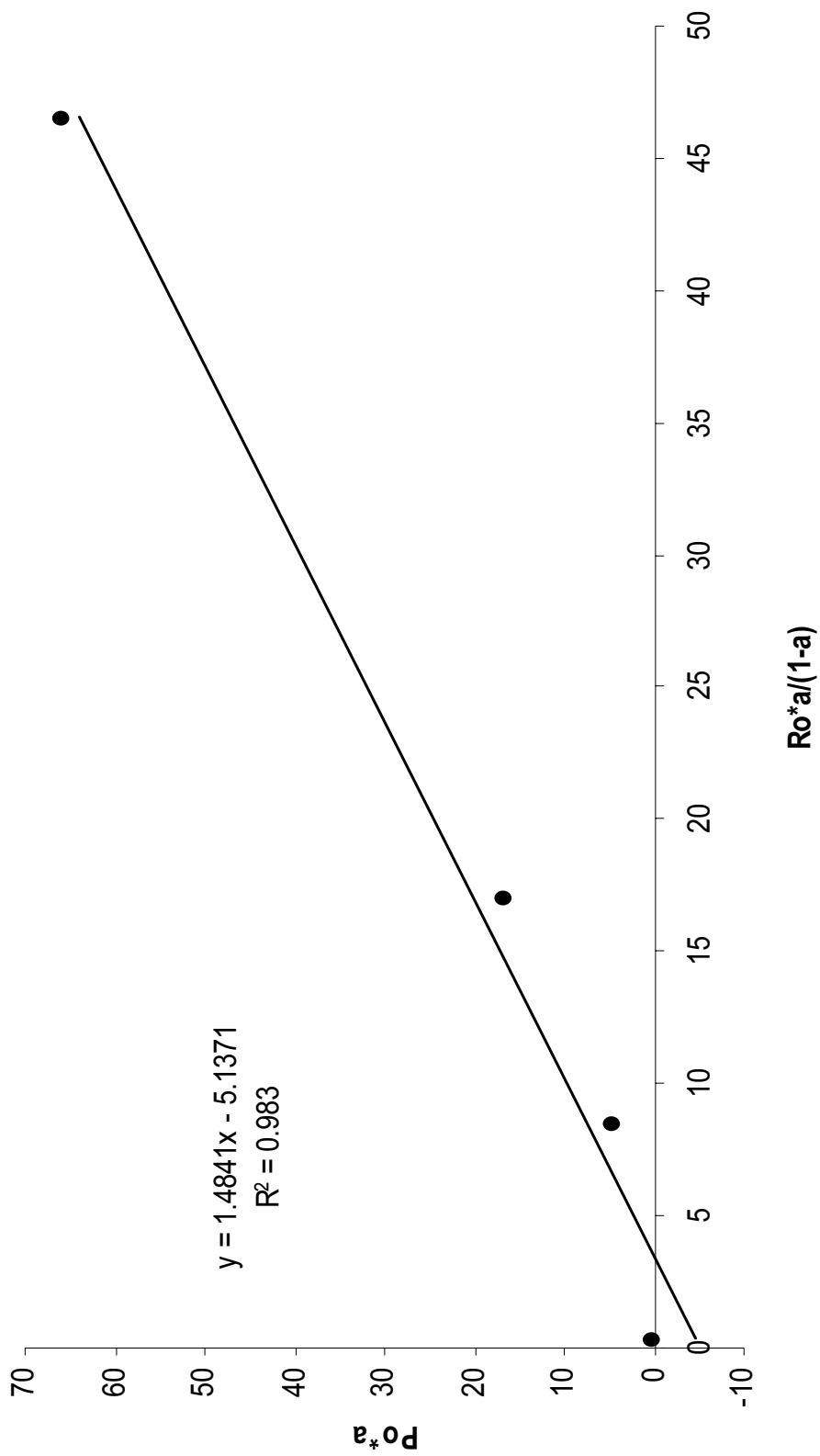
Figure 23. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP treated (600 MPa) BLG with 32 min holding time at pH 5.0.
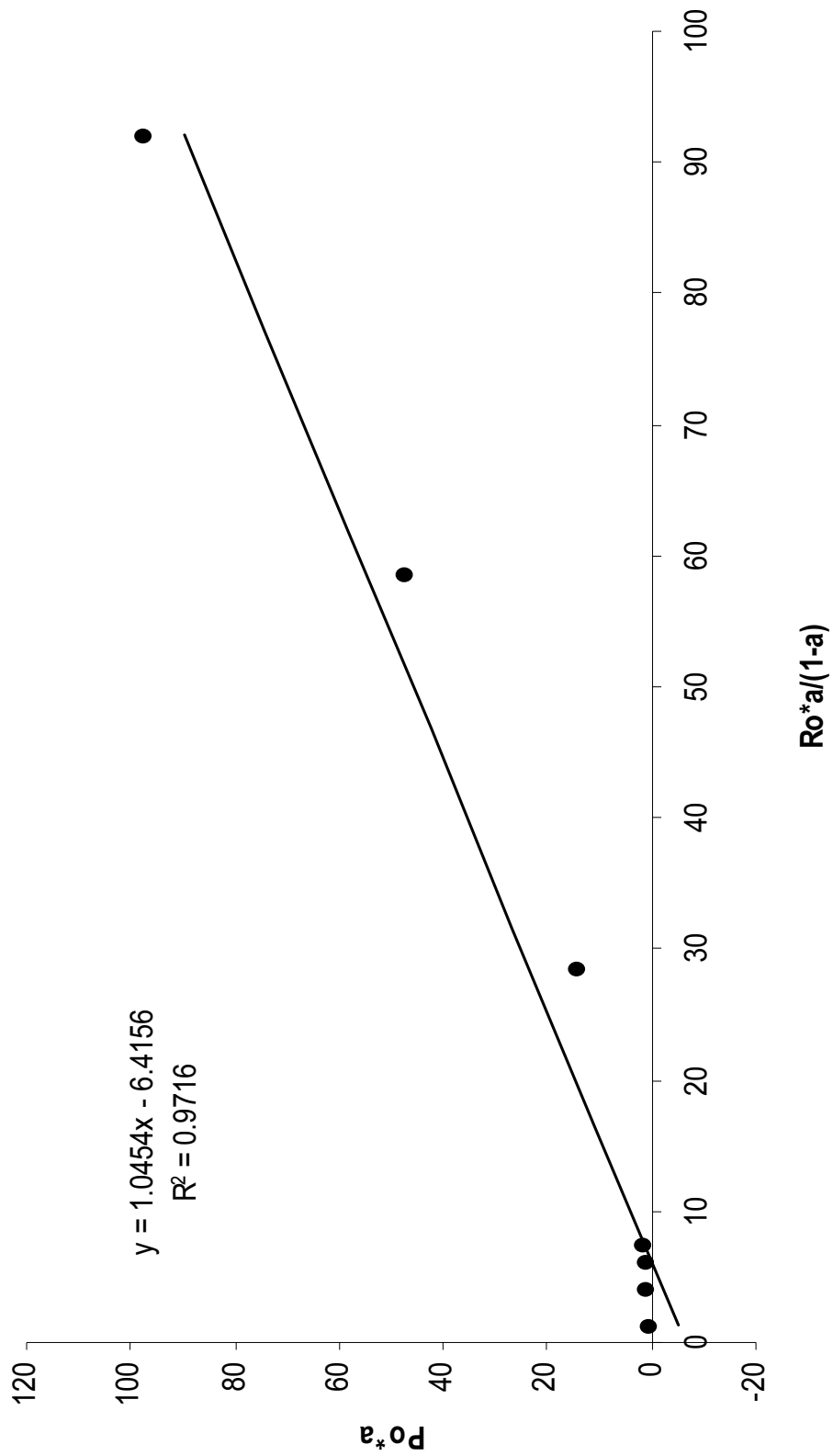
Figure 24. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for native BLG at pH 7.0.

The plot shows the regression line with equation $y = 1.0454x - 6.4156$ and $R^2 = 0.9716$. The x-axis is labeled **Ro*a/(1-a)** and the y-axis is labeled **Po*a**.
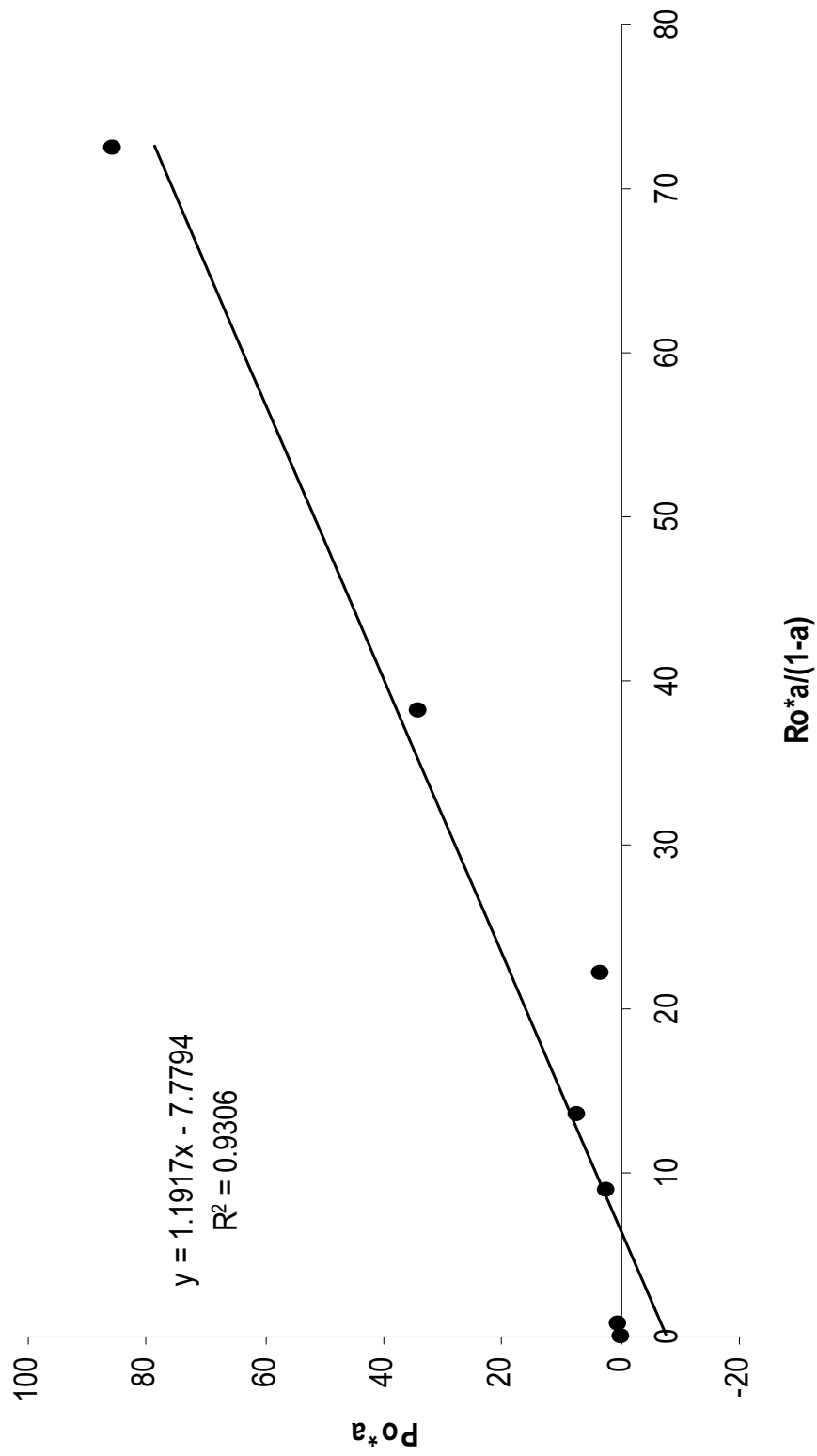
Figure 25. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP treated (600 MPa) BLG with 0 min holding time at pH 7.0.
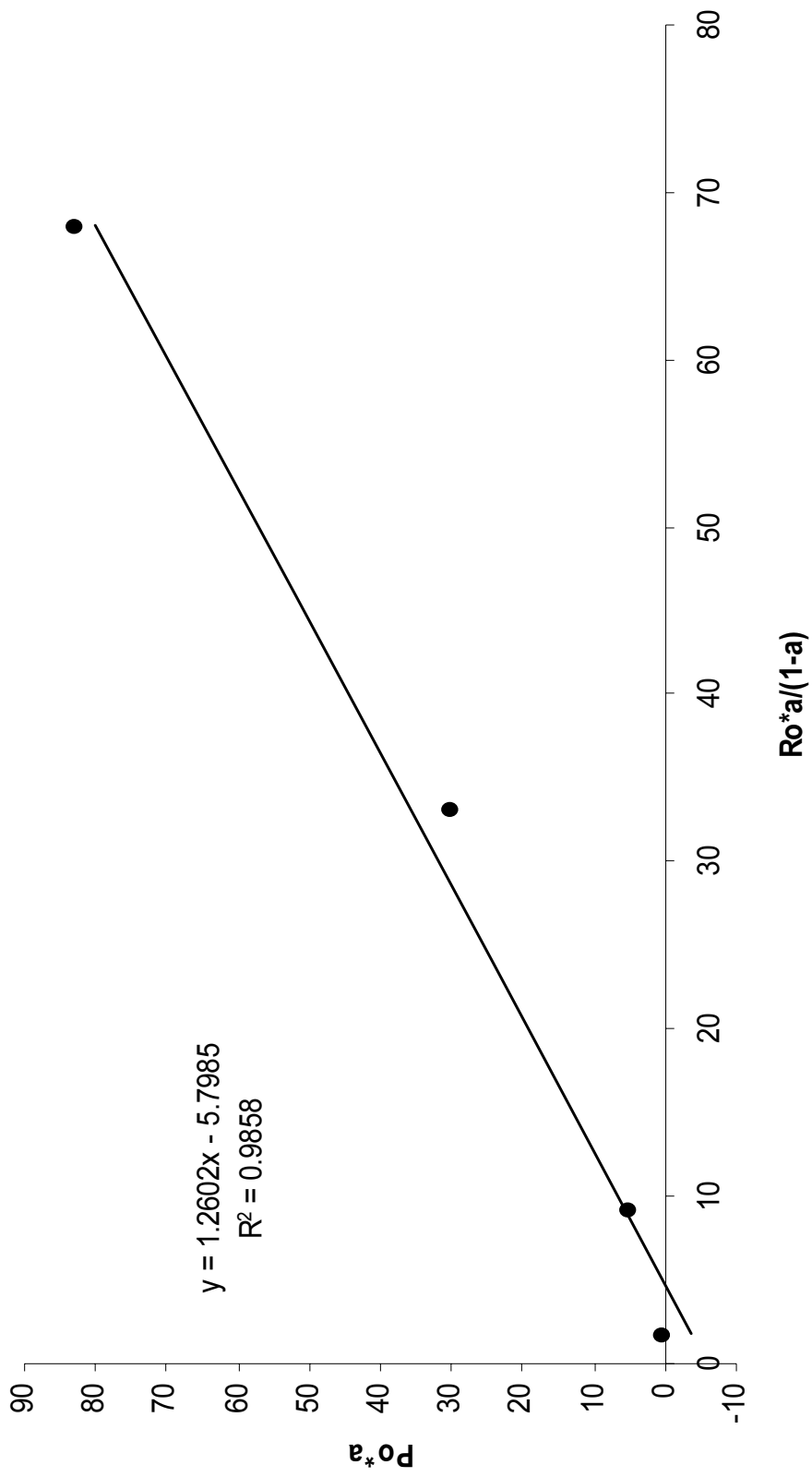
Figure 26. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP treated (600 MPa) BLG with 4 min holding time at pH 7.0.

The plot shows data with trendline equation:

$y = 1.2602x - 5.7985$

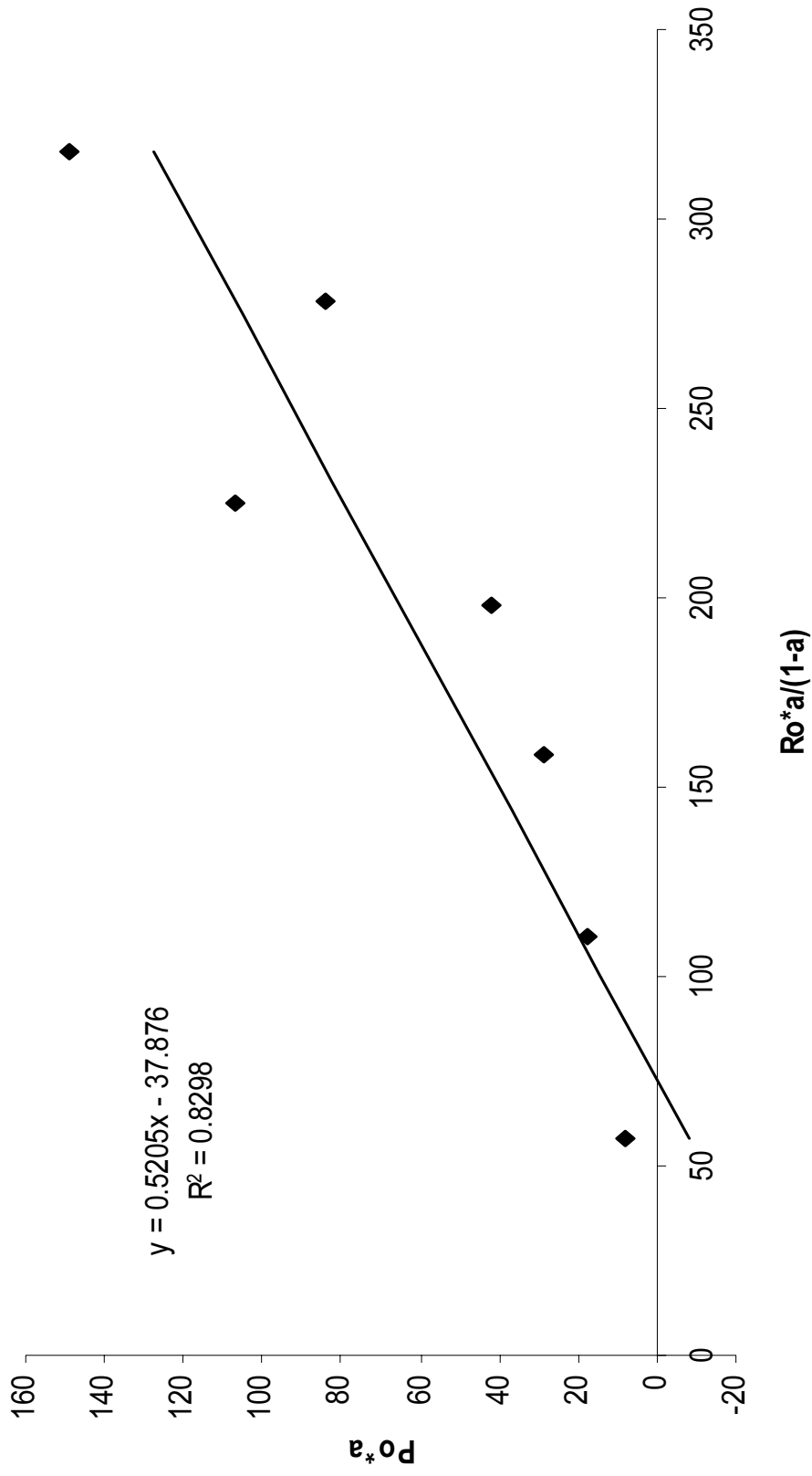$R^2 = 0.9858$

X-axis: Ro*a/(1-a)

Y-axis: Po*a
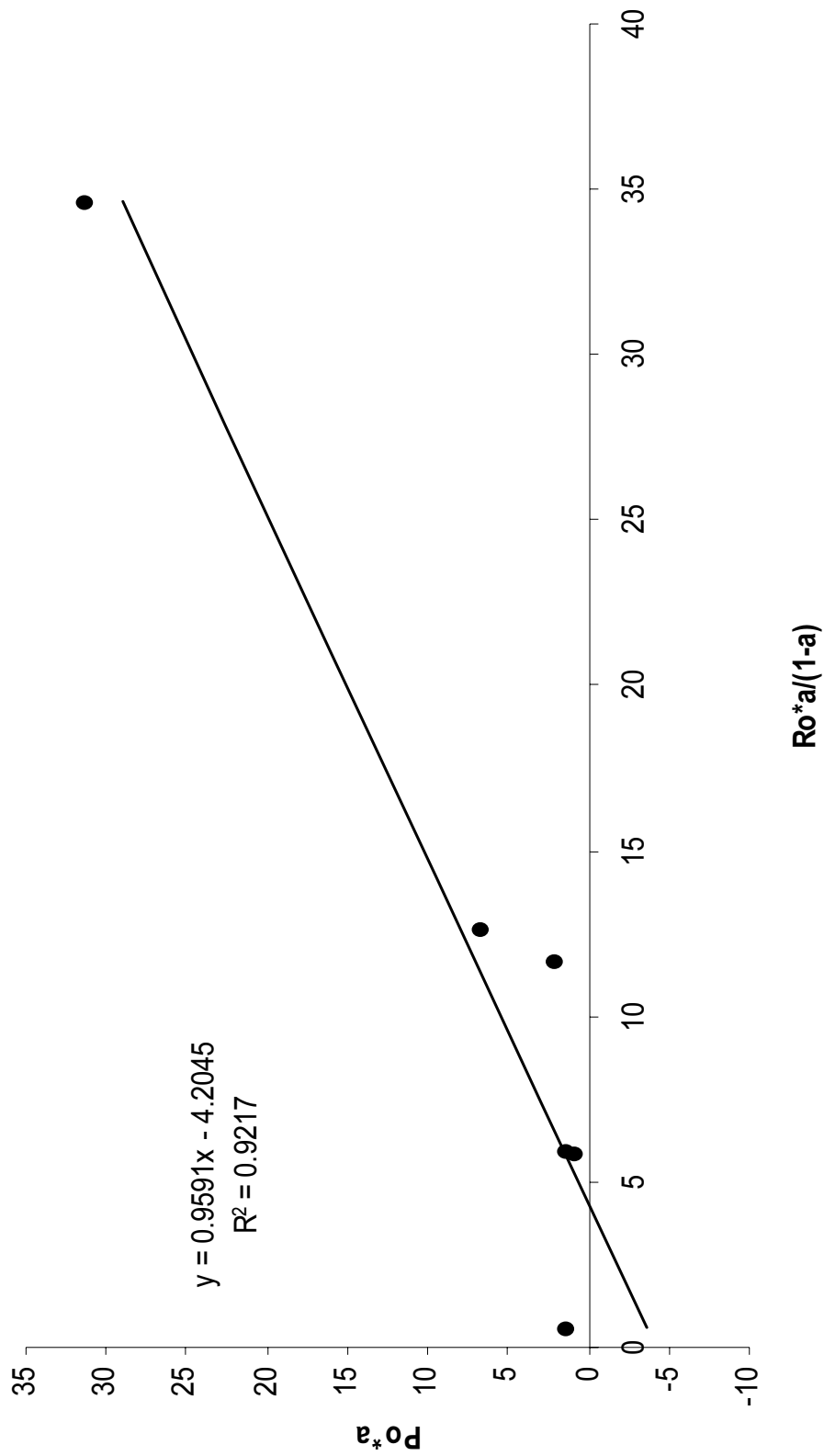
Figure 27. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP treated (600 MPa) BLG with 8 min holding time at pH 7.0.

$y = 1.1078x - 5.3498$
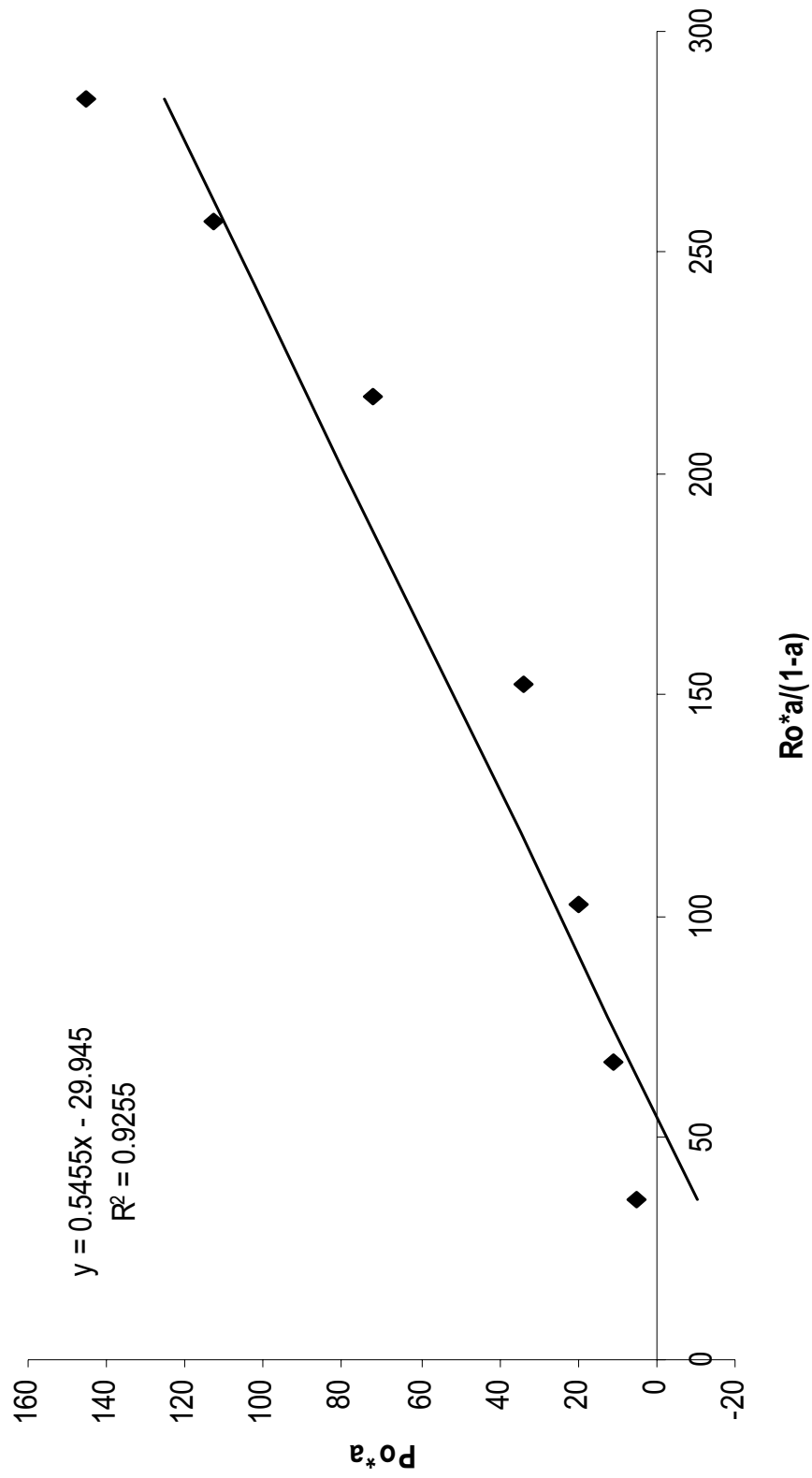$R^2 = 0.9786$
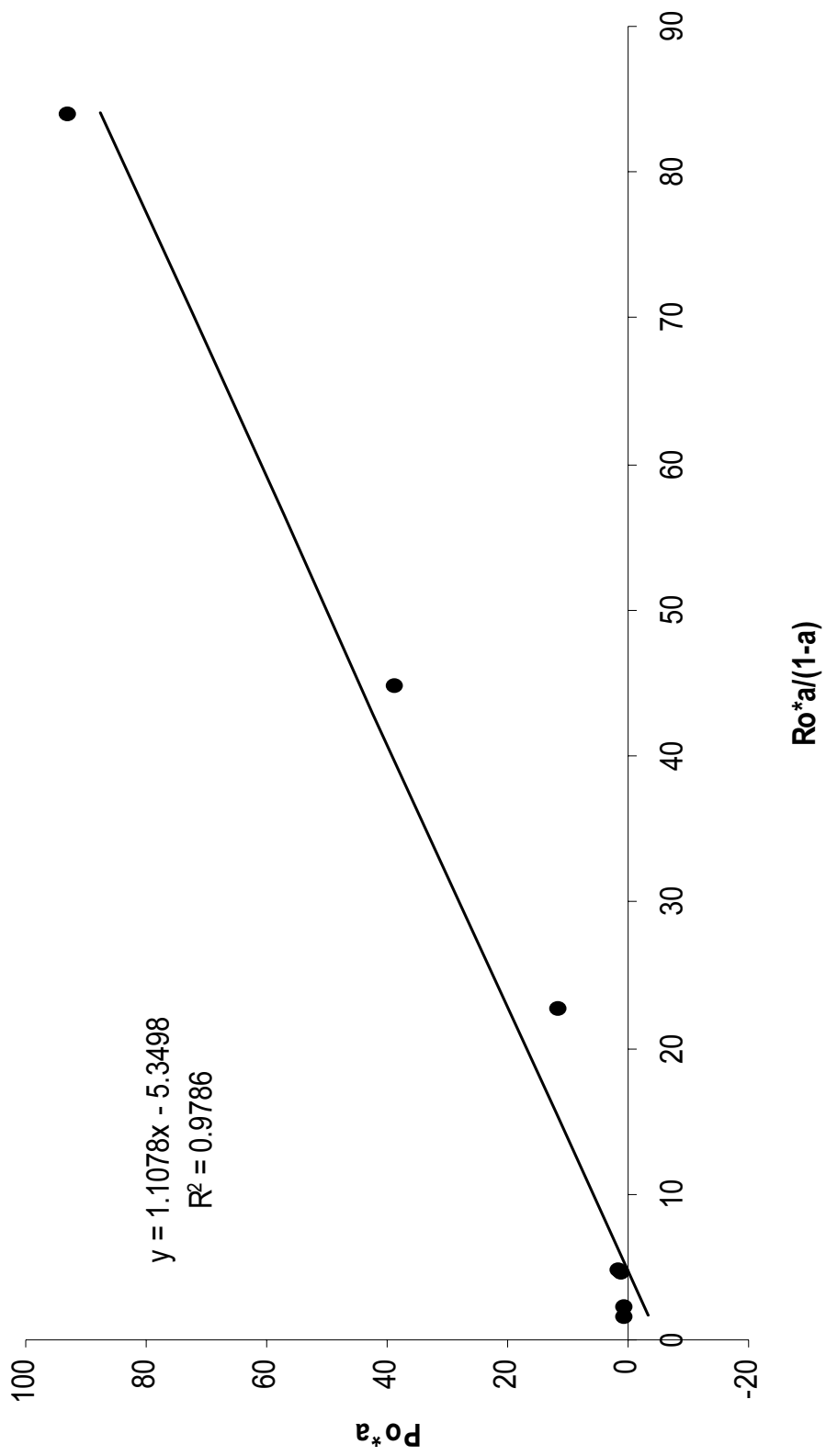
Figure 28. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP treated (600 MPa) BLG with 16 min holding time at pH 7.0.
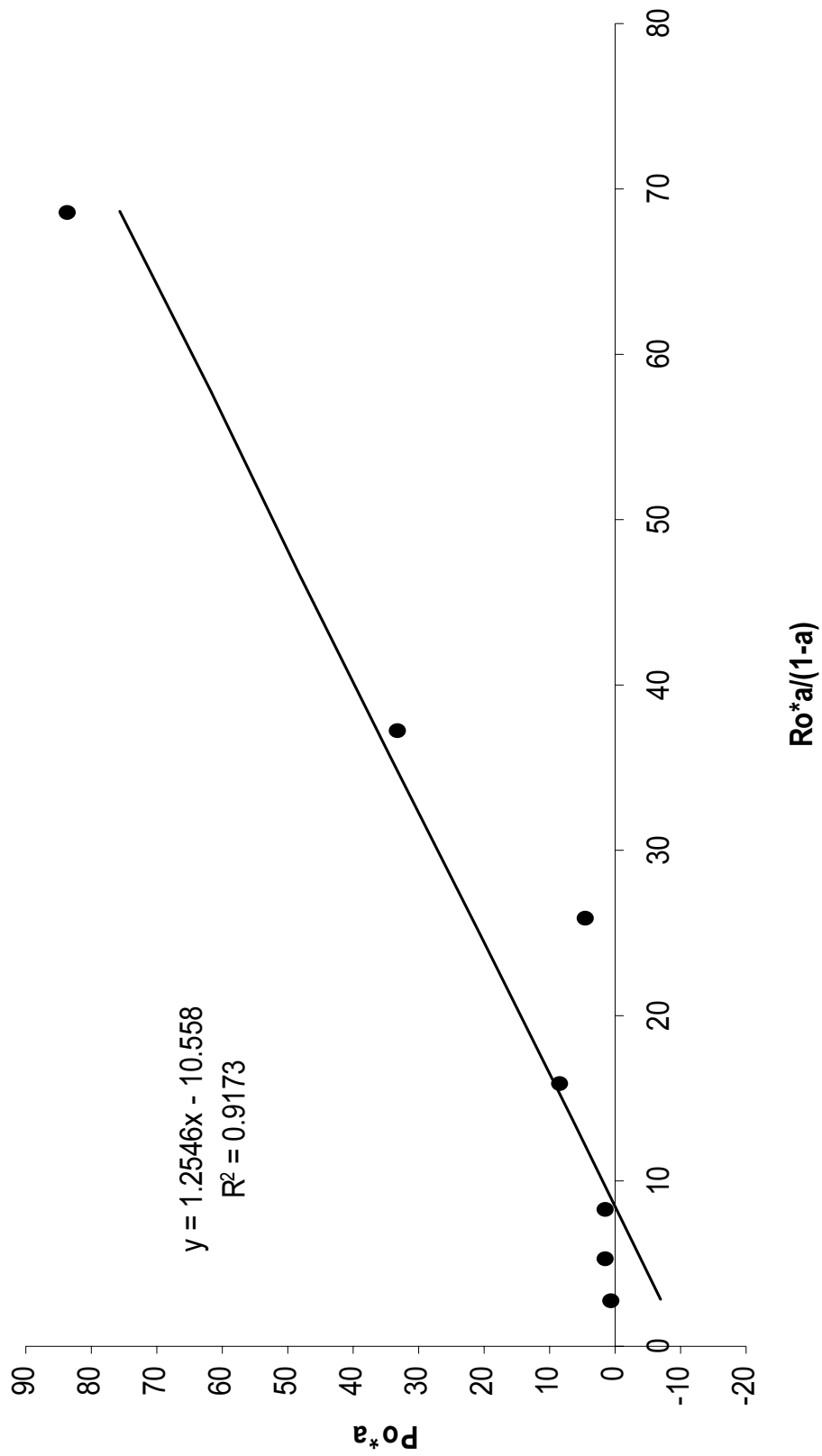
Figure 29. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP-treated (600 MPa) BLG with 32 min holding time at pH 7.0.

The plot shows data with the fit line:

$$y = 1.2546x - 10.558$$
$$R^2 = 0.9173$$

x-axis: Ro*a/(1-a)

y-axis: Po*a
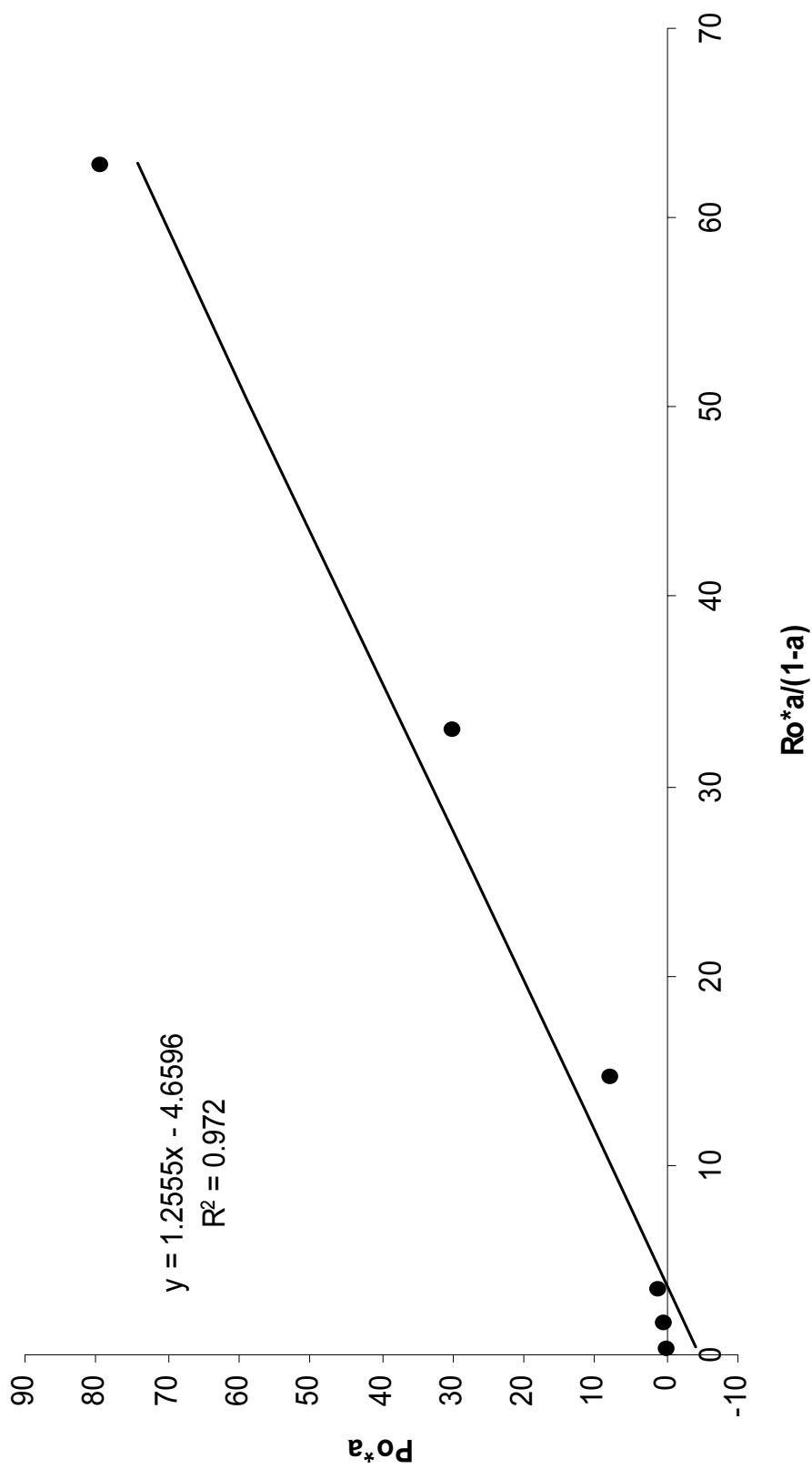
Figure 30. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for native BLG at pH 9.0.

The plot shows:
- X-axis: Ro*a/(1-a)
- Y-axis: Po*a
- $y = 1.2555x - 4.6596$
- $R^2 = 0.972$
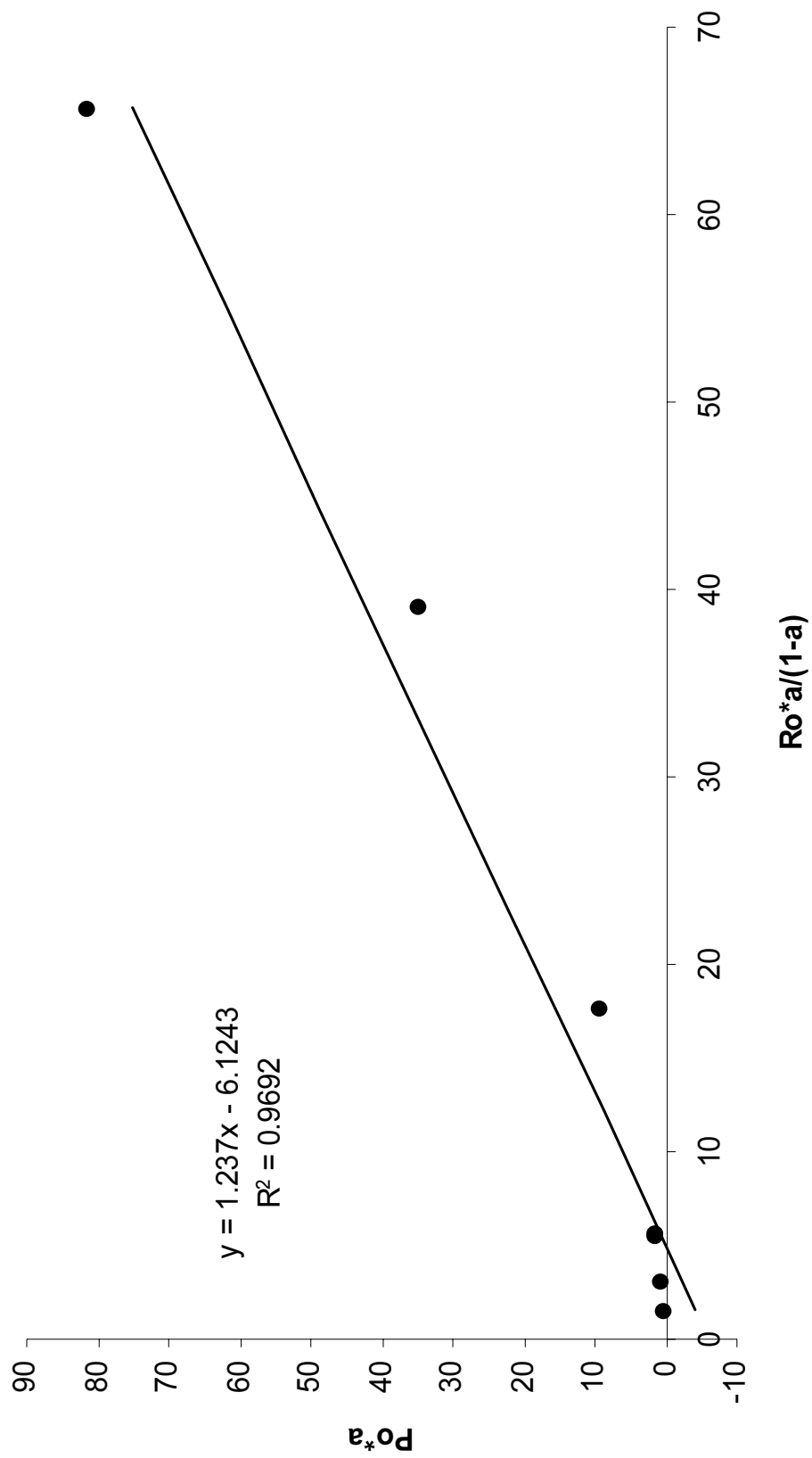
Figure 31. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP treated (600 MPa) BLG with 0 min holding time at pH 9.0.
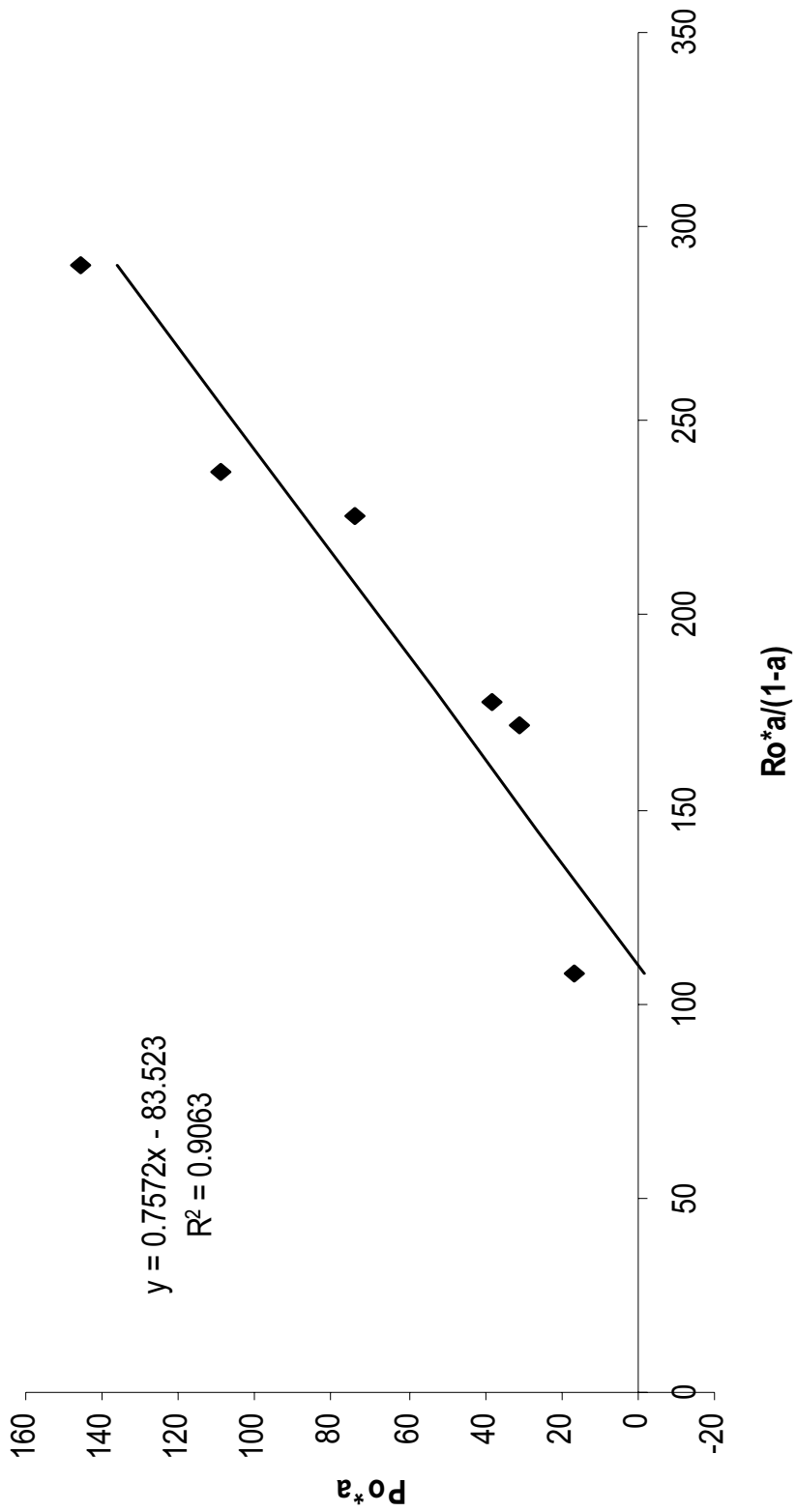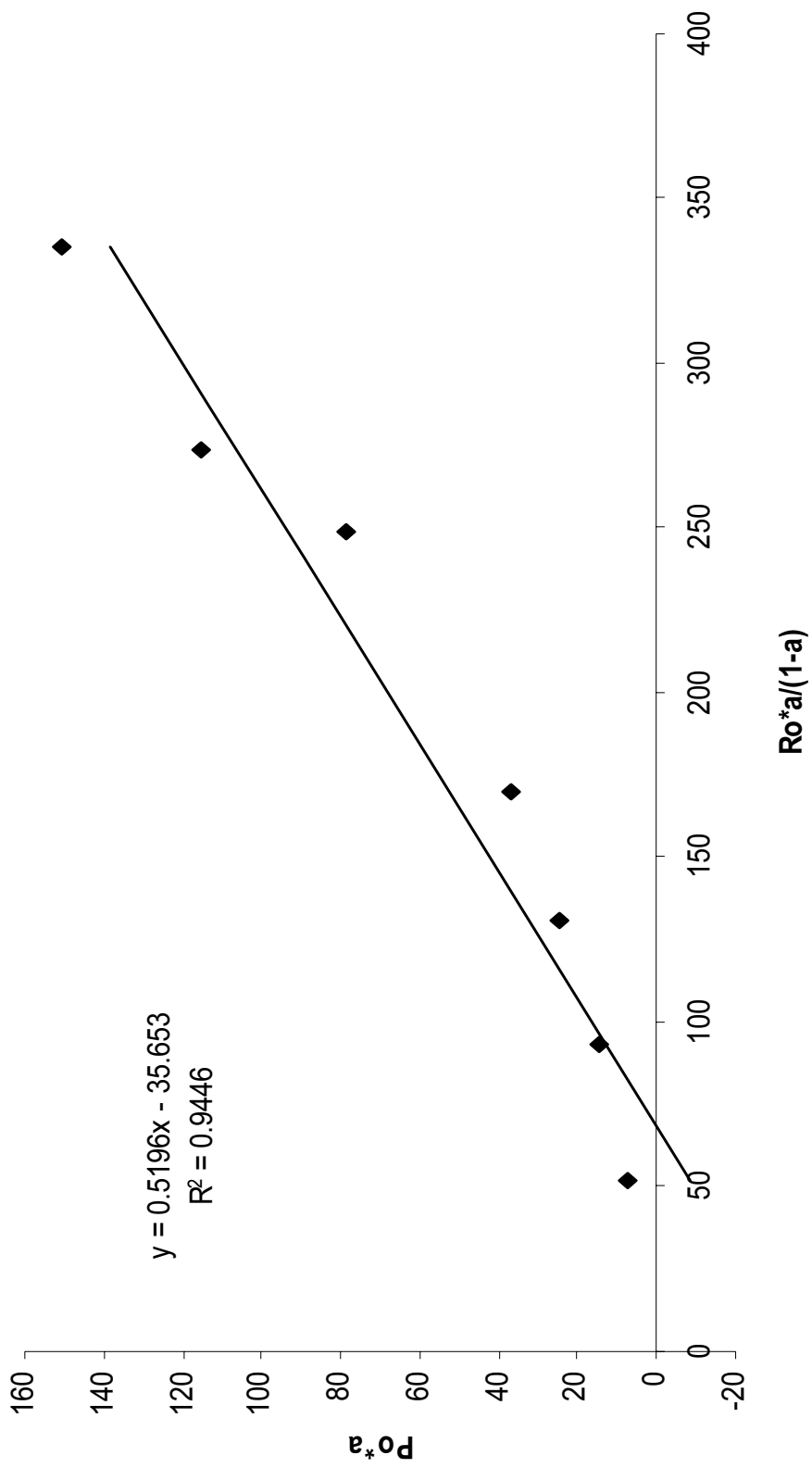
$y = 1.237x - 6.1243$
$R^2 = 0.9692$

Ro*a/(1-a)

Po*a
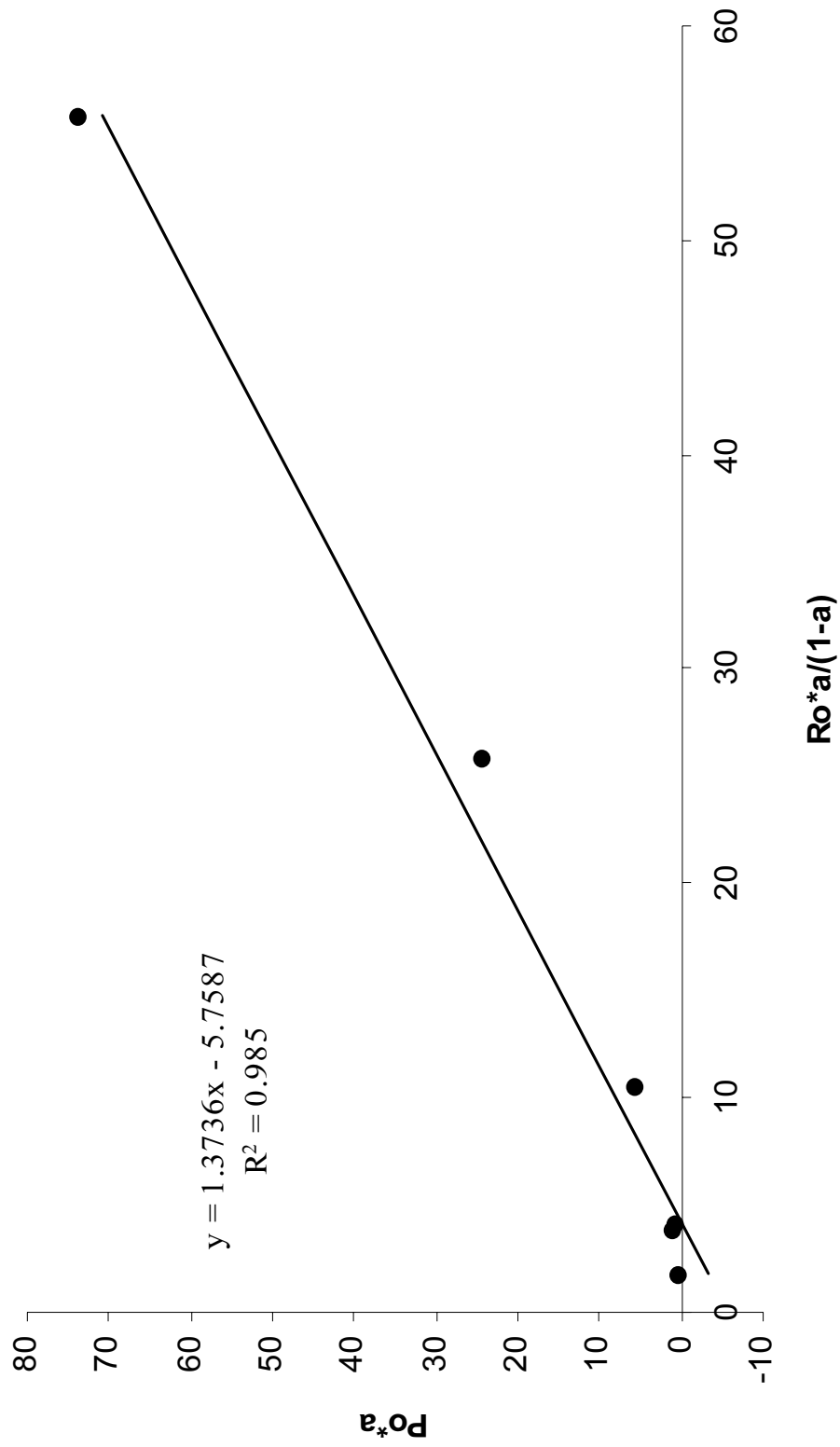
Figure 32. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP treated (600 MPa) BLG with 4 min holding time at pH 9.0.
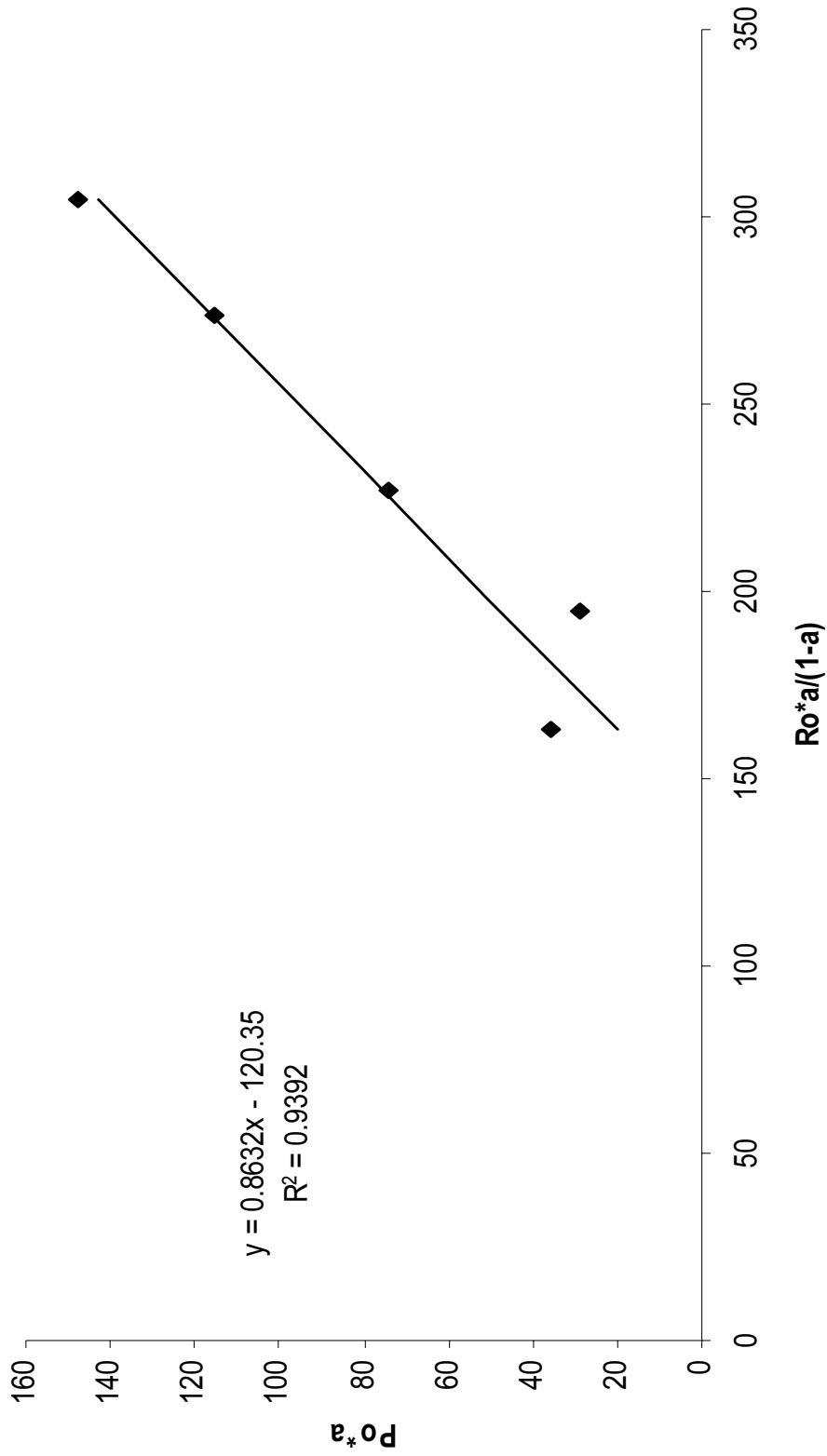
$y = 1.3736x - 5.7587$
$R^2 = 0.985$
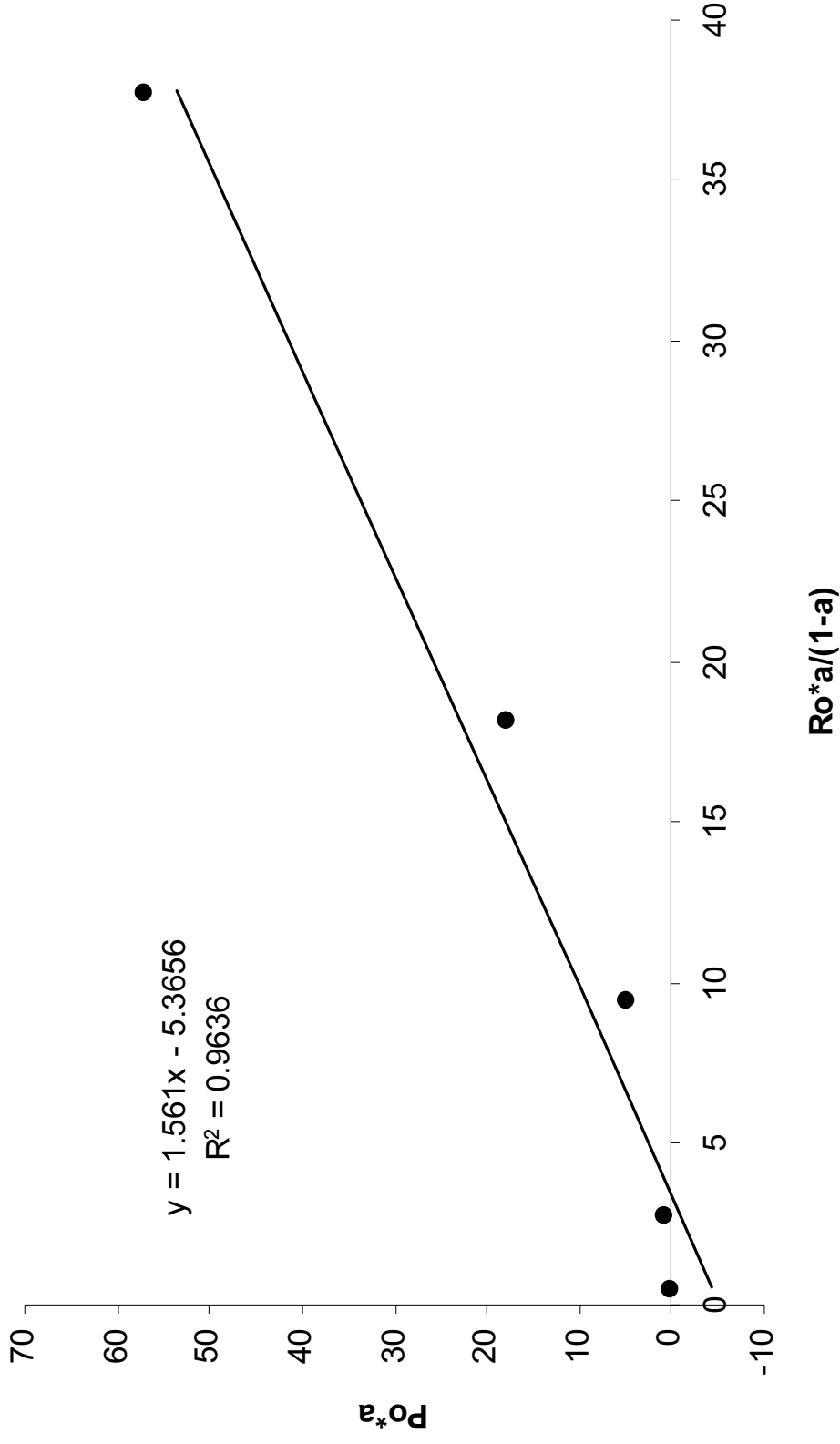
Figure 33. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP-treated (600 MPa) BLG with 8 min holding time at pH 9.0.

The plot shows Po^*a versus $Ro^*a/(1-a)$ with the linear fit:

$$y = 1.561x - 5.3656$$
$$R^2 = 0.9636$$
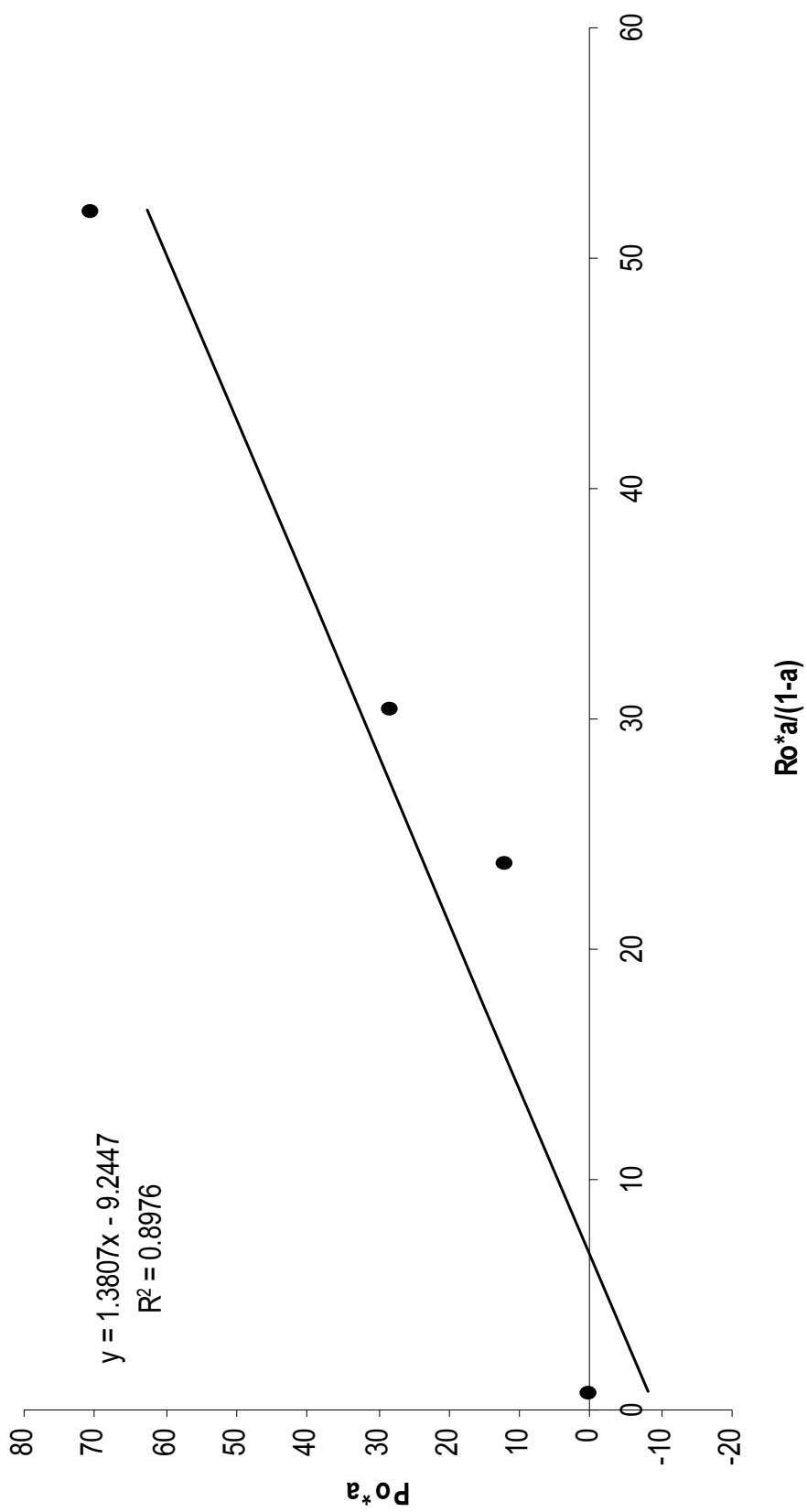
Figure 34. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP treated (600 MPa) BLG with 16 min holding time at pH 9.0.
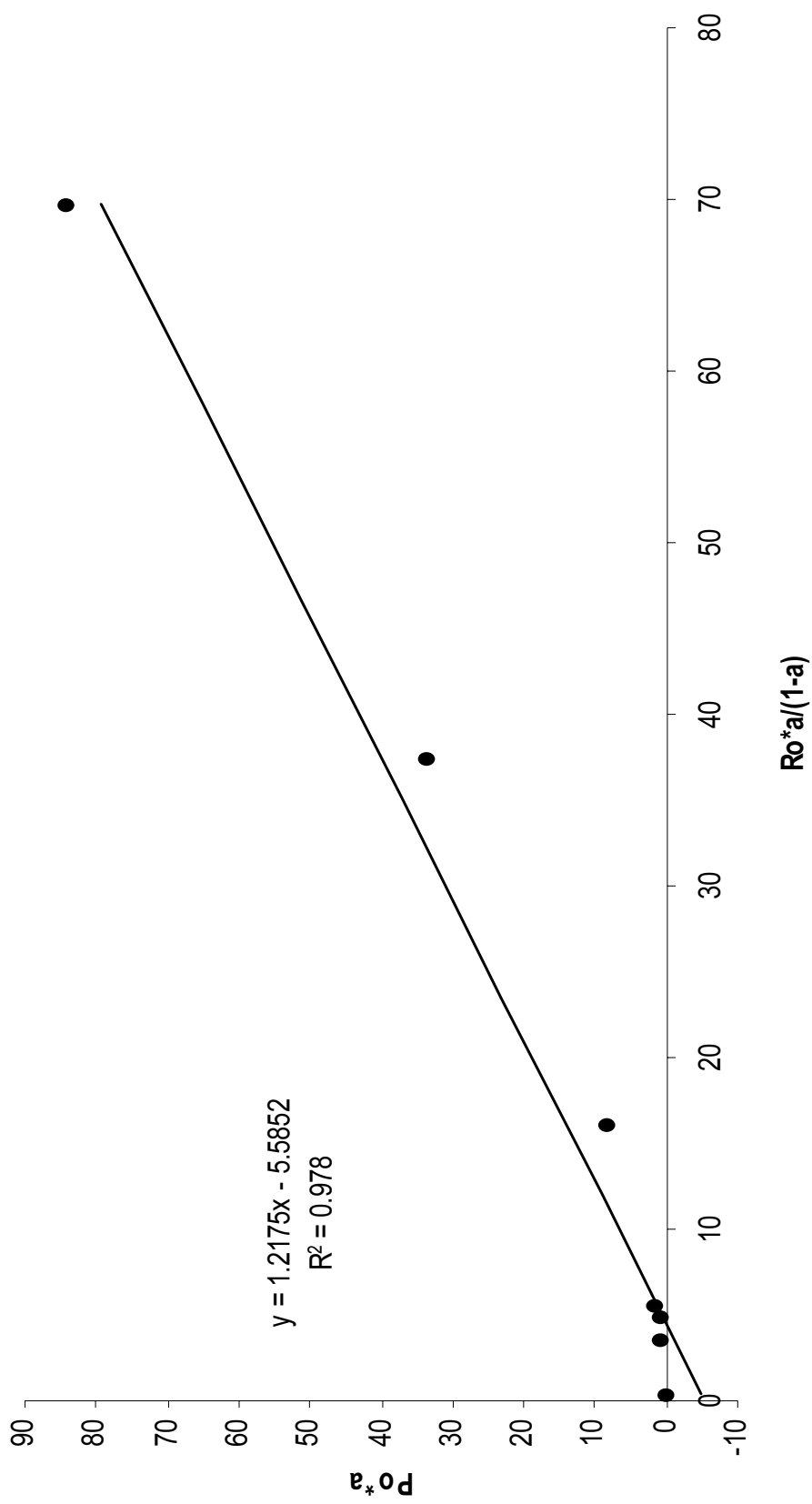
Figure 35. PRODAN binding to BLG plotted by Cogan method (1976) to calculate K'_d and n for UHP-treated (600 MPa) BLG with 32 min holding time at pH 9.0.

y = 1.2175x - 5.5852
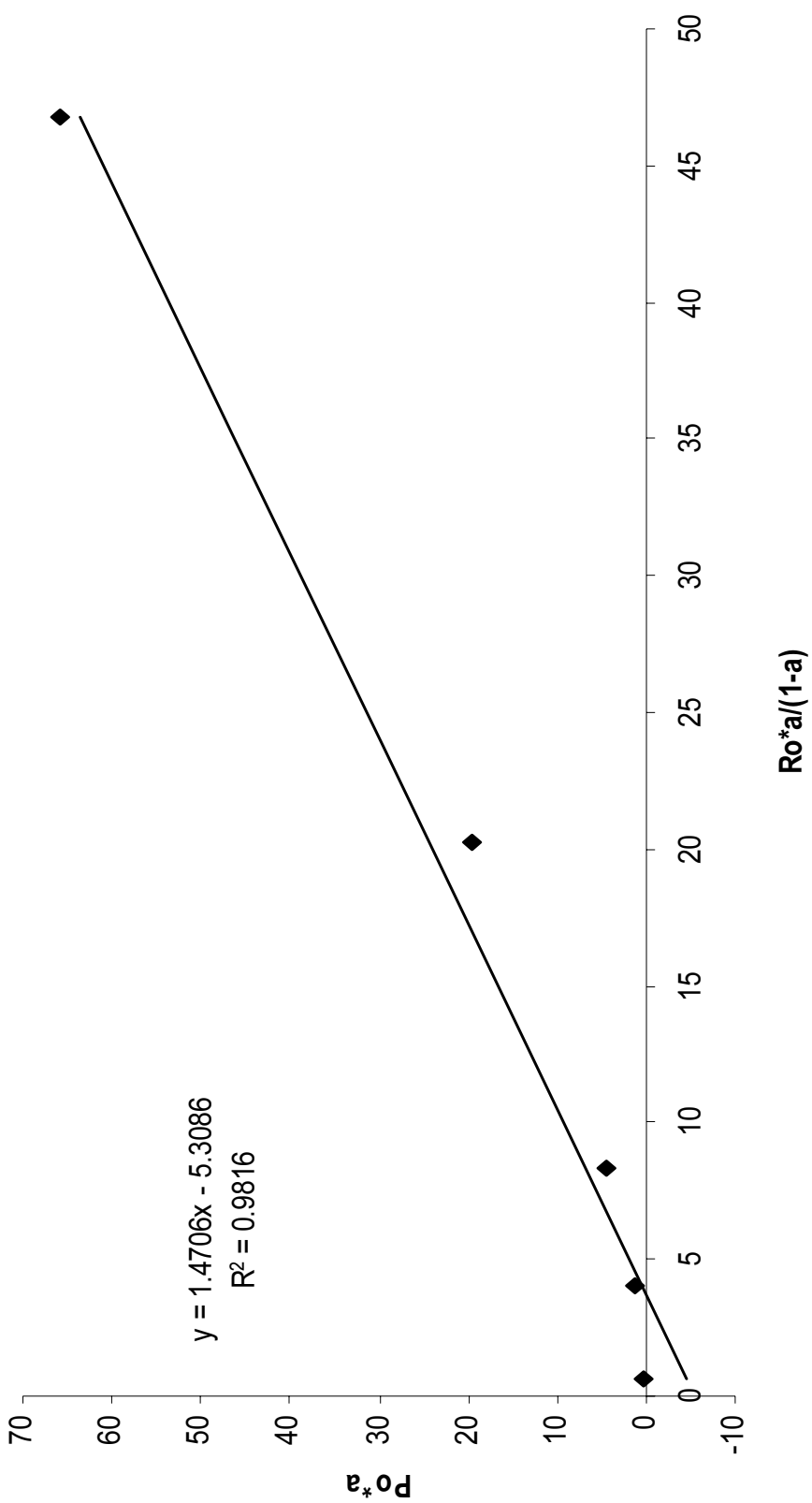R^2 = 0.978

Ro*a/(1-a)

Po*a

Table 1. Apparent dissociation constants (K'_d) and the number of binding sites (n) of BLG for PRODAN after UHP treatment (600 MPa) for selected holding times of 0 to 32 min (UHP0-UHP32) at pH 3.0-9.0 calculated using the method by Cogan and others (1976)*.

	pH3		pH5		pH7		pH9	
	K'_d (10^{-7} M)[1]	n[2]	K'_d (10^{-7} M)[1]	n[2]	K'_d (10^{-7} M)[1]	n[2]	K'_d (10^{-7} M)[1]	n[2]
BLG	112.14[a]	2.25[a]	5.55[a]	1.04[a]	6.15[a]	0.95[a]	3.71[a]	0.89[a]
UHP0	72.53[b]	1.86[b]	3.55[b]	0.78[b]	6.75[b]	0.86[b]	4.63[b]	0.79[b]
UHP4	53.99[c]	1.80[c]	5.09[c]	0.74[c]	4.51[c]	1.05[c]	4.10[c]	0.75[c]
UHP8	110.42[d]	1.25[d]	3.84[d]	0.72[d]	4.86[d]	1.04[d]	3.31[d]	0.62[d]
UHP16	67.79[e]	1.90[e]	7.14[e]	0.62[e]	4.84[e]	0.95[e]	6.70[e]	0.73[e]
UHP32	138.87[f]	1.16[f]	3.41[f]	0.69[f]	8.44[f]	0.78[f]	4.57[f]	0.84[f]

*: Data are means of three analyses calculated using method by Cogan and others (1976).
[1,2]: Means with different letters in the column are significantly different (p < 0.05).

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CHAPTER THREE

Application of solid-phase microextraction with headspace gas chromatography for the analysis of diacetyl, 2-methylbutyraldehyde, ethyl lactate, and δ-decalactone in Model Buffer Solution

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ABSTRACT

A headspace solid phase microextraction (HS-SPME) method was developed for the detection of selected flavor compounds, diacetyl, δ-decalactone, 2-methylbutyraldehyde, and ethyl lactate, in sodium phosphate buffer (0.01M, pH 9). The method required a total analysis time of 1.7 h for both extraction (10 min) and detection. PDMS/DVB and CAR/PDMS SPME fibers were used to examine their extraction efficiencies for the flavor compounds tested. Extraction time was investigated to determine the analytical performance of these fibers for the selected flavor compounds. The calibration plots were reproducible and linear ($R^2 > 0.954$) for the selected flavor compounds with the PDMS/DVB fiber tested. δ-Decalactone was not detected when using the CAR/PDMS fiber due to the long chain length and presence of a hydrocarbon tail of δ-decalactone under the current GC conditions.

INTRODUCTION

The extraction of flavor compounds from food involves concentrating the flavor compounds using headspace, purge and trap, liquid-liquid extraction, solid phase extraction, or simultaneous distillation/extraction techniques (Cai and others 2001; Cullere and others 2003; Apps and Tock 2005; Blythe and others 2006), followed by quantitation. The challenges of flavor compound analysis include extensive preparation time or excessive use of organic solvents. Static headspace using a gas-tight syringe and headspace solid-phase microextraction (SPME) are simple techniques, which are portable and inexpensive, that eliminate these drawbacks (Saraulio and others 1997). SPME requires no solvents or complicated apparatus that may introduce errors, can be used to concentrate volatile and nonvolatile compounds in liquid or vapor phases, provides linear results over wide concentrations of analytes, and can be coupled with any gas chromatograph or gas chromatograph-mass spectrometer system for the identification and quantitation of volatile compounds (Pawliszyn 2001).

With headspace (HS)-SPME, equilibrium involves the partitioning of analytes between the aqueous phase of sample, the vapor phase and the extraction phase of the SPME fiber. Extraction conditions must therefore be systematically optimized to increase the partitioning of analytes in the coated fiber. Besides extraction conditions and analyte properties, the choice of fiber coating is one of the most important aspects of optimization, because the coating is dependent on analyte properties. Several SPME fiber coatings are commercially available for extraction of nonpolar and polar compounds from liquid or gaseous samples. Pawliszyn (2001) suggested considering polydimethylsiloxane (PDMS) first, because of its ability to withstand high injection

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temperatures. Although PDMS is a nonpolar liquid phase, it can successfully be applied to extracting more polar compounds after optimizing extraction conditions (Pawliszyn 2001). Factors that improve the extraction of analytes by SPME are agitation, addition of salt to the analyte solution, sample volume, headspace volume, pH or temperature of the analyte solution, or sampling mode (immersion vs. headspace).

Gas chromatography (GC) is an important tool in analytical chemistry. Capillary columns increase separating capabilities of GC so that complex compounds such as flavors can be successfully resolved from solutions containing hundreds of components (Braithwaite and Smith 1999). The factors that influence the retention of flavor compounds are composition and properties of the mobile phase; type and properties of the stationary phase; the intermolecular forces between the compound(s) and stationary and mobile phases; and temperature of the column. Electrostatic interactions between molecules include polar van der Waal's retention forces arising from interaction between molecules having a surface charge; and nonpolar dispersion forces between neutral molecules or functional groups. Polar van der Waal's retention forces are dipole-dipole interactions and hydrogen bonding between molecules. Hydrogen bonded solvents will attract polar solute molecules but will exhibit varying degrees of repulsion to nonpolar molecules. Thus, solute molecules will be attracted towards the phase of similar polarity in the GC system. In nonpolar solvents or stationary phase, London's dispersion forces are the main interactions between molecules. London dispersion forces are weak intermolecular forces that arise from the attractive force between transient multipoles of nonpolar molecules without permanent multipole moments. Multipole moments are created when the electron densities are not evenly distributed throughout the nonpolar

molecule. Therefore, multipoles of one nonpolar molecule may interact with multipoles of other nonpolar molecules (French 2000).

Diacetyl, 2-methylbutyraldehyde, ethyl lactate, and δ-decalactone were selected based on the flavor profiles of foods perceived to contain or are rich, such as dairy products (Badings and Neeter 1980; Milo and Reineccius 1997), chocolate, or cream-based foods.

Diacetyl, or 2,3-butanedione, is one of the major flavor compounds in dairy products and wine. Diacetyl contributes a characteristic buttery aroma and complexity to the final sensory impact of food. Diacetyl is naturally produced upon the conversion of lactose to lactic acid by lactic acid bacteria in cultured milks and fresh cheeses (Urbach 1995). After alcoholic fermentation ceases when fermentative yeasts die, malolactic fermentation continues, either naturally or by inoculation, in wines and produces diacetyl among other aroma compounds (Hayasaka and Bartowsky 1999). Diacetyl is an important flavor compound in many dairy products and wine, yet is not studied or extensively subjected to quantitative analyses due to analytical difficulties in the quantitation from its high volatility. Diaz and others (2004) evaluated fiber selection, extraction time, temperature and ionic strength for headspace SPME analysis of diacetyl from water samples of the Llobregat River in Spain. They determined that extraction of diacetyl for 30 min with a CAR/PDMS fiber and polar column at 60 °C resulted good linearity ($r^2 > 0.999$) and good reproducibility (R.S.D. < 20%).

2-Methylbutyraldehyde (2MB) is a Strecker aldehyde that contributes a characteristic cocoa aroma. 2MB is formed from the deamination and decarboxylation of branched chain amino acid isoleucine by brewing yeast, and can be reduced

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enzymatically to 'fusel' alcohols (Ford and Ellis 2002). The 'fusel' alcohols and their

subsequent esters are important in the development of flavor during the brewing process.

Foese and others (1999) were able to detec 2-methylbutyraldehyde quantitatively in

disinfected water by purging water samples with N2 and trapping on Tenax-TA ™

adsorbent. The adsorbent was thermally desorbed by heating to 200 C, transferring the

analyte into a nonpolar GC column. Guillen and Ibargoitia (1999) used dichloromethane,

an organic solvent, to extract liquid smoke components, such as 2-methylbutyraldehyde,

and concentrated the components by evaporation prior to direct injection into a GC. Little

research is available on 2-methybutyraldehyde as a flavor ingredient in foods.

Ethyl lactate (EL) contributes a characteristic fruity, buttery and butterscotch

aroma. EL is used in yeast fermentations to produce roasted aromas that contribute to the

desirable flavor of thermally processed foods such as meat and bread (Rhlid and others

2002). Ethyl lactate was identified as a flavor compound in fresh coconut sap via direct

injection of a solvent concentrate into a GC-MS (Borse and others 2006). According to

the official method of the Office International de la Vigne et du Vin (OIV) (1994), ethyl

lactate is quantitatively determined using gas chromatography with a polar capillary

column. Soufleros and others (1994) modified the mthod by OIV in which analytes were

extracted by a mixture of solvents, mixed with an internal standard, and injected directly

into a GC with a polar capillary column.

Although fruits such as peaches, apricots and nectarines contain the pleasant

aromas of δ-lactones (Tamura and others 2005), δ-decalactone (dDL) imparts a creamy

and coconut note to foods. Guillot and others (2006) used headspace-SPME-GC to

quantitate various flavor compounds, including γ-decalactone, from apricot puree. They

reported that γ-decalactone was extracted in more quantities by a PDMS fiber than by Carboxen/PDMS fiber when using a polar (DB-WAX) capillary column. Tamura and others (2005) assessed the authenticity of δ-decalactone from peach, apricot, and nectarine fruits by direct injection of analytes containing the flavor compound into a DB-WAX column using a temperature program of 5-200 °C at a rate of 5 °C/min.

The objective of this research was to develop one method that can accommodate the extraction and quantitation of diacetyl, ethyl lactate, δ-decalactone, and 2-methylbutyraldehyde, separately and together, using HS-SPME coupled with gas chromatography.

MATERIALS & METHODS

Diacetyl, 2-methylbutyraldehyde, δ-decalactone, and ethyl lactate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The SPME fibers were purchased from Supelco (Bellefonte, PA, USA). Polydimethylsiloxane-divinylbenzene (PDMS/DVB) of 65 μm thickness and carboxen- polydimethylsiloxane (CAR/PDMS) of 75 μm thickness SPME fibers were selected based upon their affinity for the flavor. Fibers were conditioned prior to use according to the manufacturer's instructions: PDMS/DVB was inserted into the GC injector at 250 °C for 0.5 h and CAR/PDMS at 300°C for 1-1.5 h.

SPME Procedure

Individual standard stock solutions of selected flavor compounds were prepared at a concentration of 1 μg/mL in 10% ethanol and water to solubilize the flavor compounds. An aliquot of each standard stock solution was mixed with 2 mL of sodium phosphate buffer (0.01 M, pH 9.0) and 0.65 g NaCl to selected concentrations (0.001-0.01 μg/mL) in a 4 mL amber vial sealed with a silicone septum.

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An amber vial containing buffer, individual or combined flavor compounds, and salt was placed on a magnetic hot stir plate (model PC-220, Corning, NY) and stirred at 400 rpm for 5 min to pre-equilibrate volatiles for each headspace (HS)-SPME analysis. A pre-equilibration temperature of 37°C was chosen to mimic the temperature of the mouth when flavor compounds are released during eating. The buffer containing the flavor compounds and salt continue to stir during adsorption. The SPME needle pierced the septum and the fiber was extended through the needle to bring the stationary phase in contact with the assay headspace above selected concentrations of flavor solutions. The fiber was withdrawn into the needle after an exposure time from 1 to 60 min. The volatiles adsorbed to the SPME fiber were thermally desorbed into the injection port of a Hewlett-Packard HP 5890A (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph connected to a FID detector in splitless mode. A DB-WAX column (60 m x 0.32 mm i.d., 0.5 um film thickness, J&W Scientific, Folsom, CA) was chosen according to methods by Diaz and others (2004), OIV (1994), Guillot and others (2006), and Tamura and others (2005). Helium was used as the carrier gas. The injector temperature was 250°C, according to the method of Adahchour and others (2005). The oven temperature program was adopted from Tamura (2005) with a change at which the oven was maintained at 33 °C, for 5 min before increasing to 50 °C, at a rate of 2 °C/min, then to 200 °C, at a rate of 5 °C/min, and held for 37 min.

A standard curve, to assure a linear response of HS-SPME, was prepared by adding selected flavor compounds in 10% ethanol to amber vials containing 2 mL sodium phosphate buffer (0.01M, pH 9.0) and 0.65 g NaCl to yield solution concentrations from 4.02 μg/mL to 20.62 μg/mL of the flavor compounds. Volatile compounds in the vial

headspace were extracted using HS-SPME from 1 to 60 min at 37°C, and a calibration curve was prepared by plotting peak area against solution concentration of specific flavor compounds.

The characterization of chemical compounds using absolute gas chromatographic retention times, or even specific retention volumes, is problematic, due to changes in most of the critical gas chromatographic parameters for a given column that can occur gradually with time. Thus, marked differences often exist between one chromatographic system and the next (Peppard and Ramus 1988). Consequently the Kovats' retention index (KI) overcomes such problems. The KI relates the retention of compounds during isothermal GC to the retention of a series of *n*-alkanes analyzed under identical conditions. Each compound is thus bracketed by two hydrocarbons, both of which are assigned a retention index value corresponding to the number of carbon atoms in the molecule multiplied by 100. The KI for each flavor compound for the selected temperature GC program was based on the following equation:

$$KI = 100 * \left(\frac{t_{R(x)} - t_{R(y)}}{t_{R(z)} - t_{R(y)}} \right) + 100y$$

where t_R is retention time, x is the solute of interest (i.e. flavor compound), y is the normal alkane with y number of carbon atoms eluting before x, and z is the normal alkane with z number of carbon atoms eluting after x.

Statistical Analysis

Data were analyzed by using one-way analysis of variance (ANOVA) procedure using the General Linear Model, with further analysis using Tukey's pairwise comparison test to determine significance at $p < 0.05$ (SAS for Windows, version 9.1 TS Level, 1M2, SAS Institute., Cary, NC, USA).

RESULTS & DISCUSSION

Extraction Time

The extraction time profiles of the PDMS/DVB and CAR/PDMS SPME fibers were established by plotting detector response against extraction time. The equilibration time is reached when a further increase in extraction time does not result in a significant increase in detector response. Figures 1 and 2 present the time profiles for the PDMS/DVB and CAR/PDMS fibers, respectively. Different extraction time profiles were observed for the analytes on the tested coatings. The signals obtained increased with increasing extraction time for all analytes; equilibration time was, however, different for each coating. The PDMS/DVB fiber is a made of a blend of porous divinylbenzene polymer particles with a liquid polydimethylsiloxane polymer. The PDMS polymer has a high degree of porosity (1.5 mL/g) that can physically retain analytes, producing a strong retention of analytes that fit tightly into the pores. DVB is mesoporous with micropores that are fairly larger than Carboxen particles, and is ideal for trapping C_6-C_{15} analytes (Pawliszyn 1999). The combination of PDMS and DVB has better affinity for polar analytes. CAR/PDMS, on the other hand, is ideal for the analyses of molecules in the C_2-C_{12} range, and has unique pores that pass completely through the particles so that small analytes can be rapidly desorbed (Pawliszyn 1999).

The equilibration time for diacetyl, δ-decalactone, and ethyl lactate was 10 min, and for 2-methylbutyraldehyde was 20 min for the PDMS/DVB fiber. The semipolar nature of PDMS/DVB allows for the extraction of the selected flavor compounds, including dDL, which has a polar head and a 5-carbon tail. Higher uptake of diacetyl, ethyl lactate, and 2-methylbutyraldehyde was observed for the CAR/PDMS fiber,

because the polar fiber has a higher affinity for the low molecular mass compounds, and Diaz and others (2004) have reported that the fiber presented greater efficiency than other polar fibers, such as CAR-DVB-PDMS. This observation indicated that, though dDL is within the detectable limits of CAR/PDMS, dDL is not detectable under the GC conditions set in the method, because of its chain length and polarity (Figure 2).

For quantitative analysis, it is not necessary for the analytes to reach equilibrium, as long as the extractions are carefully timed and the mixing conditions and volumes remain constant (Ai 1997). An extraction time of 10 min was selected to keep the total extraction time comparable with the chromatographic run time. The equilibrium conditions for the selected flavor compounds reached after about 20 min, and the slope of the curve is small. Therefore, a 1-min deviation in the exposure of the fiber results in about the same absolute error independent of the extraction time (Pawliszyn 1997). The PDMS/DVB fiber was chosen over the CAR/PDMS fiber, because of higher recovery of diacetyl, ethyl lactate, 2-methylbutyraldehyde, and δ-decalactone and decreased extraction time.

Analytical Characteristics

To check the performance of the procedure, SPME was applied to the analysis of sodium phosphate buffer solutions containing known concentrations of flavor compounds using the PDMS/DVB fiber. The linearity of the method was investigated by determining calibration plots over the concentration range 5-20 μg/mL. Series of three concentrations were obtained by spiking sodium phosphate buffer with the flavor compounds to generate calibration plots. Each solution was extracted with the PDMS/DVB fiber, in replicates of two, and analysis was performed by GC-FID. The line of best fit for the relationship

between the mean peak area and the concentration of analyte in the buffer was determined by linear regression. The procedure revealed linear behavior over the whole concentration range tested with correlation coefficients $R^2 > 0.954$ for selected flavor compounds (Figure 3). The calculated KI for diacetyl, 2-methylbutyraldehyde, ethyl lactate, and δ-decalactone are presented in Table 1.

CONCLUSIONS

The SPME adsorption-GC detection method developed is a convenient quantitative analytical technique for diacetyl, ethyl lactate, 2-methylbutyraldehyde, and δ-decalactone in a model buffer solution. The small amount of time and materials required for SPME concentration and extraction, and GC separation and detection resulted in an inexpensive and simple method for analysis. The combination of extraction and concentration into one SPME adsorption step significantly decreased the time required for analysis. SPME adsorption-GC detection method described required 1.7 h for both extraction and detection of selected flavor compounds. The selectivity of the SPME method versus more common methods such as liquid-liquid extraction, excludes necessary clean-up procedures which may introduce errors and extend the time necessary for an efficient and sensitive analytical method.

REFERENCES

Ai J. 1997. Solid phase microextraction for quantitative analysis in nonequilibrium situations. Anal. Chem. 69: 1230-1236.

Apps P, Tock MLA. 2005. Enhanced flavor extraction in continuous liquid-liquid extractors. J. Chrom. A 1083: 215-218.

Badings HT, Neeter R. 1980. Recent advances in the study of aroma compounds of milk and dairy products. Neth. Milk Dairy J. 34(1): 9-30.

Blythe JW, Heitz A, Joll CA, Kagi RI. 2006. Determination of trace concentrations of bromophenols in water using purge-and-trap in situ acetylation. J. Chrom. A 1102: 73-83.

Borse BB, Rao LJM, Ramalakshmi K, Raghavan B. 2006. Chemical composition of volatiles from coconut sap (neera) and effect of processing. Food Chem. In Press.

Braithwaite A, Smith FJ. 1999. Chromatographic methods. Dordrecht: Kluwer Academic Publishers. 559p.

Cai J, Liu B, Su Q. 2001. Comparison of simultaneous distillation extraction and solid-phase microextraction for the determination of volatile flavor components. J. Chrom. A 930: 1-7.

Cullere L, Aznar M, Cacho J, Ferreira V. 2003. Fast fractionation of complex organic extracts by normal-phase chromatography on a solid-phase extraction polymeric sorbent optimization of a method of fractionate wine flavor extracts. J. Chrom. A 1017: 17-26.

Diaz A, Ventura F, Galceran MT. 2004. Identification of 2,3-butanedione (diacetyl) as the compound causing odor events at trace levels in the Llobregat River and Barcelon's treated water (Spain). J. Chrom. A 1034: 175-182.

Ford G, Ellis EM. 2002. Characterization of Ypr1p from *Saccharomyces cerevisiae* as a 2-methylbutyraldehyde reductase. Yeast 19: 1087-1096.

French RH. 2000. Origins and applications of London dispersion forces and Hamaker constants in ceramics. J. Am. Ceram. Soc. 83: 2117-2146.

Hayasaka Y, Bartowsky EJ. 1999. Analysis of diacetyl in wine using solid-phase microextraction combined with gas chromatography-mass spectrometry. J. Agric. Food. Chem. 47: 612-617.

Milo C, Reineccius GA. 1997. Identification and quantification of potential odorants in regular-fat and low-fat mild cheddar cheeses. J. Agric. Food Chem. 45(9): 3590-3594.

Pawliszyn J. 1997. Solid phase microextraction: theory and practice. New York: Wiley-VCH, Inc. 247p.

Pawliszyn J. 1999. Applications of solid phase microextraction. Cambridge: The Royal Society of Chemistry. 655.

Pawliszyn J. 2001. Solid phase microextraction. In: Rouseff R and Cadwallader K. Headspace analysis of foods and flavors. New York: Kluwer Academic/Plenum Publishers. p 73-87.

Peppard TL, Ramus SA. 1988. Use of Kovats' gas chromatographic retention indices in beer flavor studies. J. ASBC 46: 26-30.

Rhlid RB, Fleury Y, Blank I, Fay LB, Welti DH, Vera FA, Juillerat MA. 2002. Generation of roasted notes based on 2-acetyl-2-thiazoline and its precursor, 2-(1-hydroxyethyl)-4,5-dihydrothiazole, by combined bio and thermal approaches. J. Agric. Food Chem. 50(8): 2350-2355.

Saraulio A, Martos PA, Pawliszyn J. 1997. Water analysis by solid phase microextraction based on physical chemical properties of the coating. Anal. Chem. 69: 1992-1998.

Tamura H, Appel M, Richling E, Schreier P. 2005. Authenticity assessment of γ- and δ-decalactone from Prunus fruits by gas chromatography combustion/pyrolysis isotope ratio mass spectrometry (GC-C/P-IRMS). J. Agric. Food Chem. 53: 5397-5401.

Urbach G. 1995. Contribution of lactic acid bacteria to flavour compound formation in dairy products. Int. Dairy J. 5: 877-903.
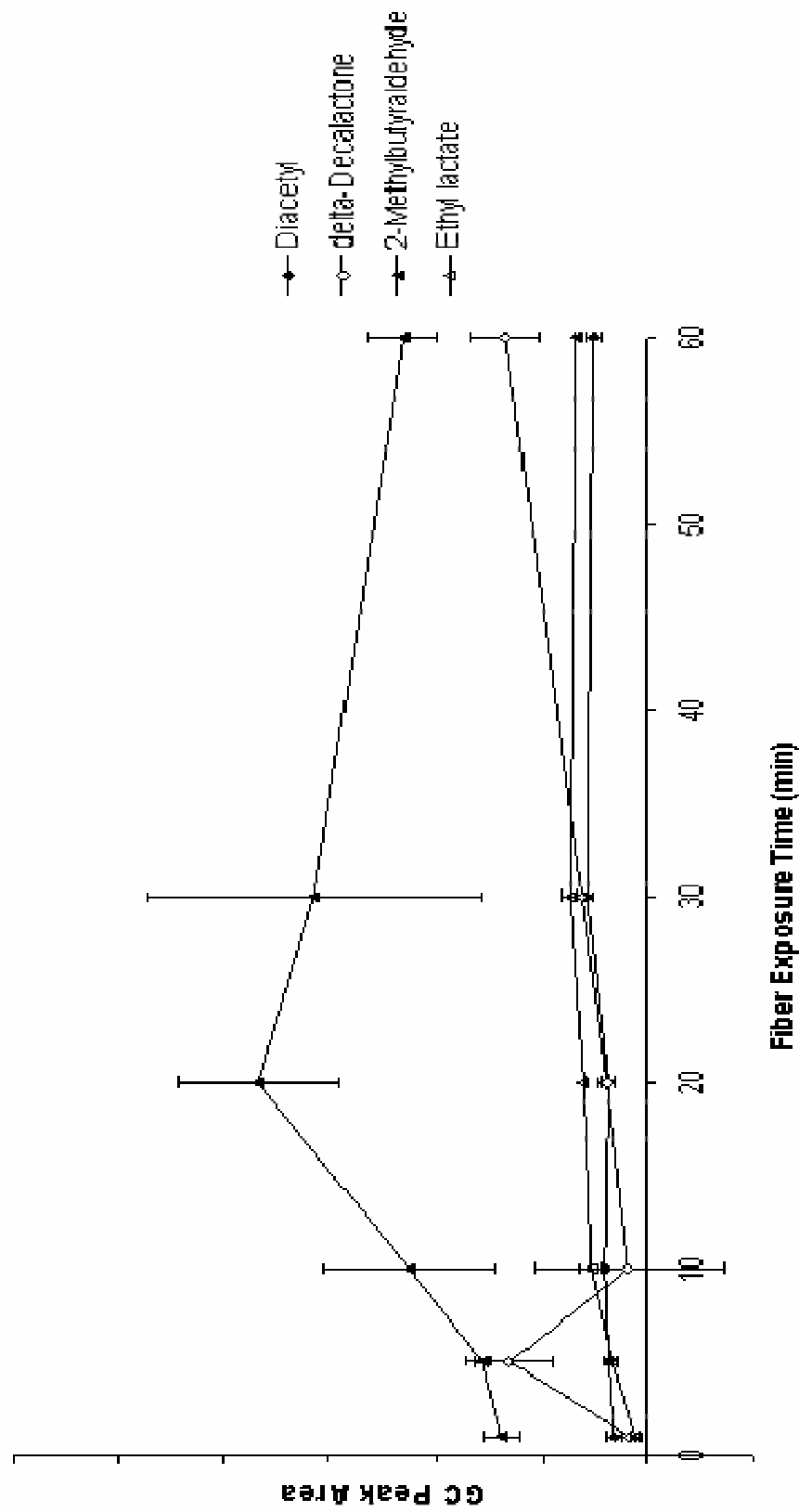
Figure 1. Exposure time of selected compounds adsorbed by a PDMS/DVB fiber. The GC peak area of selected flavor compounds compounds at 1, 5, 10, 20, 30, and 60 min were the mean areas of two replicated runs.

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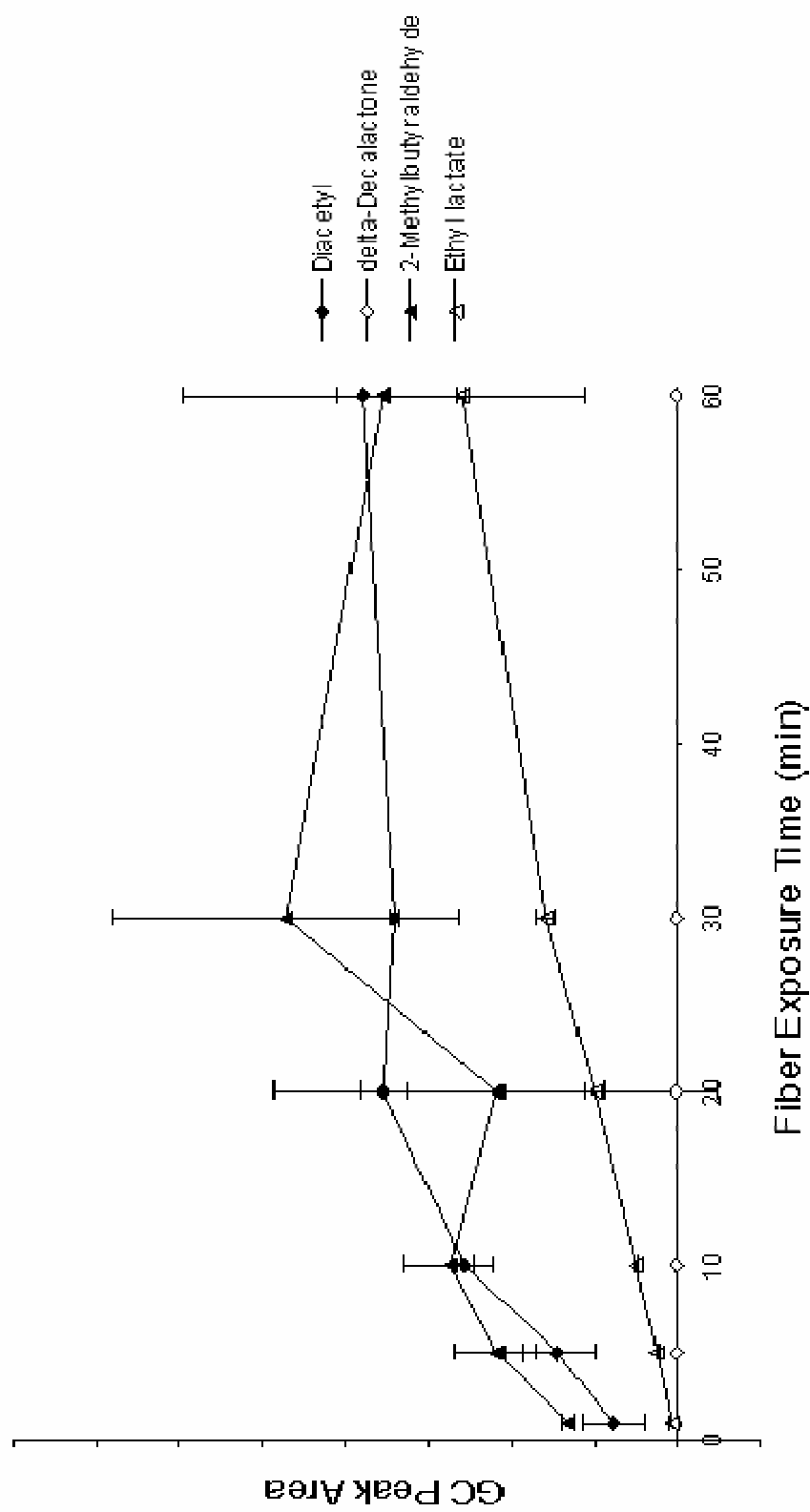
Figure 2. Exposure time of selected compounds adsorbed by a CAR/PDMS fiber. The GC peak area of selected flavor compounds compounds at 1, 5, 10, 20, 30, and 60 min were the mean areas of two replicated runs.
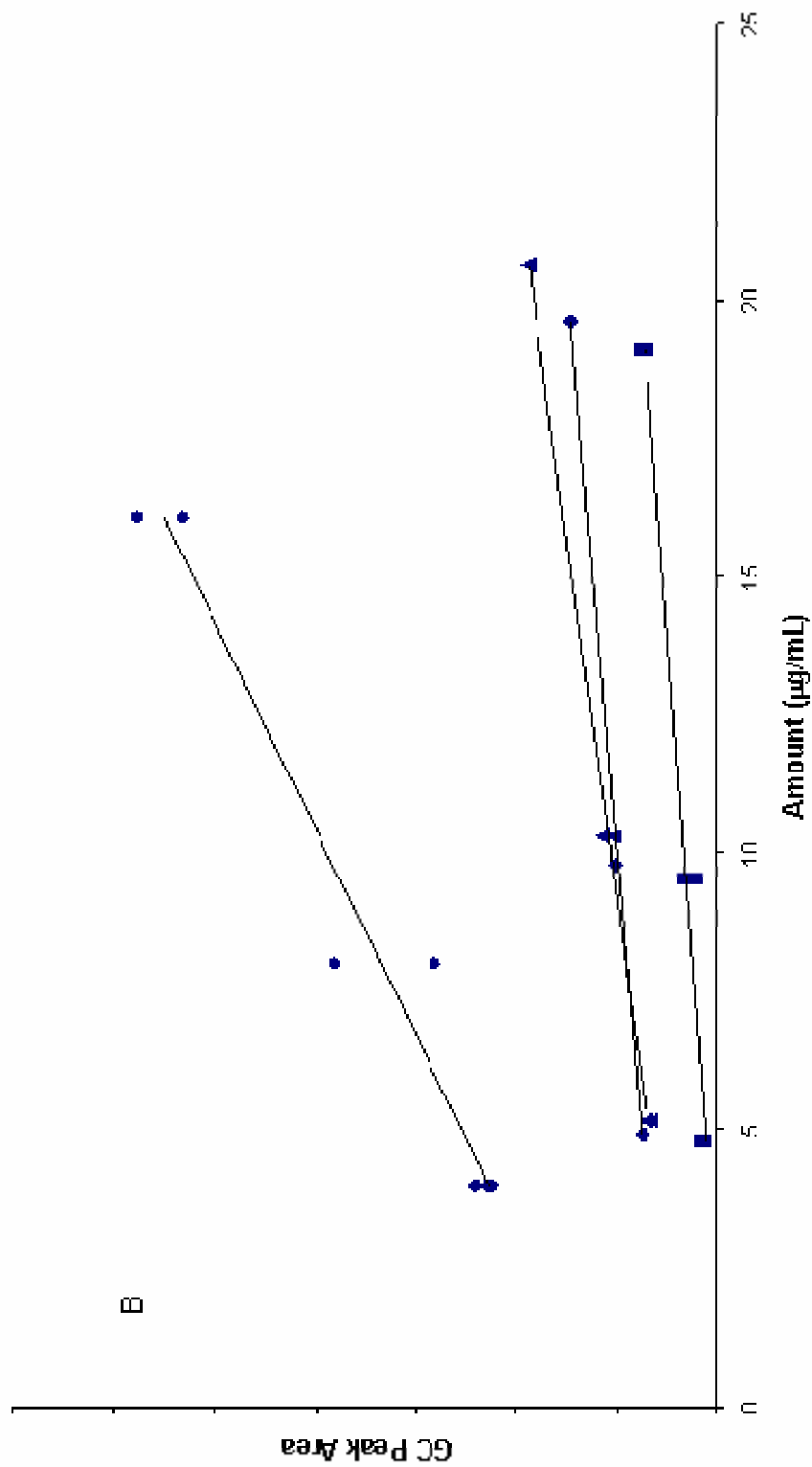
Figure 3. Linearity data of ethyl lactate (▲, R^2=0.995), diacetyl (♦, R^2=0.997), d-decalactone (■, R^2=0.951), and 2-methylbutyraldehyde (●, R^2=0.954) in sodium phosphate buffer (0.01 M, pH 9.0) extracted by HS-SPME.

Table 1. Volatile compounds identified using HS-SPME GC-FID. Calculated Kovats retention indices relative to a *n*-alkane series on a 60 m DB-WAX column.

Compound	Kovats Indices
2-Methylbutyraldehyde	913
Diacetyl	980
Ethyl lactate	1391
d-Decalactone	2278

CHAPTER FOUR

Application of solid-phase microextraction with headspace gas chromatography to the analysis of diacetyl, 2-methylbutyraldehyde, ethyl lactate, and δ-decalactone in ultra high pressure-treated β-lactoglobulin solutions

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ABSTRACT

The effects of ultra high pressure (UHP) treatment on the flavor-binding properties of β-lactoglobulin (BLG) were determined with selected flavor compounds diacetyl, ethyl lactate, 2-methylbutyraldehyde, and δ-decalactone. Following UHP treatment (600 MPa, for 0, 8, 32 min) of BLG, intrinsic tryptophan fluorescence quenching, static headspace solid-phase microextraction (HS-SPME) and GC analysis were used to study the flavor binding properties of BLG. Fluorescence quenching data indicated an increase in the fractional number of binding sites of BLG for diacetyl, δ-decalactone, and ethyl lactate with high dissociation constant values. As observed by HS-SPME GC, native BLG retained approximately 50% of the concentration of the selected flavor compounds added to BLG solutions. UHP treatments of BLG resulted in significant increases ($p < 0.005$) in the flavor retention of the selected flavor compounds, with the greatest retention for diacetyl following UHP of 32 min.

INTRODUCTION

Volatile constituents in food are contributing factors to flavor, exhibited by the observation that elimination of our sense of smell (e.g. by a common cold), results in many foods tasting bland. The food industry is aware of the importance of flavor as an essential factor in food selection and acceptance. Flavor is one of the most important attributes of a food, driving consumer acceptance.

In the process of perception, food is first assessed by appearance, and then followed by aroma, consistency and texture, and flavor evaluation, yet these attributes tend to overlap (Meilgaard and others 1999). Aroma is the odor perceived by the olfactory system when volatiles enter the nasal passage, either orthonasally or retronasally. A sufficient concentration of a flavor compound in the vapor phase (nasal) or aqueous phase (saliva) must be achieved to elicit olfactory and taste receptor responses. The quantity of volatiles released from a food is dependent upon the temperature and chemical properties of the flavor compounds. Flavor is defined as the sum of perceptions resulting from stimulation of the sensory receptors grouped together at the entrance of the alimentary and respiratory tracts (Meilgaard and others 1999), otherwise perceived in the mouth.

Food is a complex system in which equilibrium of flavors between the aqueous, gaseous, and solid phases is rarely reached during preparation or eating, but physical chemistry provides tools useful for understanding and predicting flavor behavior. Equilibrium partitioning theory can explain the interaction of flavor compounds in the gas phase and aqueous phase or their combinations. The partition coefficient (K) of flavor compounds between the gas phase and the aqueous phase in a closed system is described

by $K = C_s/C_g$, where C_s is the concentration of the flavor compound in the aqueous phase and C_g is the concentration of the flavor compound in the gas phase. The partition coefficients represent the quantity of flavor compounds in the gas phase and are useful in the quantitative determination of the availability of flavor compounds to be detected by sensory receptors.

Flavor perception in foods, which is an important determinant of food acceptance, is influenced by flavor binding and release between flavor compounds and a variety of food components, such as protein, carbohydrates, and lipids. Proteins in foods often decrease the volatility of flavor compounds. Proteins interact with volatile flavor compounds, both reversibly and irreversibly, with a decrease in flavor perception for the latter (Fabre and others 2002). In general, proteins retain volatile compounds by reversible hydrophobic adsorption or absorption, resulting in chemical affinities of various strengths. BLG is one of the best known and most studied of proteins. The whey protein is extensively characterized, exhibits good emulsification, and can interact with many flavor compounds such as aldehydes and ketones (van Ruth and Villeneuve 2002), ionones (Jung and Ebeler 2003), and hydrocarbons (Wishnia and Pinder 1966).

Analytical techniques, including exclusion chromatography, equilibrium dialysis, static headspace, fluorometry, affinity chromatography, as well as sensory evaluation, are used to detect the binding of proteins to flavor compounds (Damodaran and Kinsella 1980; Steffen and Pawliszyn 1996; Pelletier and others 1998; Guichard and Langourieux 2000; Fabre and others 2002; Apps and Tock 2005). Fluorescence methods are important tools to investigate the structure, function and reactivity of proteins and other biological molecules. Fluorescence quenching is defined as a process which decreases the

fluorescence intensity of a given substance (Lakowicz 1983), and is widely used to study

the accessibility and localization of intrinsic fluorophores of proteins and the

permeability of membranes to quenchers (Lakowicz 1983). Busti and others (2002) have

used external quenching of intrinsic protein fluorescence by acrylamide to monitor BLG

denaturation, and reported that BLG denatures with the dissociation of dimers preceding

the unfolding of monomers at both pH 6.8 and pH 2.5. Equilibrium headspace analysis of

volatile components in the headspace equilibrated over a food using solid-phase

microextraction (SPME) gives the association or binding of flavor to food constituents

(Damodaran and Kinsella 1980; Guichard and Langourieux 2000). However, collection

of headspace volatile flavors compounds using a syringe is not sensitive enough to detect

small concentrations resulting in irreproducible chromatography (Rouseff and

Cadwallader 2001).

Studies have been done to understand the effects of thermal denaturation on

binding of flavors to whey proteins (Burova and others 2003; Patel and others 2006).

Damodaran and Kinsella (1980) reported that the affinity of ketones for bovine serum

albumin increased with chain length, the binding is hydrophobic in nature, and the

thermodynamic driving force for binding is entropic. Whey protein was found to have

higher affinity to vanillin than soy or casein proteins, and binding was enthalpy driven

(Li and others 2000). There are many research studies that try to understand the structural

changes BLG undergoes during ultra high pressure treatment (UHP). Increases in the

surface hydrophobicity (Knudsen and others 2004) and aggregation (Futenberger and

others 1995) of BLG were observed when BLG was treated with UHP between 200 and

400 MPa. Yang and others (2001) reported that the native ordered state of BLG contains

an intrinsic pocket that binds 1-anilinonaphthalene-8-sulfonate (ANS). An increase in the extrinsic fluorescence of ANS after high pressure treatment (600 MPa, 50C) of BLG follows the transition from the ordered to molten globular state. Also, a decrease in near-UV circular dichroism intensity from aromatic side chains occurs during the transition of BLG from native to molten globule states (Dolgikh and others 1981). At pH 2.0, pressure-induced denaturation of BLG is reversible (Ikeuchi and others 2001). Proteins in the molten globule state usually retain the secondary structure of the native state and exhibit a compact tertiary structure, but exhibit increased mobility and looser packing of the protein chain (Ptitsyn 1995). Kolakowski and others (2001) reported that using low pressure (300 MPa) and low temperature (4 °C) minimizes the loss of native structure, decreases undesirable aggregation, and enhances the exposure of the hydrophobic regions of BLG to the solvent. Limited research has been done on the effects of ultra high pressure (UHP) treatment of BLG on binding of flavor compounds with the purpose of enhancing food flavors and perception of reduced-fat foods. Liu and others (2005) studied the effects of UHP on the flavor-binding properties of whey protein concentrate (WPC), and reported that there is not one pressure treatment for WPC that increases the number of binding sites or apparent dissociation constant of any flavor compound. A pressure treatment of 600 MPa for 0, 10, or 30 min holding time on WPC increased the number of binding sites and apparent dissociation constant for benzaldehyde, but only a 0-min hold time was required to increase the number of binding sites and apparent dissociation constant for heptanone and octanone (Liu and others 2005). Currently there is no research that focuses on pre-incubating a mixture of flavor compounds and proteins,

subjecting the mixture to UHP treatment, and then analyzing the mixture through extraction and detection procedures.

The objectives of this study were to investigate the binding properties of selected flavor compounds with UHP-treated β-lactoglobulin. Intrinsic fluorescence quenching of tryptophan and headspace SPME-GC analysis were employed in the present research.

MATERIALS & METHODS

Materials

β-Lactoglobulin (BLG) (90% pure) was purchased from Sigma Chemical Co. (St. Louis, MO, no. L-0130). BLG with the same lot number was used throughout the experiments.

Pre-Incubation

δ-Decalactone, diacetyl, ethyl lactate and 2-methylbutyraldehyde were flavor compounds selected to bind with BLG. These flavor compounds were selected because they are associated with fat-rich foods, such as dairy products (Badings and Neeter 1980; Milo and Reineccius 1997), chocolate, or cream-based foods , and also for their characteristic physical properties. Each flavor compound (in 10% aqueous ethanol) was added to BLG solutions to reach final concentrations of 1-200 ppm and held at 4 °C prior to UHP treatment.

Ultra High Pressure Treatment

BLG solutions, at a concentration of 0.35 mg/mL in sodium phosphate buffer (0.01 M, pH 7), that contained selected flavor compounds were treated with UHP of 600 MPa for holding times of 0 (come-up time), 8, or 32 min in the presence of ice to reduce effects of temperature. Temperature was measured before and after UHP treatment was

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5°C and 8°C, respectively. The come-up time (5.45 min) was the compression time required to reach a pressure of 600 MPa. After exposure to UHP, BLG solutions were studied immediately or stored at 4 °C for fewer than 10 days, otherwise the protein precipitated.

Flavor Compound Fluorescence Binding Assay

Stern-Volmer analysis

Flavor binding experiments were performed in 0.01 M sodium phosphate buffer (pH 7.0, adjusted with 0.01 N HCl or NaOH) with selected flavor concentrations. Intrinsic tryptophan fluorescence was assayed using an excitation wavelength of 287 nm and emission at a wavelength of 332 nm. The quantitative binding of ligands was determined from the decrease of the protein tryptophan fluorescence at 332 nm (Dufour and Haertle 1991). Since the flavor-BLG complex is responsible for the fluorescence quenching of tryptophan in BLG, we assume that static quenching is responsible for the decrease of BLG fluorescence upon addition of flavor compounds. Stern-Volmer analysis for static quenching predicts that the ratio F_0/F between the fluorescence of BLG in the absence of selected flavor compounds (F_0) and the fluorescence of BLG in the presence of selected flavor compounds (F) is, for small amounts of flavor compounds, a linear function of the concentration of the flavor compound (Q) (Tian and others 2006).

$$\frac{F_0}{F} = K_{SV}[Q] + 1 \tag{1}$$

The slope obtained from the linear regression provides the static quenching constant, a relevant binding parameter.

Double logarithmic analysis

Double logarithmic analysis predicts a linear relationship between

$Log\left[\dfrac{F_0 - F}{F - F_{inf}}\right]$ and $Log[Q]$, where F is the fluorescence of BLG in the presence of

selected flavor compounds, F_0 is the fluorescence of BLG in the absence of selected

flavor compounds and F_{inf} is the residual fluorescence of BLG saturated with selected

flavor compounds. The slope of the double Log plot yields the number of binding sites in

BLG and the intercept at $Log\left[\dfrac{F_0 - F}{F - F_{inf}}\right] = 0$ provides the dissociation constant (Tian and

others 2006).

Headspace Analysis

δ-Decalactone, diacetyl, ethyl lactate and 2-methylbutyraldehyde were chosen to

investigate the effects of UHP on flavor retention by BLG. Analyses were conducted in

duplicate in amber flasks (4 mL) with a flavor concentration of 5 ppm in 10% ethanol and

a BLG concentration of 0.35 mg/mL in sodium phosphate buffer (0.01 M, pH 7.0).

Analyzed solutions containing 0.65g NaCl were stirred at 400 rpm on a magnetic hot stir

plate (model PC-220, Corning, NY) for 5 min at 37 °C to pre-equilibrate volatiles in the

headspace. A polydimethylsiloxane-divinylbenzene (PDMS/DVB) solid-phase

microextraction (SPME) needle was used to adsorb volatiles in the headspace of the

flavor-BLG solution. The PDMS/DVB SPME fiber was used because the fiber can

adsorb the high molecular weight (170.25 MW) δ-decalactone flavor compound. The

SPME fiber was allowed to extract volatiles for 10 min. Volatile flavor compounds

adsorbed to the SPME fiber were thermally desorbed into the injection port of a Hewlett-

Packard HP 5890A (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph

connected to a flame ionization detector, equipped with a SPME inlet liner, and a DB-WAX column (60 m x 0.32 mm i.d., 0.5 μm film thickness, J&W Scientific, Folsom, CA). Helium was used as the carrier gas. The injector and detector temperatures were the same at 250 °C according to Adahchour and others (2005). The column oven program temperature was initially maintained at 33 °C for 5 min before increasing to 50 °C at a rate of 2 °C/min and then to 200 °C at a rate of 5 °C/min and maintained at 200 °C for 45 min.

Statistical Analysis

Data were analyzed by 2-way ANOVA followed by Tukey's pairwise comparison using SAS 9.1, and $p \leq 0.05$ was defined as significance.

RESULTS AND DISCUSSION

Quenching of BLG by Selected Flavor Compounds

UHP treatment of BLG solutions pre-incubated with selected flavor compounds at selected UHP hold times produced quenching of the BLG intrinsic fluorescence. Figures 1-3 show that for diacetyl, ethyl lactate, or 2-methylbutyraldehyde, at all UHP treatment times, the Stern-Volmer plots were linear in the 0-2 mM range. The Stern-Volmer plot for the quenching of native or UHP treated BLG by δ-decalactone was not linear suggesting that the number of replications were not adequate to justify linearity (Figure 4). The quenching constant of BLG for diacetyl increased as UHP treatment increased from come-up time to 32 min (Table 1). The quenching constants of native BLG for δ-decalactone, 2-methylbutyraldehyde, or ethyl lactate were low. Following UHP treatment of 32 min, the quenching constant showed a 500%, 33%, and 78% increase, respectively,

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indicating that the unfolding of BLG structure and exposure of the hydrophobic interior allowed for the binding of the selected compounds.

In the present study, the dissociation constants (K_d) determined from the double logarithmic plots for native BLG with diacetyl, ethyl lactate, 2-methylbutyraldehyde, and δ-decalactone were 2.1×10^{-3} M, 1.6×10^{-3} M, 0.8×10^{-3} M, and 0.1×10^{-3} M, respectively. The affinity of BLG for a flavor compound or a ligand is dependent upon the molecular structure of the flavor compound or ligand (Damodaran and Kinsella 1980). The high K_d exhibited by native BLG for diacetyl results from the high polar nature of the two carbonyl groups present. Ethyl lactate may also be excluded from binding to BLG because of a hydroxyl group and carbonyl group present. On the other hand, 2-methylbutyraldehyde and δ-decalactone have short hydrocarbon tails that may fit in the hydrophobic pockets on native BLG while the polar heads (carbonyl groups) are exposed to the solvent.

The highest dissociation constants of UHP treated BLG occurred after 32 min for δ-decalactone and 2-methylbutrladehyde (Table 1). Since Yang and others (2001) have reported that pressure and temperature of 600 MPa and 50 °C induce BLG into the molten globule state, it is possible that the increased mobility and looser packing of the protein chain and further exposure of hydrophobic sites may prevent these flavor compounds from further binding to BLG. Liu and others (2005) reported apparent dissociation constants for benzaldehyde, heptanone, octanone, and nonanone on the order of 1.8-6.2 $\times 10^{-8}$ M of whey protein concentrate for aliphatic methyl ketones. These findings indicated that BLG has smaller affinity for the selected flavor compounds in this study than aliphatic methyl ketones selected by Liu and others (2005), probably due to

the hydrophobic binding sites on interior of the proteins favoring nonpolar flavor compounds. However, small K_d values were observed after UHP come-up time for diacetyl and ethyl lactate, 1.2 and 0.9 x 10^{-3} M, respectively, suggesting that a short UHP treatment may improve the retention of certain flavor compounds.

The fractional number of binding sites determined by the slope of the double log plot shows an increase in binding sites of BLG after UHP come-up time for diacetyl, δ-decalactone, and ethyl lactate. A decrease in the fractional number of binding sites of native BLG following UHP was observed with 2-methylbutyraldehyde (Table 1). The fractional number of binding sites did not increase with increasing pressure treatment indicating that ultra high pressure does not improve binding.

Headspace Analysis

The effect of UHP on the retention of flavor by BLG was studied by HS-SPME GC analysis. Static headspace analysis determines the quantity of volatiles contained in the gas phase above a sample, usually in a sealed system at equilibrium. The composition of the headspace depends on the partitioning of volatiles between the gas phase and aqueous phase. The headspace composition may be influenced by many other factors, such as temperature, the addition of salts, headspace volume, and vial shape (Pawliszyn 1997).

The quantity of flavor compounds detected in the headspace significantly decreased ($p < 0.05$) in the presence of UHP treated BLG compared to the quantity of 2-methylbuyraldehyde (Figure 5), δ-decalactone (Figure 6), diacetyl (Figure 7), and ethyl lactate (Figure 8) in the presence of native BLG, resulting from hydrophobic and polar interactions between the flavor compounds and BLG (Guichard 2006). The retention of

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the selected flavor compounds by native BLG in decreasing order is: δ-decalactone > ethyl lactate > diacetyl > 2-methylbutyraldehyde. The retention of the selected flavor compounds after UHP-treatment of BLG in decreasing order is: diacetyl > ethyl lactate > δ-decalactone > 2-methylbutyraldehyde. The percentage of retention of 2-methylbutyraldehyde, δ-decalactone, diacetyl, and ethyl lactate were 30.6%, 40.9%, 55.8%, and 56.9%, respectively. These findings demonstrate larger retentions with a pre-incubation step than previously reported when selected flavor compounds were added to BLG already treated with UHP (Yang and others 2002; Liu and others 2005). The increase in BLG retention of selected flavor compounds was attributed to the pre-incubation period of flavor compounds with BLG prior to UHP treatment. Significant increases of 88-97% in retention were observed with UHP treatment of BLG over native BLG with diacetyl, ethyl lactate and 2-methylbutyraldehyde. Small, yet significant (p ≤ 0.05), increases in retention (56-65%) were observed for δ-decalactone, consistent with previously reported results for the retention of ketones in BLG solutions (Jouenne and Crouzet 2000). Although decreases in headspace concentration of selected flavor compounds may not be reflected in the results from fluorescence quenching experiments, flavor compounds may bind to sites on BLG far from tryptophan not detected by the fluorescence quenching experiments.

CONCLUSIONS

Fluorescence quenching data that were used to generate double logarithmic plots for the determination of dissociation constants suggested that UHP-treated BLG had low affinity for the selected flavor compounds. However, HS-SPME GC analysis exhibited a high degree of retention of BLG following UHP treatment. It may be that the

fluorescence quenching technique may not be appropriate in delineating the binding

properties of BLG to flavor compounds.

REFERENCES

Adahchour M, Wiewel J, Verdel R, Vreuls RJJ, Brinkman UAT. 2005. Improved determination of flavour compounds in butter by solid-phase (micro)extraction and comprehensive two-dimensional gas chromatography. J. Chrom. A 1086: 99-106.

Adahchour M, Wiewel J, Verdel R, Vreuls RJJ, Brinkman UAT. 2005. Improved determination of flavour compounds in butter by solid-phase (micro)extraction and comprehensive two-dimensional gas chromatography. J. Chrom. A 1086: 99-106.

Apps P, Tock MLA. 2005. Enhanced flavor extraction in continuous liquid-liquid extractors. J. Chrom. A 1083: 215-218.

Badings HT, Neeter R. 1980. Recent advances in the study of aroma compounds of milk and dairy products. Neth. Milk Dairy J. 34(1): 9-30.

Burova TV, Grinberg NV, Grinberg VY, Tolstoguzov VB. 2003. Binding of odorants to individual proteins and their mixtures. Effects of protein denaturation and association. A plasticized globule state. Colloids Surfaces. A. Physico. Eng. Aspects 213: 235-244.

Damodaran S, Kinsella JE. 1980. Flavor protein interactions. Binding of carbonyls to bovine serum albumin: thermodynamic and conformation effects. J. Agric. Food Chem. 28: 567-571.

Dolgikh DA, Gilmanshin RI, Brazhnikov EV, Bychkova VE, Semisotnov SY, Ptitsyn OB. 1981. α-Lactalbumin: compact state with fluctuating tertiary structure? FEBS Lett. 136: 311-315.

Dufour E, Haertle T. 1991. Binding of retinoids and β-carotene to β-lactoglobulin. Influence of protein modifications. Biochim. Biophys. Acta 1079: 316-320.

Fabre M, Aubry V, Guichard E. 2002. Comparison of different methods: static and dynamic headspace and solid-phase microextraction for the measurement of interactons between milk proteins and flavor compounds with an application to emulsions. J. Agric. Food Chem. 50: 1497-1501.

Futenberger S, Dumay E, Cheftel JC. 1995. Pressure-induced aggregation of β-lactoglobulin in pH 7.0 buffers. Lebensm.-Wiss.-Technol. 28: 410-418.

Guichard E. 2006. Flavour retention and release from protein solutions. Biotech. Adv. 24: 226-229.

Guichard E, Langourieux S. 2000. Interactions between β-lactoglobulin and flavor compounds. Food Chem. 71: 301-308.

Ikeuchi Y, Nakagawa K, Endo T, Suzuki A, Hayashi T, Ito T. 2001. Pressure-induced denaturation of monomer β-lactoglobulin is partially irreversible: comparison of monomer form (highly acidic pH) with dimer form (neutral pH). J. Agric. Food Chem. 49: 4052-4059.

Jouenne E, Crouzet J. 2000. Effect of pH on retention of aroma compounds by β-lactoglobulin. J. Agric. Food Chem. 48: 1273-1277.

Jung D, Ebeler SE. 2003. Investigation of binding behavior of α- and β-ionones to β-lactoglobulin at different pH values using a diffusion-based NOE pumping technique. J. Agric. Food Chem. 51: 1988-1993.

Knudsen JC, Lund M, Bauer R, Qvist KB. 2004. Interfacial and molecular properties of high-pressure-treated β-lactoglobulin B. Langmuir 20: 2409-2415.

Lakowicz JR. 1983. Principles of fluorescence spectroscopy. New York: Plenum Press. 496p.

Li Z, Grun IU, Fernando LN. 2000. Interaction of vanillin with soy and dairy proteins in aqueous model systems: a thermodynamic study. J. Food Sci. 65: 997-1001.

Liu X, Powers JR, Swanson BG, Hill HH, Clark S. 2005. Modification of whey protein concentrate hydrophobicity by high hydrostatic pressure. Innov. Food Sci. Emerg. Technol. 6(3): 310-317.

Meilgaard M, Civille GV, Carr BT. 1999. Sensory evaluation techniques. Boca Raton: CRC Press LLC.

Milo C, Reineccius GA. 1997. Identification and quantification of potential odorants in regular-fat and low-fat mild cheddar cheeses. J. Agric. Food Chem. 45(9): 3590-3594.

Patel HA, Singh H, Anema SG, Creamer LK. 2006. Effects of heat and high hydrostatic pressure treatments on disulfide bonding interchanges among the proteins in skim milk. J. Agric. Food Chem. 54: 3409-3420.

Pawliszyn J. 1997. Solid phase microextraction: theory and practice. New York: Wiley-VCH, Inc. 247p.

Pelletier E, Sostmann K, Guichard E. 1998. Measurement of interactions between b-lactoglobulin and flavor compounds (esters, acids, and pyrazines) by affinity and exclusion size chromatography. J. Agric. Food Chem. 46(4): 1506-1509.

Ptitsyn OB. 1995. Molten globule and protein folding. Adv. Prot. Chem. 47: 83-229.

Rouseff R, Cadwallader K. 2001. Headspace techniques in food, fragrances, and flavors: an overview. In: Rouseff R and Cadwallader K. Headspace analysis of foods and flavors: theory and practice. New York: Klumer Academic/Plenum Publishers. p 1-8.

Steffen A, Pawliszyn J. 1996. Analysis of flavor volatiles using headspace solid-phase microextraction. J. Agric. Food Chem. 44: 2187-2193.

Tian F, Johnson K, Lesar A, Moseley H, Ferguson J, Samuel IDW, Mazzini A, Brancaleon L. 2006. The pH-dependent conformational transition of β-lactoglobulin modulates the binding of protophyrin IX. Biochim. Biophys. Acta 1760: 38-46.

van Ruth SM, Villeneuve E. 2002. Influence of β-lactoglobulin, pH and presence of other aroma compounds on the air/liquid partition coefficients of 20 aroma compounds varying in functional group and chain length. Food Chem. 79: 157-164.

Wishnia A, Pinder TWJ. 1966. Hydrophobic interactions in proteins. The alcane binding sites of β-lactoglobulin A and B. Biochem. 5: 1534-1542.

Yang J, Powers JR, Clark S, Dunker AK, Swanson BG. 2002. Hydrophobic probe binding of β-lactoglobulin in the native and molten globule state induced by high pressure as affected by pH, KIO_3 and N-ethylmaleimide. J. Agric. Food Chem. 50: 5207-5214.

Yang J, Powers JR, Clark S, Dunker AK, Swanson BG. 2002. Hydrophobic probe binding of β-lactoglobulin in the native and molten globule state induced by high pressure as affected by pH, KIO_3 and N-ethylmaleimide. J. Agric. Food Chem. 50: 5207-5214.
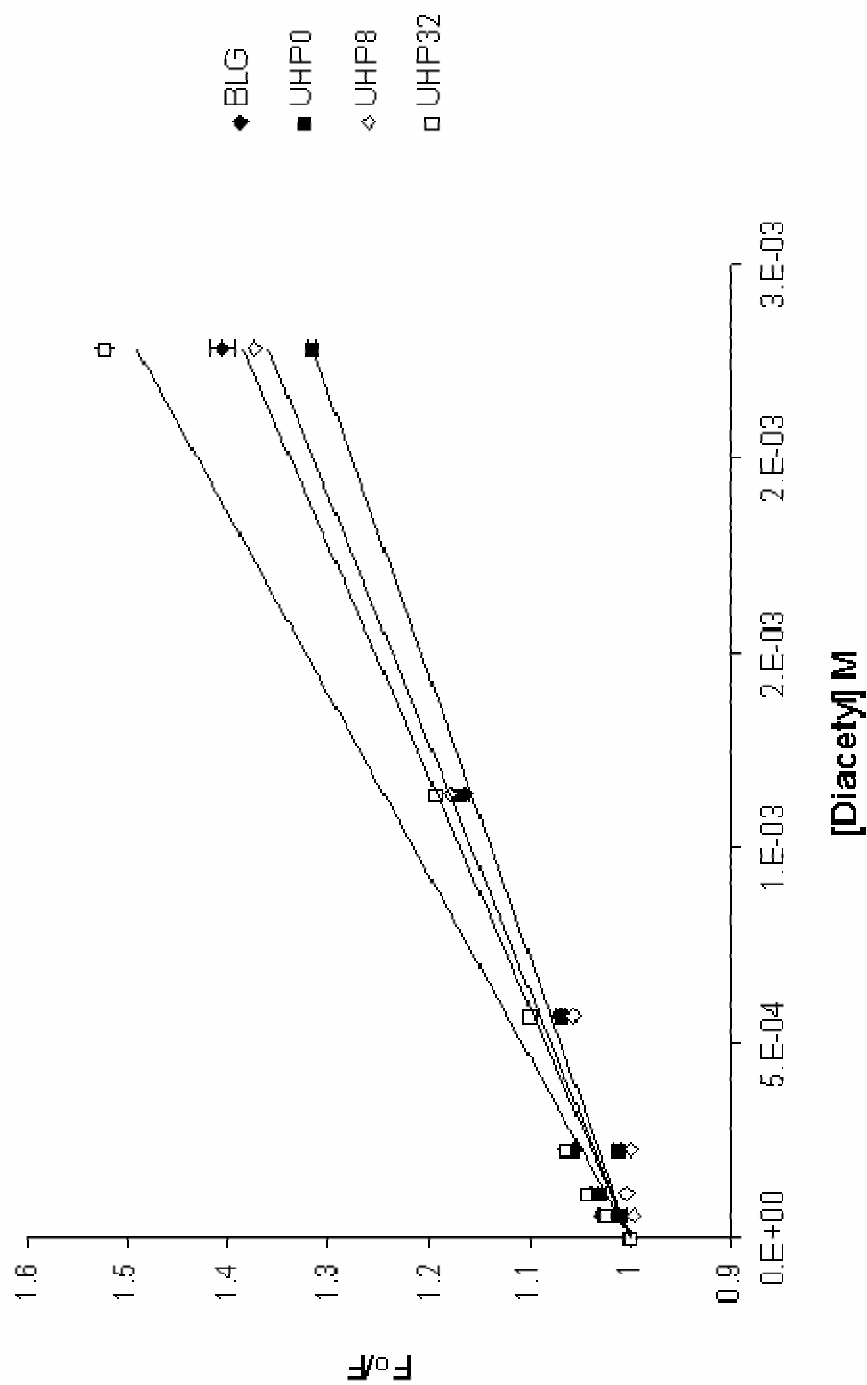
Figure 1. Stern-Volmer plots of the fluorescence quenching of native and UHP treated BLG after 0, 8, or 32 min by diacetyl. All points are the mean of three measurements.
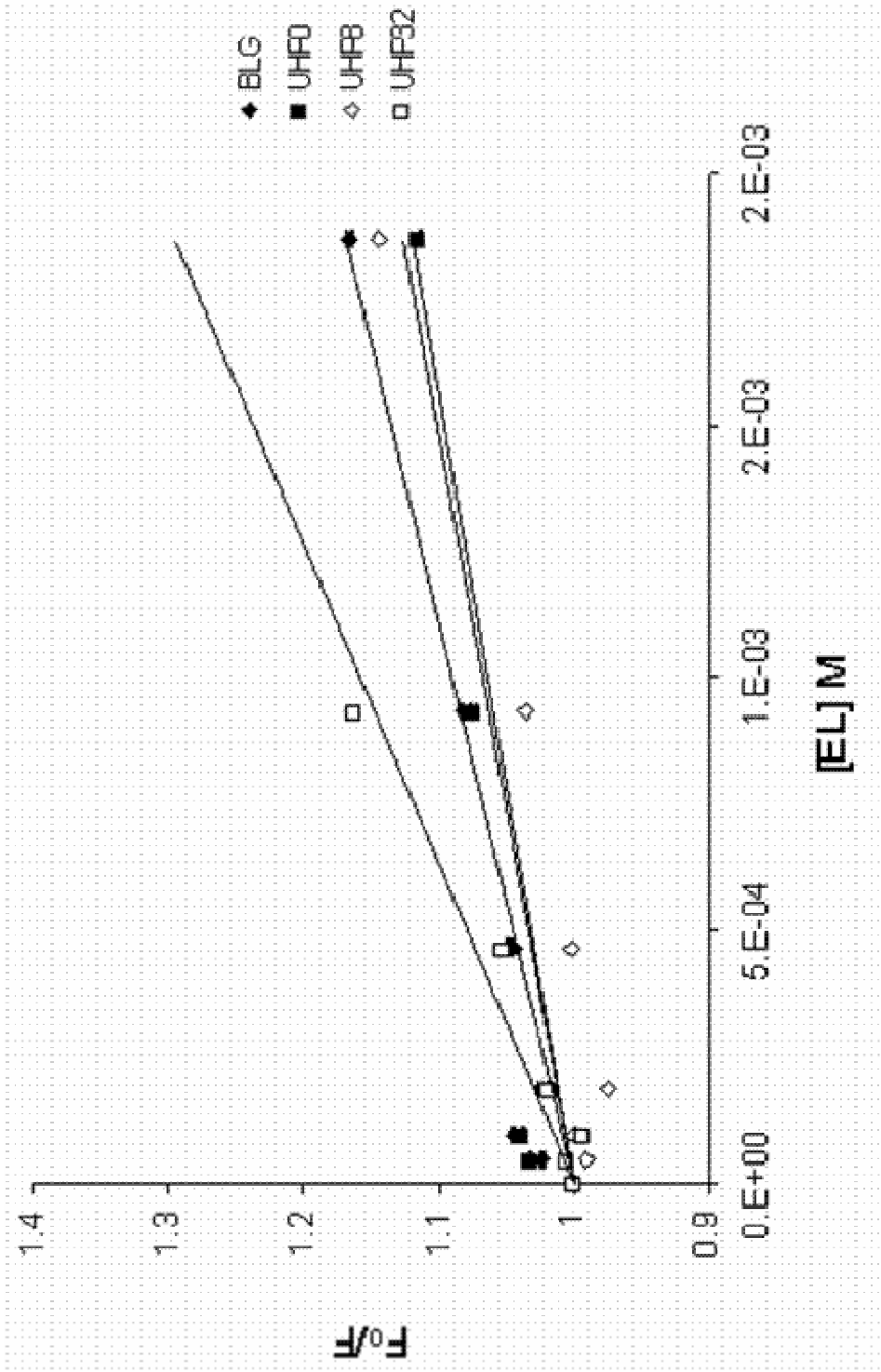
Figure 2. *Stern-Volmer* plots of the fluorescence quenching of native and UHP treated BLG after 0, 8, or 32 min by ethyl lactate. All points are the mean of three measurements.
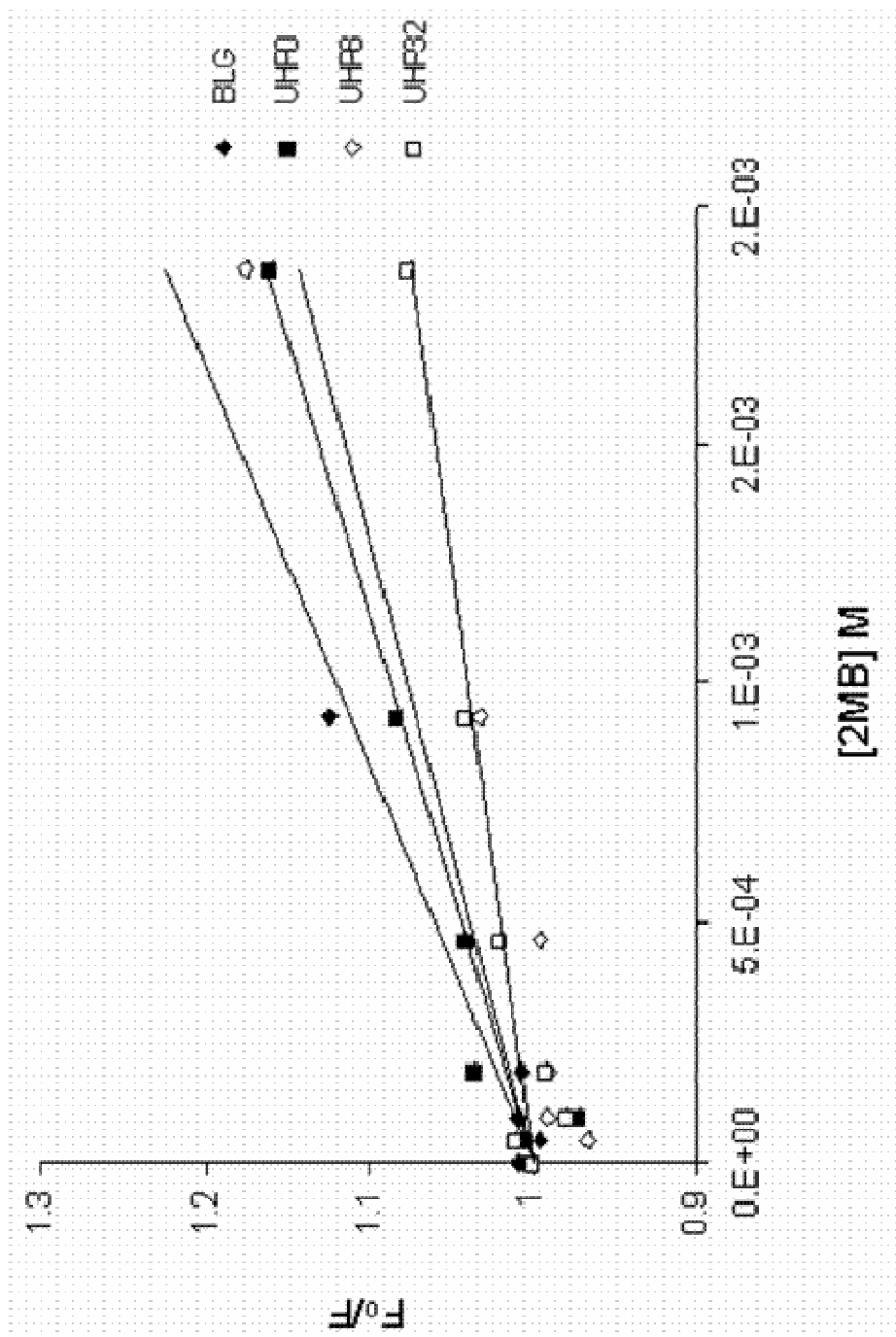
Figure 3. Stern-*Volmer* plots of the fluorescence quenching of native and UHP treated BLG after 0, 8, or 32 min by 2-methylbutyraldehyde. All points are the mean of three measurements.
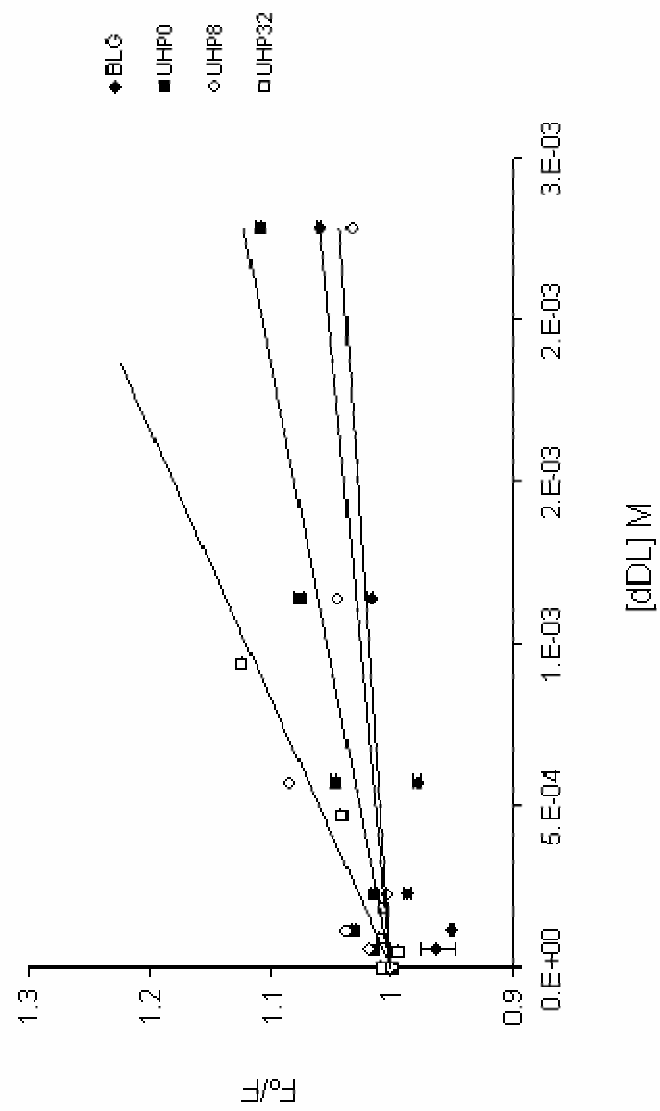
Figure 4. Stern-Volmer plots of the fluorescence quenching of native and UHP treated BLG after 0, 8, or 32 min by δ-decalactone. All points are the mean of three measurements.

Table 1. Static quenching constant (KSV), fractional number of binding sites (n), and dissociation constant (Kd) of BLG for selected flavor compounds as a function of UHP holding times (0, 8, 32 min)

	Diacetyl			δ-Decalactone			2-Methylbutyraldehyde			Ethyl Lactate		
	$K_{sv} \times 10^2$ (M-1)	n	$K_d \times 10^3$ (M)	$K_{sv} \times 10^2$ (M-1)	n	$K_d \times 10^3$ (M)	$K_{sv} \times 10^2$ (M-1)	n	$K_d \times 10^3$ (M)	$K_{sv} \times 10^2$ (M-1)	n	$K_d \times 10^3$ (M)
BLG	1.7	0.6	2.1	0.2	ND	0.1	0.9	0.8	0.8	0.9	0.5	1.6
UHP0	1.4	1.2	1.2	0.5	0.9	0.6	0.8	0.5	5.5	0.7	0.8	0.9
UHP8	1.6	0.4	3.3	0.3	ND	ND	0.4	0.4	0.8	0.6	ND	ND
UHP32	2.2	0.8	1.6	1.2	0.3	1.1	1.2	0.3	11.1	1.6	0.8	0.9

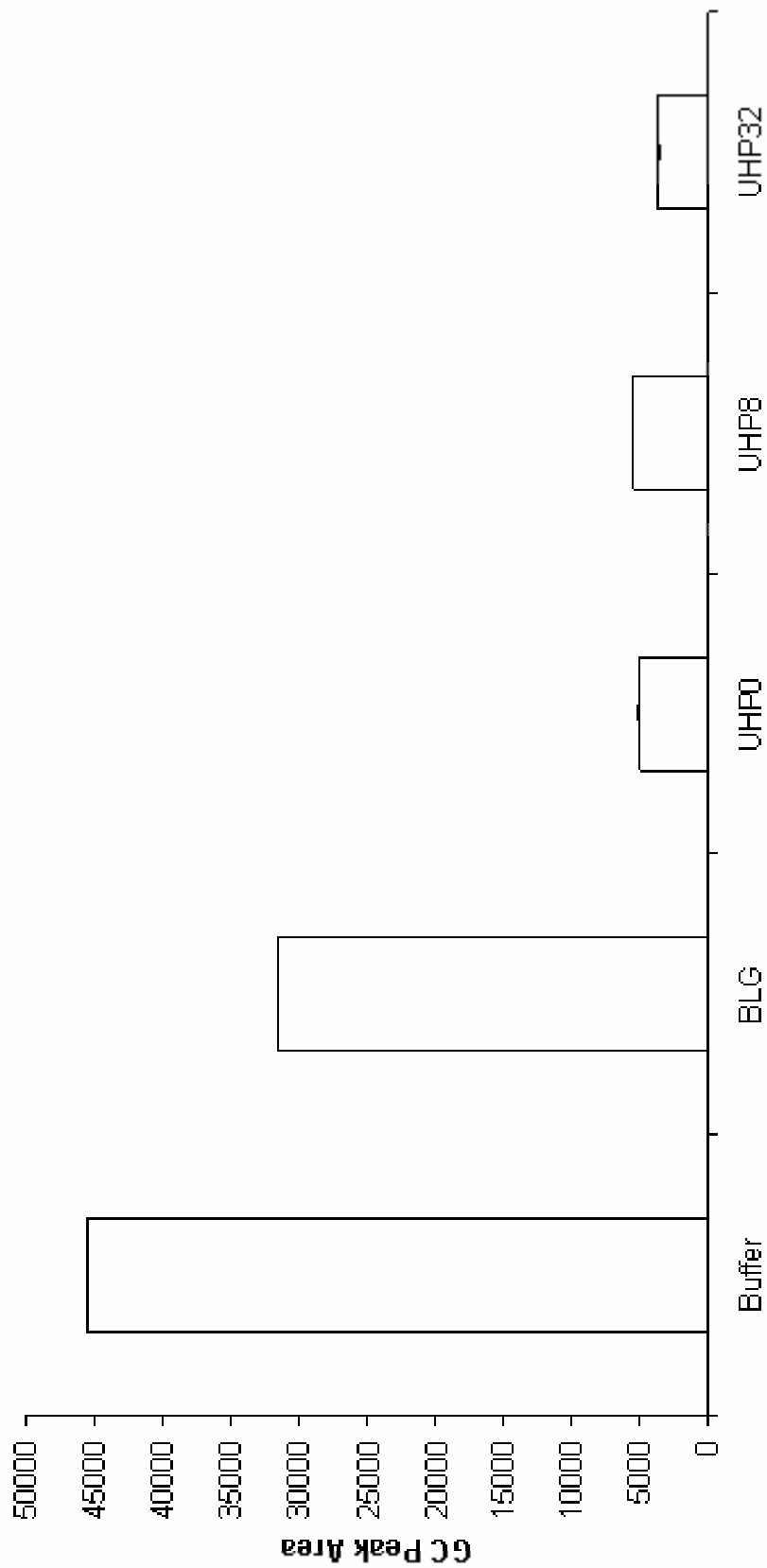ND, not detectable; K_{sv} and K_d are the means of three replicates

Figure 5. Static headspace analysis of 2-methylbutyraldehyde (5 ppm) in BLG or UHP treated BLG (600 MPa) with 0, 8, or 32 min holding time (UHP0, UHP8, and UHP32)
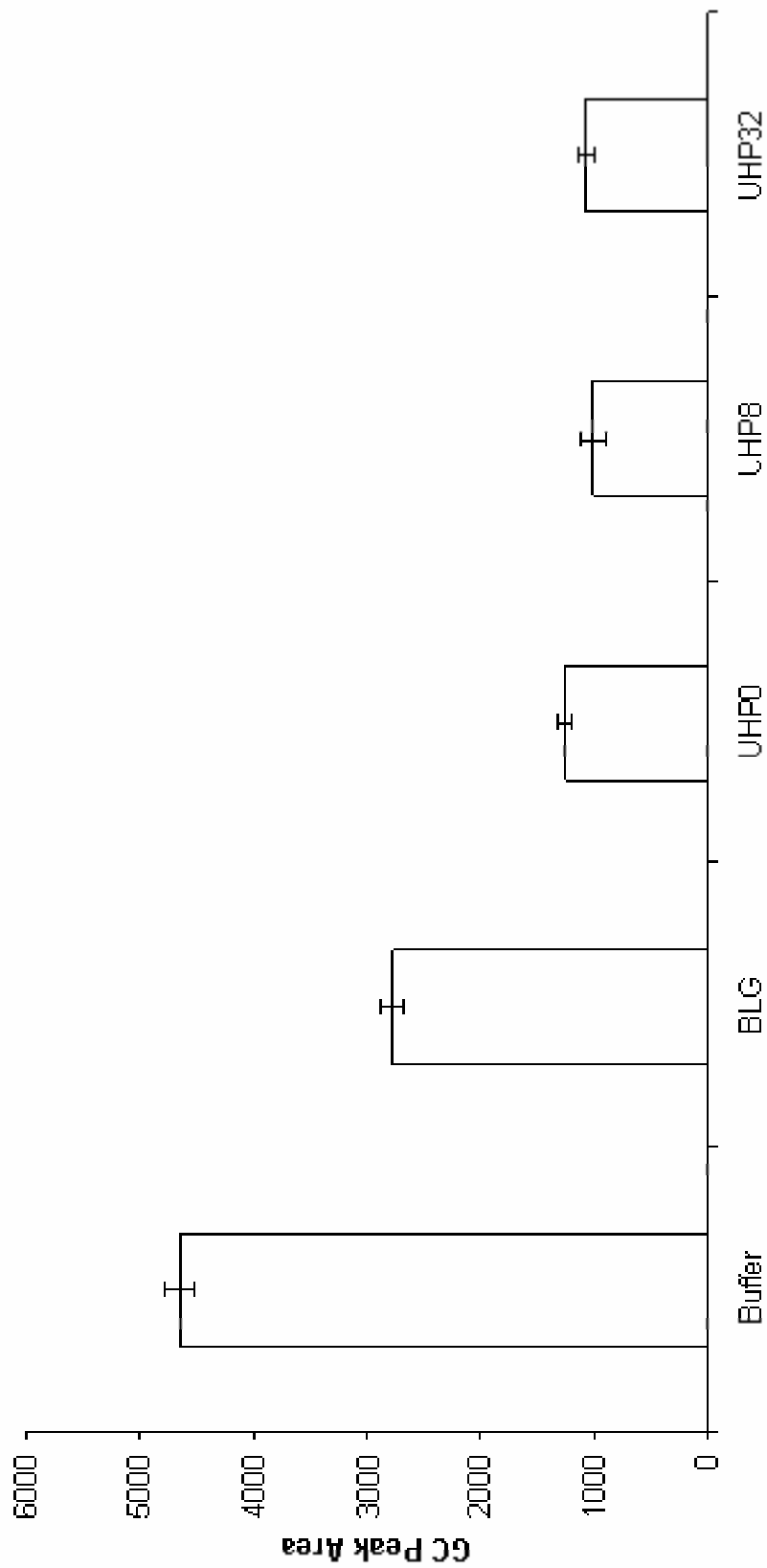
Figure 6. Static headspace analysis of δ-decalactone (5 ppm) in BLG or UHP treated BLG (600 MPa) with 0, 8, or 32 min holding time (UHP0, UHP8, and UHP32)
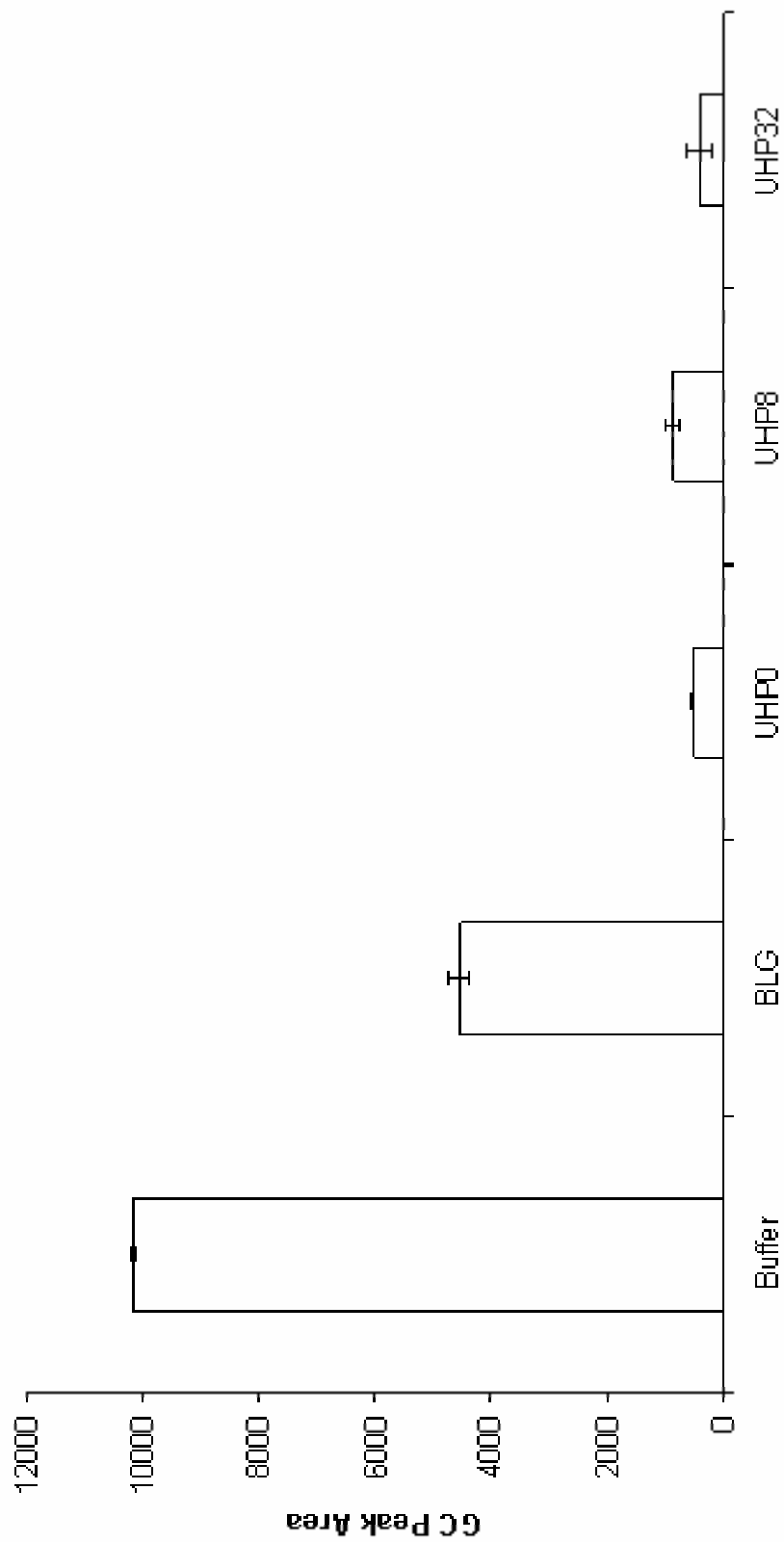
Figure 7. Static headspace analysis of diacetyl (5 ppm) in BLG or UHP treated BLG (600 MPa) with 0, 8, or 32 min holding time (UHP0, UHP8, and UHP32)
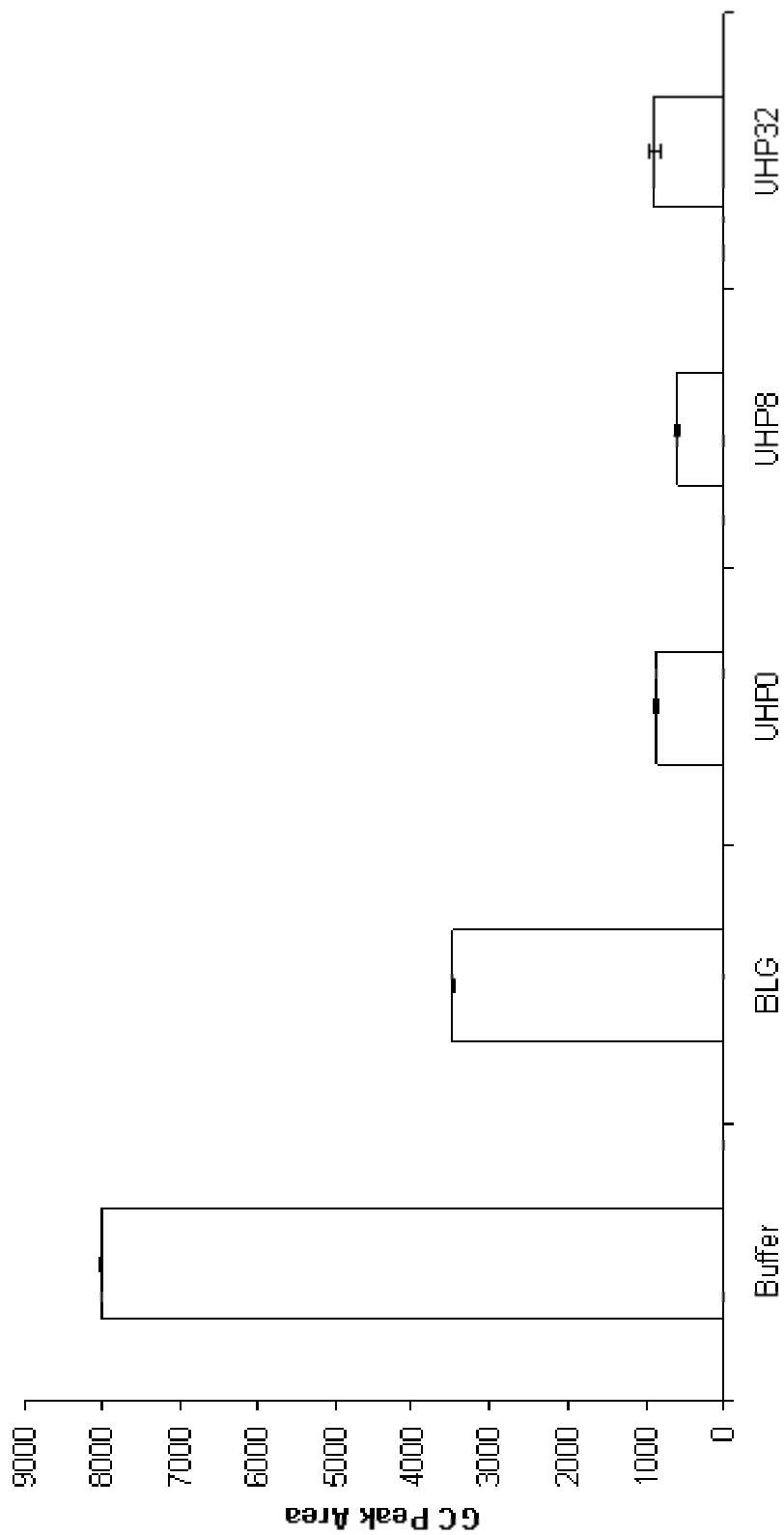
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Figure 8. Static headspace analysis of ethyl lactate (5 ppm) in BLG or UHP treated BLG (600 MPa) with 0, 8, or 32 min holding time (UHP0, UHP8, and UHP32)

CHAPTER FIVE

CONCLUSIONS

Ultra high hydrostatic pressure (UHP) treatment of BLG at 600 MPa resulted in an increase in tryptophan intrinsic fluorescence intensity at pH 7.0. A red shift in the emission wavelength occurred at pH 7.0, indicating a polarity shift from a less polar to a more polar tryptophan microenvironment and an unfolding of BLG, exposing the hydrophobic interior. These results provide evidence that UHP treatment induces BLG into the molten globule state. After come-up time at pH 9.0, BLG exhibited the greatest surface hydrophobicity, suggesting that UHP treatment increases the flexibility of the secondary structure concomitant with base-induced denaturation. Following UHP treatment (32 min) of BLG at pH 3.0, the number of binding sites for the nonpolar fluorescence probe 6-propionyl-2-(dimethylamino)-naphthalene (PRODAN) decreased from 2.26 to 1.16. These results indicate that BLG does not require long UHP treatment times to achieve more binding.

A ten minute extraction time for δ-decalactone, diacetyl, ethyl lactate, and 2-methylbutyraldehyde is adequate for headspace analysis via solid phase microextraction. The GC method described is short, requiring 1.7 h for both extraction and detection of selected flavor compounds.

UHP treatment of BLG does not increase the binding properties of BLG for diacetyl, 2-methylbutyraldehyde, ethyl lactate and δ-decalactone as observed by intrinsic fluorescence quenching, suggesting that the fluorescence technique may not be appropriate for the determination of BLG binding properties. BLG has low binding affinity for selected flavor compounds with polar groups. On the other hand, as observed

by headspace analysis, UHP treatment of BLG resulted in increases of retention of selected flavor compounds, indicating that HS-SPME is a dependable method for quantifying headspace concentrations of flavor compounds.

β-Lactoglobulin has potential as a functional ingredient in the food industry. Fat replacers are used to provide the physical and sensory properties of fat, and when BLG is included as a flavor carrier, BLG offers consumers alternative choices of eating healthy, while maintaining the full flavor experience.