Universidade do Minho
Escola de Engenharia

Nuno Miguel Sampaio Ribeiro Magalhães da Silva

Analysis of solute-water interactions by partitioning in multiple aqueous two-phase systems

outubro de 2019

Analysis of solute-water interactions by partitioning in multiple aqueous two-hase systems
Nuno Miguel Sampaio Ribeiro Magalhães da Silva
Uminho | 2019

Universidade do Minho
Escola de Engenharia

Nuno Miguel Sampaio Ribeiro Magalhães da Silva

Analysis of solute-water interactions by partitioning in multiple aqueous two-phase systems

Tese de Doutoramento
Programa Doutoral em Engenharia Química e Biológica

Trabalho efetuado sob a orientação do
Professor Doutor José António Couto Teixeira
e do
Doutor Pedro António Palma Madeira

outubro de 2019

ACKNOWLEDGEMENTS

Correndo o risco de me esquecer de alguém que, de alguma forma, foi fundamental nesta etapa, começo por agradecer a TODOS com quem me fui cruzando durante esta viagem. A vocês, o meu mais sincero OBRIGADO!

Ao meu orientador, Professor José Teixeira agradeço por todo o apoio académico e pessoal, pela disponibilidade, troca de ideias e incentivo, e acima de tudo, pela amizade e confiança que sempre depositou em mim! To Doctor Boris Zaslavsky I do not have enough words to express my most truly and profound gratitude. Besides his exceptional supervision during my collaborative work in Cleveland Diagnostics (USA), I must thank him for all the stimulating and fruitful discussions we had. Ao meu coorientador, Doutor Pedro Madeira, gostaria de agradecer pela oportunidade que me proporcionou de trabalhar consigo no grupo LSRE (FEUP-UP) e por tudo o que aprendi com ele.

À Doutora Sara Silvério (CEB-UM) e à Doutora Luísa Ferreira (CDx) agradeço pela motivação e disponibilidade que me dedicaram, mas principalmente pela sua amizade! Foram mais do que colegas de laboratório; aprendi muito com elas, pessoal e profissionalmente!

A todo o pessoal do CEB agradeço por me terem recebido tão bem desde o primeiro dia, sempre se mostraram disponíveis para me auxiliarem quando mais precisei. I would like to express my great gratitude to all Cleveland Diagnostics team, specially to Doctor Arnon Chait, Doctor Larissa Mikheeva (ex CDx) and Doctor Sasha for receiving and integrating me in the lab, for the scientific contribution and the encouragement to keep pursuing my journey in science.

À Fundação para a Ciência e Tecnologia (FCT) agradeço pela atribuição da bolsa de doutoramento (ref. SFRH/BD/111825/2015) e por todo o apoio financeiro disponibilizado.

Os meus agradecimentos mais especiais: aos meus amigos, os de sempre - JAGs, PEPC, HF - e os de agora para sempre, não há palavras suficientes para lhes agradecer. Nunca duvidaram que eu seria capaz de alcançar esta meta. Adoro-os! São a família que eu escolhi, e que bela seleção eu fui fazer! À minha família, particularmente aos meus pais. Sou muito grato pelo amor e apoio incondicional com que sempre me presentearam. E serei eternamente agradecido por tudo o que me ensinaram. Serão sempre parte essencial da minha vida! Este trabalho é dedicado a cada um de vocês.

Nuno

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

SUMÁRIO

ANÁLISE DE INTERAÇÕES SOLUTO-ÁGUA POR PARTIÇÃO

EM MÚLTIPLOS SISTEMAS DE DUAS FASES AQUOSAS

O objetivo principal do presente trabalho é contribuir para a melhor compreensão dos mecanismos envolvidos na partição de solutos em sistemas de duas fases aquosas (SDFAs). Para este trabalho, foram selecionados sistemas PEG–Na_2SO_4 e PEG–DEX e foi estudado o efeito da adição de diferentes sais aditivos (NaCl, $NaClO_4$, NaSCN e NaH_2PO_4) e/ou osmólitos (Sorbitol, Sacarose, Trealose e TMAO) nas propriedades do solvente no meio aquoso nas fases e na partição de solutos.

Os parâmetros solvatocrómicos, polaridade (π^*), capacidade do solvente em participar como dador (α) e como aceitador (β) de pontes de hidrogénio foram determinados e usados para caracterizar as fases em equilíbrio dos SDFAs. Os sistemas foram também caracterizados em termos das diferenças de hidrofobicidade relativa e das propriedades eletrostáticas das fases. Os coeficientes de partição (K) de várias biomoléculas (compostos orgânicos de baixo peso molecular iónicos e não iónicos e proteínas) foram determinados em todos os SDFAs. Verificou-se que os coeficientes de partição destes compostos podiam ser correlacionados de acordo com a equação de Collander. A partição dos solutos e as interações soluto-solvente foram descritas como uma combinação linear dos descritores específicos dos solutos, por uma relação linear de energia livre (LFER) baseada no modelo de Abraham, e previamente modificada. Os descritores específicos dos solutos obtidos em SDFAs com e sem aditivos foram comparados para verificar a existência de interações diretas aditivo-solutos ou se a presença dos aditivos aumentava a estabilidade estrutural dos solutos apenas através do seu efeito nas propriedades da água como solvente.

O uso da equação de Collander e/ou da LFER para determinar os descritores específicos de moléculas biológicas e, consequentemente, para prever o comportamento da partição de solutos em SDFAs previamente caracterizados, mostrou ser um contributo importante para um conhecimento mais profundo dos SDFAs. Os resultados apresentados contribuem para uma melhor interpretação dos efeitos de diferentes aditivos na partição de solutos. Espera-se que estes contribuam para o *design* de SDFAs mais eficientes que possam ser usados numa vasta gama de aplicações.

PALAVRAS-CHAVE: Interações soluto-água; Osmólitos e sais aditivos; Partição de solutos; Propriedades do solvente; Sistemas de Duas Fases Aquosas.

ABSTRACT

ANALYSIS OF SOLUTE-WATER INTERACTIONS BY PARTITIONING

IN MULTIPLE AQUEOUS TWO-PHASE SYSTEMS

The main purpose of the present work is to contribute for a better understanding of the mechanisms governing solute partitioning in aqueous two-phase systems (ATPSs). For this work, PEG–Na_2SO_4 and PEG–DEX systems were selected and the effect of addition of different salts additives (NaCl, $NaClO_4$, NaSCN and NaH_2PO_4) and/or osmolytes (Sorbitol, Sucrose, Trehalose and TMAO) on phases solvent properties and in solute partitioning was assessed.

The solvatochromic parameters characterizing the solvent's dipolarity/polarizability (π^*), solvent hydrogen-bond donor acidity (α), and solvent hydrogen-bond acceptor basicity (β) of aqueous media were measured in the coexisting phases of each of the systems used. Also, all the systems were characterized in terms of the difference between the relative hydrophobicity and the electrostatic properties of the phases. Partition coefficients (K-values) of several biomolecules (ionic and nonionic small organic compounds and proteins) were obtained in all the ATPSs. It was found that the partition coefficients of the compounds were correlated according to the so-called Collander equation. Solutes partitioning and solutes-solvent interactions were described by a Linear Free Energy Relationship (LFER) based on the Abraham model, which was previously modified, as a linear combination of the so-called solute specific descriptors. The solute specific descriptors values obtained in ATPSs containing different additives were compared to those obtained in ATPSs without additives to verify if there were direct additive-solutes interactions or whether the additive presence enhances the solute structure stability due to its effect on the water solvent properties only.

The use of the Collander equation and/or the LFER to determine solute specific descriptors for biological molecules and, consequently, to predict solute partitioning behavior in previously characterized ATPSs, was shown to be a valuable contribution to the understanding of ATPSs technology. The results presented not only contribute for better interpreting the effects of different additives on the solute partition in ATPSs but will also result in the design of more efficient ATPSs to be used in a wide range of applications.

KEYWORDS: Solute-water interactions; Osmolytes and salts additives; Solute partitioning; Solvent properties; Aqueous Two-Phase Systems.

TABLE OF CONTENTS

ABBREVIATIONS

A

A	Overall solute hydrogen bond acidity
ABS	Aqueous Biphasic Systems
ADME	Adsorption, Distribution, Metabolism and Excretion
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ASW	Automated Signature Workstation
ATP	Adenosine 5'-triphosphate
ATPS(s)	Aqueous Two-Phase System(s)

B

B	Overall solute hydrogen bond basicity
BBB	Blood-brain barrier

C

C	Total contribution of the non-alkyl part of the structure of a DNP-AA
CDT	Carbohydrate-deficient transferrin
CHTG	α-chymotrypsinogen A

D

DEX	Dextran
DEX75	Dextran with average molecular weight 75,000
DEX500	Dextran with average molecular weight 500,000
DI	Deionized
$d_{i,o}$	Normalized Euclidian distance between the solvent properties
DNA	Deoxyribonucleic acid
DNP-aa (or DNP-AA)	Dinitrophenylated amino acids
DNP-Ala	Dinitrophenylated-alanine
DNP-NVal	Dinitrophenylated-norvaline
DNP-NLeu	Dinitrophenylated-norleucine
DNP-AO	Dinitrophenylated-α-amino-*n*-octanoic acid

E

E	Average lnK (or logK) increment per CH_2 group
ECM	Cells-extracellular matrix
EO30PO70	Random copolymer with 30% ethylene oxide and 70% propylene oxide

F

F	Ratio of variance
FDA	Food and Drug Administration
fPSA	Free PSA in serum

G

GC	Gas Chromatography
GDP	Guanosine 5'-diphosphate
Glucoside	*p*-nitrophenyl-α-D-glucopyranoside
GMP	Guanosine 5'-monophosphate
GTP	Guanosine 5'-triphosphate

H

HbA1c	Glycated hemoglobin
HEL	Lysozyme
HPLC	High-Performance Liquid Chromatography

K

K (or K-value(s))	Partition coefficient(s)
K/NaPB	Sodium/potassium phosphate buffer

L

LFER	Linear Free Energy Relationship
logD	Lipophilicity in octanol–water systems
logK (or lnK)	Logarithmic of the partition coefficient of a certain solute
LSER	Linear Solvation Energy Relationship

M

m/z	Mass-to-charge ratio
mAbs	Monoclonal antibodies
MSA	Mean Spherical Approximation
MUM	Mycology collection of University of Minho

Mw (or Mn)	Molecular weight

N

N	Number of compounds
$n(CH_2)$	Average number of equivalent methylene groups
NaPB	Sodium phosphate buffer
N_c	Equivalent number of CH_2 groups in the aliphatic side-chain of DNP-AA

O

OPA	*o*-phthaldialdehyde reagent complete

P

PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PEG600	Polyethylene glycol with average molecular weight 600
PEG2000	Polyethylene glycol with average molecular weight 2,000
PEG4000	Polyethylene glycol with average molecular weight 4,000
PEG6000	Polyethylene glycol with average molecular weight 6,000
PEG8K	Polyethylene glycol with average molecular weight 8,000
PEG10K	Polyethylene glycol with average molecular weight 10,000
PSA	Prostate specific antigen
PSA-ACT	PSA-α-1-antichymotrypsin complex
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone

Q

QSAR	Quantitative Structure-Activity Relationship
QSPR	Quantitative Structure-Property Relationship

R

R	Universal gas constant
r^2	Correlation coefficient
R_2	"Excess molar refraction" of a solute
RNA	Ribonucleic acid
RP-HPLC	Reversed Phase HPLC

S

S	Polarity/polarizability descriptor of a solute
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate Denaturing Polyacrylamide Gel Electrophoresis
SP	Property of a solute
S_s; B_s; A_s; C_s	Solute-specific coefficients
STL	Slope of the tie-line
S-value	Selectivity

T

T	Absolute temperature (in Kelvin)
TLL	Tie-line length
TMAO	Trimethylamine *N*-oxide
tPSA	Total serum PSA

U

UB	Universal Buffer
UNIQUAC	Universal Quasichemical Model
UV (UV-VIS)	Ultraviolet (Ultraviolet-Visible)

V

V	Group-contributable solute volume descriptor

Δ

ΔG°	Standard free energy of transfer a solute
ΔG°(CH2)	Gibbs free energy of transfer a methylene group
ΔX1	Differences between component X1 in the coexisting phases
ΔX2	Differences between component X2 in the coexisting phases
$\Delta\alpha$	Differences between solvent hydrogen bond donor acidity
$\Delta\beta$	Differences between solvent hydrogen bond acceptor basicity
$\Delta\pi^*$	Differences between solvent dipolarity/polarizability

FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

TABLES

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

1.1. Relevance and motivation

Permanent and considerable advances in the modern biotechnology sector have increased the need for new biological products since the number of novel applications and uses for these materials have risen drastically. The developing and improvement of highly effective and low-cost downstream processing techniques for these products has been the major challenge for the biotechnology industry. Despite being an important field in demand, extraction-based purification techniques have developed too much slowly relatively to the upstream or production technologies. It is estimated that between 50 and 90% of the total production cost of any biological product is associated with its purification. Hence the need for new, fast, robust, effective and economical separation technologies is imperative [1].

Extraction in Aqueous Two-Phase Systems (ATPSs) is well-known to be an alternative and promising approach, as liquid-liquid extraction methodology, that can easily replace the conventional methods, such as chromatography [2]. Partition in ATPSs has emerged as an attractive separation process due to their exceptional features, they are environmentally friendly, easy to scale-up, they provide an innocuous environment for the treatment of the biologic materials and the operational costs and equipment required are not expensive. So far, ATPSs formed by two polymers mainly Polyethylene glycol (PEG)–Dextran (DEX) or DEX–Ficoll are the most widely used for the separation of biomolecules [3]–[6]. But systems composed by mixing a single polymer and a salt, such as PEG and an inorganic salt, such as sodium sulfate or phosphate, have also been successfully used for the extraction and purification of a wide range of bioproducts [1].

There are almost no restrictions regarding the nature or type of bioproduct that is aimed to be isolated or separated using ATPSs extraction. However, for the successful use of ATPSs partitioning, especially at the industrial scale, it is imperative to understand the mechanisms of solute distribution in the systems as well as the properties of the systems at the molecular level. This information is crucial for prediction of optimal extraction conditions and to design optimized strategies of extraction and purification processes [7]. However, due to the lack of knowledge regarding these topics, and

despite all their advantages, ATPSs have faced a massive skepticism by the industry. There is no company that takes on the risk of submitting an ATPS downstream process to Food and Drug Administration (FDA) approval. This fact is also due to some fears over quality control of the phase forming components, the difficulty of removing and recycling them and the need of coupling further downstream processing steps to get better purification degrees [7].

Nevertheless, ATPSs may be successfully applied in a different field of biotechnology, at the (bio)analytical level. Once these systems are sensitive to the surface properties of the solutes, they present a high potential for the surface characterization, identification and differentiation of several bioproducts, namely proteins, for detection of changes in protein molecular structure and conformation, for quantitative structure-activity analysis (QSAR), among other analytical applications such as diagnostic tools or even cell biopatterning [8]–[13]. At bioanalytical level, the widespread application seemed to be easier. But, once again, ATPSs underutilization may be due to the lack of a general theory for solute partitioning [7], [8].

Thus, even for different reasons, to understand the mechanisms behind solute partition in ATPSs is extremely important no matter their final application. Some work has been done by Zaslavsky research group regarding solute partitioning phenomenon. The work carried out by this author represents an attempt to examine, interpret and understand how solute partition occurs and how can it be affected and/or manipulated.

It has been shown by Zaslavsky that solute partitioning in polymer–polymer and polymer–salt ATPSs is affected by similar factors, among them the use of salts as additives. Also, clear similarities between partitioning of solutes in ATPSs and in water–organic solvent biphasic systems were demonstrated when the Collander equation and the Linear Solvation Energy Relationship (LSER) (empirical and semi-empirical approaches frequently used to describe solute partitioning in water–organic solvent biphasic systems) were successfully employed to correlate the partition coefficients for various biomolecules in different polymer–polymer and polymer–salt ATPSs [3], [6], [14]–[16].

The remarkable conclusions of the vast work of Zaslavsky and coworkers have been used to support and to validate his theory about solute partitioning. From Zaslavsky point of view, the unique role of the water as a solvent, which mediates interactions among solutes, should be the focus of the new studies aiming to understand solute partitioning phenomenon.

It is well recognized that interactions of any substance with aqueous environment are fundamentally important for their functions *in vivo* playing an important role in many biological processes [17]–[19]. The understanding of these solute-water interactions on a molecular level will

help to gain better insight into fundamental mechanisms of many different biological processes and also to disclose the mechanisms behind solute partitioning in ATPSs.

The solute-solvent interactions are generally described by Linear Free Energy Relationships, particularly by the Abraham model as a linear combination of the so-called solute descriptors and corresponding solvent descriptors. Currently solute descriptors for multiple compounds are determined by separate physicochemical measurements. Unfortunately, this approach is usually successful only for nonionic organic compounds, hardly applicable to ionized compounds and cannot be used for analysis of biomacromolecules [15], [20]–[23].

An alternative approach suggested by Madeira *et al.* is to characterize solvent descriptors in different solvent systems using solvatochromic measurements. This approach was already used for the effective determination of solute descriptors of nonionic and ionizable organic compounds, free amino acids and proteins. The most important distinction of this approach is that ATPSs, suitable for analysis of biological macromolecules and consequently are more adequate to simulate solvent environment *in vivo*, are used instead of common organic solvent–water biphasic systems [15], [20]–[24].

1.2. Main objectives

The mechanisms of solute partitioning in ATPSs are difficult to explore and its comprehension is among the major current challenges for the scientific community in this field.

Thus, the main goal of this thesis is to add to the knowledge on solute partition in ATPSs, by demonstrating how the presence of several additives can affect the solvent properties of the coexisting phases of different systems and consequently, the partition of solutes.

In order to successfully accomplish the general objective of this work, we (i) evaluated and compared the solvent features, as the relative hydrophobicity and the solvatochromic parameters of ATPSs composed by the combination of different polymers (PEG–DEX), and one polymer and one inorganic salt (PEG–Na_2SO_4), with and without osmolytes and salt additives; (ii) analyzed solute-water interactions by ATPS partitioning; (iii) attempted to increase the efficiency of the method by exploring the possibility to reduce the number of ATPSs necessary for solute-water interactions analysis; and

(iv) investigated and analyzed different types of solute-water interactions for a series of different solutes, including proteins.

1.3. Outline

This thesis is divided in 8 main chapters. In Chapter 2 - State of the art - a brief bibliographic review focusing on the history of ATPSs and their novel bioanalytical applications is presented. Also, the most well-known and established theories found in literature explaining two-phases formation and solute partitioning phenomena are described. The chapter ends with a subsection concerning solute-solvent interactions analysis, where their relevance is presented and briefly discussed.

Chapter 3 focuses on the partition of a homologous series of dinitrophenylated amino acids (DNP-AA) with aliphatic side chains in aqueous PEG8K–Na_2SO_4 systems with the additives NaSCN, $NaClO_4$, and NaH_2PO_4 at concentrations ranging from 0.025 M up to 0.54 M. The differences between the relative hydrophobicities and electrostatic properties of the two phases in all ATPSs were estimated. Partitioning of adenine, adenosine mono-, di- and tri-phosphates was also examined in all ATPSs, including those with NaCl additive. Partition coefficients for these compounds and for nonionic organic compounds previously reported were analyzed in terms of linear solvent regression relationship, LSER.

Chapter 4 presents the partition behavior of nine small organic compounds and six proteins in PEG8K–Na_2SO_4 containing 0.5 M of osmolyte (sorbitol, sucrose, trehalose, trimethylamine N-oxide (TMAO)) and in PEG10K–Na_2SO_4 systems, all in 0.01 M NaPB, pH 6.8. The differences between the solvent properties of the coexisting phases (solvent dipolarity/polarizability, hydrogen bond donor acidity, and hydrogen bond acceptor basicity) were characterized with solvatochromic dyes using the solvatochromic comparison method. Differences between the electrostatic properties of the phases were determined by analysis of partitioning of sodium salts of DNP-AA with aliphatic alkyl side-chain.

In Chapter 5, the partition behavior of eight small organic compounds and six proteins was examined in PEG8K–Na_2SO_4 systems containing 0.215 M NaCl and 0.5 M osmolyte (sorbitol, sucrose and TMAO) and in PEG10K–Na_2SO_4 with 0.215 M NaCl systems, all in 0.01 M sodium phosphate buffer, pH 6.8. The differences between the solvent properties of the coexisting phases (solvent dipolarity/polarizability, hydrogen bond donor acidity, and hydrogen bond acceptor basicity) were

characterized with solvatochromic dyes using the solvatochromic comparison method. Differences between the electrostatic properties of the phases were also determined by analysis of partitioning of sodium salts of DNP-AA with aliphatic alkyl side-chain. The partition coefficients of all compounds examined (including proteins) were described in terms of solute-solvent interactions.

Chapter 6 focuses on the partition behavior of adenosine and guanine mononucleotides in aqueous PEG–DEX and PEG–Na_2SO_4 two-phase systems. The partition coefficients for each series of mononucleotides were analyzed as a function of the number of phosphate groups. The data obtained were analyzed together with the results for other organic compounds and proteins previously reported and the linear interrelationship between logarithms of partition coefficients in PEG–DEX, PEG–Na_2SO_4 and PEG–Na_2SO_4-0.215 M NaCl (all in 0.01 M Na- or K/Na-phosphate buffer, pH 7.4 or 6.8) was established.

In Chapter 7, the effects of two salt additives, NaCl and $NaClO_4$, at the fixed concentrations of 0.215 M are examined on the properties of ATPSs formed by DEX and PEG. Similarly, the effects of $NaClO_4$ at 0.215 M are tested on the properties of ATPSs formed by PEG and Na_2SO_4. In each system, with a given salt additive, 0.5 M sorbitol, 0.5 M sucrose, and 0.5 M and 1.5 M TMAO were also used as additives. Moreover, the effects of these salt additives on the partitioning of 12 small organic compounds and five proteins in the above ATPSs were studied. The results obtained were compared with those previously reported for the PEG–DEX and PEG–Na_2SO_4 ATPSs without salt additives and in the presence of 0.215 M NaCl.

Finally, in Chapter 8 the major conclusions of the work are summarized as well as future considerations and prospects.

References

[1] E. Omidinia, H. Shahbaz Mohamadi, R. Dinarvand, and H.-A. Taherkhani, "Investigation of chromatography and polymer/salt aqueous two-phase processes for downstream processing development of recombinant phenylalanine dehydrogenase," *Bioprocess Biosyst. Eng.*, vol. 33, no. 3, pp. 317–329, Mar. 2010.

[2] S. A. Oelmeier, F. Dismer, and J. Hubbuch, "Application of an aqueous two-phase systems high-throughput screening method to evaluate mAb HCP separation," *Biotechnol. Bioeng.*, vol. 108, no. 1, pp. 69–81, 2011.

[3] P. P. Madeira, J. A. Teixeira, E. A. Macedo, L. M. Mikheeva, and B. Y. Zaslavsky, "'On the Collander equation': Protein partitioning in polymer/polymer aqueous two-phase systems," *J. Chromatogr. A*, vol. 1190, no. 1–2, pp. 39–43, May 2008.

[4] P. P. Madeira, J. A. Teixeira, E. A. Macedo, L. M. Mikheeva, and B. Y. Zaslavsky, "ΔG(CH2) as solvent descriptor in polymer/polymer aqueous two-phase systems," *J. Chromatogr. A*, vol. 1185, no. 1, pp. 85–92, 2008.

[5] P. P. Madeira, J. A. Teixeira, E. A. Macedo, L. M. Mikheeva, and B. Y. Zaslavsky, "Correlations between distribution coefficients of various biomolecules in different polymer/polymer aqueous two-phase systems," *Fluid Phase Equilib.*, vol. 267, no. 2, pp. 150–157, May 2008.

[6] B. Y. Zaslavsky, *Aqueous Two-Phase Partitioning: Physical Chemistry and Bioanalytical Applications*. Taylor & Francis, 1994.

[7] P. Madeira, "Protein Partitioning in Polymer/Polymer Aqueous Two-Phase Systems," Faculdade de Engenharia da Universidade do Porto, 2008.

[8] A. L. Grilo, M. Raquel Aires-Barros, and A. M. Azevedo, "Partitioning in Aqueous Two-Phase Systems: Fundamentals, Applications and Trends," *Sep. Purif. Rev.*, vol. 45, no. 1, pp. 68–80, Jan. 2016.

[9] J. V. D. Molino, D. de A. Viana Marques, A. P. Júnior, P. G. Mazzola, and M. S. V. Gatti, "Different types of aqueous two-phase systems for biomolecule and bioparticle extraction and purification," *Biotechnol. Prog.*, vol. 29, no. 6, pp. 1343–1353, Nov. 2013.

[10] B. Y. Zaslavsky, V. N. Uversky, and A. Chait, "Analytical applications of partitioning in aqueous two-phase systems: Exploring protein structural changes and protein-partner interactions in vitro and in vivo by solvent interaction analysis method.," *Biochim. Biophys. Acta*, vol. 1864, no. 5, pp. 622–644, May 2016.

[11] M. Iqbal *et al.*, "Aqueous two-phase system (ATPS): an overview and advances in its applications," *Biol. Proced. Online*, vol. 18, p. 18, Oct. 2016.

[12] A. G. Teixeira, R. Agarwal, K. R. Ko, J. Grant-Burt, B. M. Leung, and J. P. Frampton, "Emerging Biotechnology Applications of Aqueous Two-Phase Systems.," *Adv. Healthc. Mater.*, vol. 7, no. 6, p. e1701036, Mar. 2018.

[13] J. González-Valdez, K. Mayolo-Deloisa, and M. Rito-Palomares, "Novel aspects and future trends in the use of aqueous two-phase systems as a bioengineering tool," *J. Chem. Technol. Biotechnol.*, vol. 93, no. 7, pp. 1836–1844, 2018.

[14] S. C. Silvério, O. Rodriguez, J. A. Teixeira, and E. A. Macedo, "Solute partitioning in polymer–salt ATPS: The Collander equation," *Fluid Phase Equilib.*, vol. 296, no. 2, pp. 173–177, Sep. 2010.

[15] P. P. Madeira, C. A. Reis, A. E. Rodrigues, L. M. Mikheeva, and B. Y. Zaslavsky, "Solvent Properties Governing Solute Partitioning in Polymer/Polymer Aqueous Two-Phase Systems: Nonionic Compounds," *J. Phys. Chem. B*, vol. 114, no. 1, pp. 457–462, Jan. 2010.

[16] R. Collander, "The Partition of Organic Compounds Between Higher Alcohols and Water," *Acta Chem. Scand.*, vol. 5, pp. 774–780, 1951.

[17] P. Ball, "Water as a Biomolecule," *ChemPhysChem*, vol. 9, no. 18, pp. 2677–2685, 2008.

[18] P. Ball, "Biophysics: More than a bystander," *Nature*, vol. 478, no. 7370, pp. 467–468, Oct. 2011.

[19] P. Ball, "Concluding remarks: Cum grano salis," *Faraday Discuss.*, vol. 160, no. 0, pp. 405–414, 2013.

[20] P. P. Madeira *et al.*, "Solvatochromic relationship: prediction of distribution of ionic solutes in aqueous two-phase systems," *J. Chromatogr. A*, vol. 1271, no. 1, pp. 10–16, 2013.

[21] P. P. Madeira, A. Bessa, L. Alvares-Ribeiro, M. R. Aires-Barros, A. E. Rodrigues, and B. Y. Zaslavsky, "Analysis of amino acid-water interactions by partitioning in aqueous two-phase systems. l–amino acids with non-polar side-chains.," *J. Chromatogr. A*, vol. 1274, pp. 82–86, Jan. 2013.

[22] P. P. Madeira, C. A. Reis, A. E. Rodrigues, L. M. Mikheeva, A. Chait, and B. Y. Zaslavsky, "Solvent properties governing protein partitioning in polymer/polymer aqueous two-phase systems," *J. Chromatogr. A*, vol. 1218, no. 10, p. 1379—1384, 2011.

[23] P. P. Madeira *et al.*, "Study of organic compounds–water interactions by partition in aqueous two-phase systems," *J. Chromatogr. A*, vol. 1322, pp. 97–104, 2013.

[24] A. Zaslavsky, P. Madeira, L. Breydo, V. N. Uversky, A. Chait, and B. Zaslavsky, "High throughput characterization of structural differences between closely related proteins in solution," *Biochim. Biophys. Acta - Proteins Proteomics*, vol. 1834, no. 2, pp. 583–592, Feb. 2013.

2.1. Aqueous Two-Phase Systems – an overview

2.1.1. From discovery to practical applications

2.1.1.1. History of discovery and exploration of ATPSs

Aqueous Two-Phase Systems (ATPSs), also called Aqueous Biphasic Systems (ABSs), were reported for the first time in 1896 by Beijerink. He found that by combining aqueous solutions of gelatin and agar or gelatin and starch, the turbid mixtures obtained could separate and form two aqueous-based phases. Also, he concluded that the top phases of the mixtures were essentially composed by gelatin and the bottom ones enriched in agar (or starch) [1].

Later, between 1947 and 1950, Dobry and Boyer-Kawenoki also observed two-phases formation in their studies of polymers miscibility in organic solvents. These authors concluded that incompatibility between this type of compounds was a frequent phenomenon [1].

However, it was P.-A. Albertsson who is considered the pioneer of using partitioning in ATPSs as a separation process. Half a century after ATPSs discovery, in 1956, Albertsson was able to isolate chloroplasts pyrenoids by unintentionally using a mixture of a polymer (polyethylene glycol) and potassium phosphate buffer.

The potential of application of the systems was clearly demonstrated by Albertsson, who increased the popularity of the systems for separation and isolation of biological components. He also described the formation of two-phase systems by mixing aqueous solutions of a low molecular weight PEG with low molecular weight compounds such as inorganic salts (e.g. ammonium and magnesium sulfate). Moreover, he observed that in these water-based systems, top phases were polymer-rich while bottom phases were the salt-rich phases, and that different biological components were able to unevenly distribute between them [1].

2.1.1.2. Different types of ATPSs

ATPSs can spontaneously arise from the aqueous mixture of two different water-soluble compounds. Beyond some certain critical conditions, such as temperature and/or concentrations, these mixtures separate into two aqueous phases. Each one of the aqueous-based phases is enriched with one of the compounds, being clearly separated from the other one by an interfacial boundary [1]–[4].

The unique character of ATPSs is related to their high-water content, each phase can contain over 80% of water, on a molal basis, and remain immiscible [4], [5].

Traditionally, there are two types of systems, which are distinguished by their main constituents. But despite the type of the system, both phases are mainly composed by water (>70%). Each one of them is enriched in a different component, which may vary according to the type of system. Thus, the aqueous mixtures of two different hydrophilic polymers comprise the polymer–polymer systems, and mixtures of a single polymer and single low molecular compound, such as an inorganic salt, represent the polymer–salt systems [1], [6], [7].

Several polymers can be used to prepare polymer–polymer ATPSs. Due to the availability of polymers with different molecular weights, it is even possible to generate more systems with different properties.

However, the most studied pair of polymers used to obtain a polymer–polymer biphasic system is the PEG–DEX. The properties of PEG–DEX systems have been reported in literature and these systems have been used for different purposes [8]–[10]. However, the high cost of the polymers, especially when high-purity dextrans are used, is often indicated as the main limitation associated to these widely studied biphasic systems. This fact has led to the search for alternative, new and cheaper polymers to form ATPSs [11].

In the last decades, some polymers, most of them obtained from natural sources, came up as promising cheaper substitutes. Systems composed by alternative polysaccharides and their derivatives, polyelectrolytes and synthetic polymers were reported through the years [1], [12]–[22].

To obtain polymer–salt systems there is a variety of salts that can be mixed with polymers under suitable conditions. The most widely used salts are the phosphates and sulfates. Environmental issues have been associated with these salts, however, when they are released directly into natural ecosystems. And even thought new salt recycling approaches may provide promising solutions, in most cases those are not economically feasible. The biodegradable salts, such as citrate, tartrate or

oxalate, were reported as environmentally benign components to form aqueous polymer–salt systems [23], [24].

To prepare this type of ATPSs, many polymers have been used through the years, with PEG being the most used. References to PEG–salt systems and their characterization can be found in numerous studies in literature [1], [4].

As aforementioned, polymer–polymer and polymer–salt systems are the traditional types of ATPSs. Nevertheless, new types of systems have been described by using alternative components like surfactants, ionic liquids or stimuli-responsive polymers [25]–[30].

These alternative components can be used to form ATPSs just by their dissolution in water above certain temperature and concentration conditions; mixing aqueous solutions of their cationic and anionic species; or combining with aqueous solutions of traditional components (salts, polymers or carbohydrates) [27], [31]–[33].

Most of these alternative ATPSs have gained special attention due to their greener potential and easy recycling process, which reduce the associated costs and decrease their environmental impact.

2.1.1.3. Phase diagrams and ATPSs characterization

The composition of any ATPS is usually represented by a rectangular phase diagram as illustrated in Fig. 2.1. Phase diagrams are specific representations for each ATPS under given conditions (pH, temperature and/or concentrations of additives). These representations are essential to identify and quantify the two-phases concentration range and contain information about phases equilibrium. Additionally, they provide relevant information about the systems, for example, about the volume ratio of the coexisting phases in equilibrium, the concentration (or amount) of each component that is needed to prepare an ATPS, as well as the composition of top and bottom phases [1], [34].

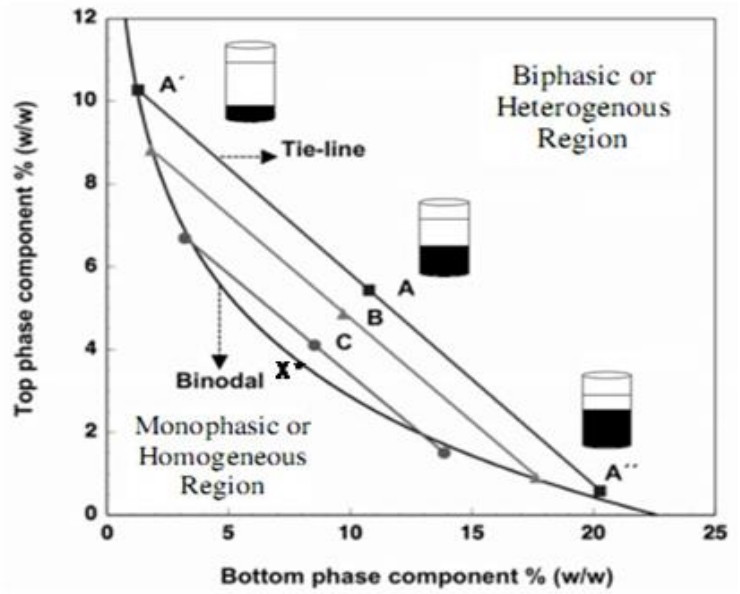


Figure 2.1. Rectangular representation of a phase diagram for top phase component : bottom phase component : water system.

Typical phase diagrams for ternary mixtures are exemplified using triangular representation, but for ATPSs simple rectangular representations are usually used, where the solvent concentration (water) is omitted. Generally, the abscises axis represents the bottom phase-rich component while the vertical axis, the ordinates, is used to represent the concentrations of component which is enriched in the top phase. The curve line on the figure is called binodal curve, and it separates the mono from the biphasic region (or the homo from the heterogenous region). Above binodal curve all the concentration points originate an ATPS, and below the curve, homogenous mixtures are obtained [1].

In this figure, for a given system the total composition is represented by A (or B or C), and that of the two phases by A' and A'' (and so on for B and C). Normally, the composition of the systems is specified in weight percentage, but any convenient units may be used.

The points A' and A'' are called nodes and the straight line connecting them is the tie-line. The tie-lines are often parallel and characterize the composition of each phase in equilibrium. With decreasing feed composition, the tie-line length decreases and eventually becomes zero at the critical point, designated by X in the figure. Along each tie-line, the total system composition and the phases volume ratio are varied, but phases compositions are the same (defined by the nodes). The critical point (X) represents the system with the same composition and volume in both phases.

Each tie-line can be characterized by two parameters frequently calculated: the tie line length (TLL) and the slope of the tie-line (STL). It was found empirically that the TLL can be used as a single numerical measure of the difference between the compositions of the two phases, for different initial compositions of the system [35]. TLL is expressed in concentration units and corresponds to the distance between the nodes (points A' and A'') and can be calculated as follows:

$$TLL = [(\Delta X1)^2 + (\Delta X2)^2]^{(1/2)}$$

(Equation 2.1)

where $\Delta X1$ and $\Delta X2$ are the differences between component X1 and X2 in the coexisting phases in equilibrium.

STL is obtained by linear regression of the top, bottom and feed compositions of the respective tie-line, and is defined as:

$$STL = \frac{\Delta X2}{\Delta X1} = \frac{X2_{Top} - X2_{Bottom}}{X1_{Top} - X1_{Bottom}}$$

(Equation 2.2)

Other systems characteristics can be determined based on information present in phase diagrams, namely phase ratio (phases weight ratio, commonly determined by the lever rule) and the phases volume ratio (calculated when the densities of the both equilibrium phases are known).

Phase diagrams can be experimentally obtained by determination of the compositions of the coexisting phases for several initial different feed compositions within the heterogeneous region of the diagrams. The determination of each component varies according to its properties, but generally the methods used are the refractive index, electrical conductivity, freeze drying, HPLC or polarimetry. The curve that connects all the tie-lines ends, or nodes, for each equilibrium composition is the binodal curve.

Alternatively, the cloud point method or turbidimetry, can be used to determine binodal curves at fixed temperatures. This method consists in dropwise addition of an aqueous solution of one of the ATPS components to an aqueous solution of another component until persistent turbidity is observed. The occurrence of turbidity corresponds to the transition from homogeneous to the two-

phase region. The cloud point can also be determined by adding small amounts of water to an ATPS of known composition, until the two phases disappear. In both cases, the binodal is the curve that connects the several cloud points obtained from different feed compositions [1], [36]. Even though cloud point method can be affected by some errors, caused by the polydispersity of the polymers and the inherent observant-associated errors once it if the visual approach is used, this method is still the most widely used for binodal curves determination.

•

There are some factors that can be manipulated to change the equilibrium compositions of the systems. These factors have been extensively studied and include the molecular weight of phase-forming polymers, the temperature and the addition of certain compounds, such as inorganic salts, osmolytes, etc. [1], [2], [4].

In general, the higher the molecular weight of a phase forming polymer, the lower the concentration required for phase separation. The chemical nature of the polymers is of paramount importance for phase separation. However, there is still a lack of sufficient experimental data preventing one from formulating general rules.

Also, generally, for polymer–polymer ATPSs the higher the temperature, the higher the concentrations of phase-forming polymers required to achieve phase separation. The inverse is true regarding temperature effect on polymer-salt ATPSs formation.

The effect of the presence of different additives varies according to the type of the compound added to the system. It is known that the addition of water-structure-breaking salts generally elevates the binodal of an aqueous two-polymer biphasic system, meaning that the threshold amounts of the polymers required for two-phases separation increase. The opposite effect is observed when water-structure-making salts are added to polymer–polymer ATPSs [4].

It is important to mention that the above general rules are applicable only to systems formed by non-ionic polymers.

2.1.1.4. ATPSs features and conventional applications

Since its discovery, many systems have been described and their physicochemical properties characterized. Thus, the diversity of systems already known, offering different features, has been used for various purposes.

Extraction in ATPSs, for example, has been clearly demonstrated as a powerful and efficient approach for the separation, recovery and purification of biological products [1]–[3], [37], [38].

Besides the inherent advantages of each type of system, ATPSs present other general characteristics over the conventional separation techniques that make them a very attractive technique. Low cost and operational times, easy operational mode, high capacity, rapid mass transfer due to low interfacial tensions, possibility of continuous operation and easy scale-up, are clear advantages of this technology. But even more important, their biocompatibility, environmental sustainability and the possibility of process integration increase the potential of applicability of the systems.

Example of successful utilization of ATPSs in the recovery of various biological products, such as cells, viruses, organelles, nucleic acids, proteins, enzymes, and other compounds were reported in the literature [1], [4], [23].

Other applications of ATPSs have been described also. Within the (bio)remediation field, ATPSs have been used for the removal of a variety of environmental pollutants and hazardous substances. Efficient removal of textile dyes from industrial textile effluents and benzenes from wastewaters using ATPSs was reported [39], [40]. Extraction of sulfonamides from waste water samples and heavy metals from electrical wastes was demonstrated [41], [42].

More recently, due to the increasing importance of pharmaceutical wastes valorization, drugs and pharmaceuticals were the target of separation and purification by ATPSs extraction, with antibiotics standing out as the compounds attracting the most attention. Besides antibiotics, non-steroidal anti-inflammatory drugs, analgesics, vasodilators, antidepressants, fibrates, hypnotics, anticonvulsants, immunosuppressants and enantiomers with pharmacological activity are among the drugs studied [43]–[47].

The integration of the upstream processes with the downstream recovery methods, particularly for industrial practices, represents the possibility of having more efficient bioprocesses. Due to their specific characteristics, ATPSs allow the combination of two or more operational steps into one. From

this process integration or process intensification point of view, some strategies based on ATPSs were developed, such as extractive fermentation and extractive conversion [34], [48]–[50].

Even though ATPSs present extraordinary advantages, their selectivity for some targets is low. Therefore, a new approach called affinity partitioning was developed as an attempt to increase ATPSs selectivity. In practice, this methodology is based on the addition of specific affinity ligands attached to phase forming polymer, which can recognize the targets [48], [49].

The intensive work developed around the upstream processes, and its consequent improvement, over the last 20 years have challenged the downstream processing sector. The development and upgrading of alternative harvesting, separation and purification techniques such as ATPS extraction increased. Though, even ATPS extraction-based processes were shown to yield competitive results in comparison with the standard procedure like chromatography, there is no driving force to make companies move into the "unknown". In general, ATPSs are not as good as the chromatographic methods inasmuch selectivity is concerned. Even with the current development of affinity ligands, allowing the implementation of ATPS affinity partitioning approaches, that can possibly allow to achieve the same performance of a chromatographic separation and the same operational costs, the industry will rather play safe and keep using chromatography. A flagrant example is the case of the use of protein-A affinity chromatography, which will hardly be replaced by another technique, to purify therapeutic proteins [51].

Thus in the near future of downstream processing, ATPSs might be applied only in preliminary steps, like cell debris clearance, and not in the more refined stages [51].

2.1.2. Bioanalytical ATPS-based applications

ATPSs can make the difference, however, being used in bioanalytical applications. In this promising field, ATPS partitioning may be used as a tool due to its unique analytical potential. As an analytical technique, its applications include, among others, label-free cell analysis; proteomic analysis, ADME (absorption, distribution, metabolism and excretion) studies and quantitative structure-property relationship (QSPR) analysis, diagnosis, cell biopatterning and microtissue engineering, and synthetic biology. Several examples providing experimental data, proof-of-concept

demonstrations and even clinical trials results have been reviewed in the literature in the last past years [51]–[56]. Some of the most relevant examples of each field are presented below.

It is important to mention that, in this wide field of applications, ATPSs have been integrated and/or coupled with some devices such as column contactors and microfluidics devices. Likewise, they can also be combined with other separation techniques, as in Aqueous Two-Phase Flotation (combination with solvent sublation), Centrifugal Partition Chromatography, or in 3D electrophoresis (combined with two-dimensional electrophoresis or dielectrophoresis), and others [54].

2.1.2.1. Label-free cell analysis

The cells surface properties characterization can be extremely important for the differentiation of cell populations, e.g. normal and cancerous cells. Cell differentiation approaches based on ATPSs, i.e., label-free methodologies, allow to reduce the process costs, work under the regulatory guidelines and to be used for a wide range of cells lacking cell-specific surface markers.

SooHoo and Walker reported for the first time, in 2009, a combined microfluidic separation device coupled with an ATPS applied to blood sample analysis. They attempted to concentrate leukocytes from whole blood samples using a laminar flow microfluidic device with ATPS. Three different patterns of three polymer streams, immiscible PEG–PEG–DEX, immiscible PEG–DEX–DEX, and miscible PEG–Buffer (PBS)–DEX, were evaluated in terms of leukocytes-erythrocytes ratio [57].

Nagel and coworkers (2013) developed a high throughput screening technology for label-free cell separation in polymer–polymer biphasic systems. The authors found the most suitable system for separate differentiated from undifferentiated HL-60 cell lines, looking at different types and concentrations of polymers and salts, pH and other separation conditions [58].

Zimmermann *et al.* (2016), described an automated robotic screening method enabling the high-throughput cell partitioning analysis in PEG8K–DEX500 ATPSs buffered with isotonic concentrations of sodium phosphate buffer and sodium chloride. In this work, the separation conditions for the differentiable promyelocytic cell line HL-60 were optimized and a counter-current distribution-model was used to investigate optimal separation conditions for a multi-stage purification process of CD11b-positive and CD11b-negative HL-60 cells [59].

In another work, Zimmermann *et al.* (2018), demonstrated how polymer molecular weight and tie-line length affected the partition of five model cell lines (from different species and tissue type) in

PEG–DEX systems. Also, the authors reported that the separability of each cell line from the mixture was highly dependent on the both factors studied [60].

In a recent study, Hazra and coworkers (2019) reported a novel label-free passive microfluidic technique for isolation of cancer cells from peripheral blood mononuclear cells in PEG–DEX ATPSs. Isolation of two different types of cancer cells was shown to be efficiently achieved with this method [61].

Worthy of mention, is the fact that there are more studies regarding cell differentiation by aqueous two-phases partitioning, but not representing label-free approaches. In these works, different types of labels, affinity ligands, or biorecognition molecules are used in order to promote the steering of the target cells to a desire phase of the system. In these cases, partition is mainly dependent of the properties of the labels and their binding to the cell, and thus it cannot be explained by the general theories of solute partitioning. For this reason, these cases are not presented and discussed here.

2.1.2.2. Proteomic analysis

Regarding their application in proteomics, ATPSs partitioning has been included in some studies of characterization of protein surface properties, assessment of changes in protein structure and conformation, and ligand binding evaluation. The main outcome of biomedical proteomic analysis is the discovery of new drug targets and biomarkers, which could finally be used in clinical and medical practices.

Particularly in this field of application, the differences between the physicochemical and/or solvent properties of the phases are used to discriminate between similar biomolecules. The analytical information provided by partitioning (and expressed by the partition coefficients) is completely related to the solute-solvent interactions. These interactions are known to be highly related to the structure of the solute, so its partition coefficient can be used as an indicator of its characteristics.

Berggren *et al.*, in 2002, studied the influence on partitioning of the surface exposed amino acid residues of eight monomeric proteins. The authors partitioned the proteins in two polymers DEX–EO30PO70 (random copolymer of ethylene oxide and propylene oxide) systems, only differing in polymers concentrations. The authors were able to describe the partitioning behavior of the proteins by the differences in surface exposed amino acid residues (partition behavior depends on the solvent

exposed amino acids residues properties such as the presence of aromatic groups, length of the aliphatic chains or the presence of charge) [62].

Lee and Forciniti (2013) have used ATPSs to analyze the effect of glycosylation on the partition of monoclonal antibodies (mAbs). They concluded that the partitioning of these proteins was governed by the presence (or absence) of polysaccharide chains, in certain ATPSs [63].

A new method for the evaluation of the ratio of two structurally different proteins (transferrin and carbohydrate-deficient transferrin) and of other low molecular weight compounds in mixtures, without any preseparation step was described by Zaslavsky and coworkers [64].

Jensen *et al.*, made use of hydrophobic affinity partitioning to create a method for studying various conformational states of the human α-macroglobulins (α2-macroglobulin and pregnancy zone protein). In this study partition coefficient was shown to be a measure of the extent of changes in the conformation of the proteins. It was concluded that the difference of the partition coefficients between a modified and an unmodified conformational state of a specific protein could be useful as a parameter to describe the apparent conformational changes in the proteins [65].

In a study involving the analysis of plasma samples from patients with posttraumatic stress disorder, and similar samples from a control group (healthy individuals), by ATPS partitioning it was verified a difference in the overall partition of total plasma proteins between the two groups [66].

The use of two aqueous phases partitioning was used as well to study the effect of the presence of a variety of ligands (Fe^{3+}, Cu^{2+}, Al^{3+}, Bi^{3+} and Ca^{2+}) on apotransferrin aqueous solutions. After partition of each protein-ligand solution in a series of different ATPSs, partition coefficients were determined and the results showed a range of partition coefficients for the binding of different ligands, reflecting the changes on apotransferrin conformational state as result of the binding of each ligand [67].

2.1.2.3. ADME studies and QSPR analysis

Comparison of several studies enrolled in the analysis of partition of diverse subsets of compounds in the traditional octanol–buffer biphasic system and in ATPSs, namely in PEG–DEX systems, has demonstrated the clear advantage of the aqueous systems for the hydrophobicity measurements. When a pH dependence of the partition coefficients in ATPSs was observed, it exposed the biggest limitation of the octanol-buffer biphasic system, commonly used in studies involving distribution and/or transport of chemical compounds in biological systems. Subsequently,

the suitability of ATPSs for quantitative structure-activity relationship (QSAR) analysis has been also proved by combining data from ATPS partitioning and information from the traditional methods (lipophilicity or logD) [68], [69].

Aqueous PEG–DEX two-phase systems with pH ranging from 2.0 up to 12.5 were used to evaluate partitioning of 15 β-blockers and structurally related compounds and estimate their relative hydrophobicity [70]. In a similarly study, PEG–DEX two-phase systems containing 0.15 M NaCl in 0.01 M sodium phosphate buffer pH 7.4 were chosen to partition of 153 peptides (di- to hexa-chains) [71]. In both cases, the method shows that the estimated relative hydrophobicity parameter (expressed in terms of equivalent numbers of methylene units) can be calculated and used as a structural descriptor in QSAR analysis [70], [71].

In 2003, Gulyaeva *et al.*, proposed a simple model to predict blood-brain barrier (BBB) distribution based on the combination of the estimated relative hydrophobicity of compounds, determined by PEG–DEX systems partitioning, and lipophilicity (calculated by gradient RP-HPLC or by octanol-buffer shake-flask techniques). The model was created using the experimental data obtained by analysis of a set of 63 compounds with known permeability through the BBB, and allows to differentiate compounds capable of crossing the barrier from those that cannot [72].

More recently, during his amino acids partitioning studies, Madeira *et al.* (2013), demonstrated that the use of solute-specific coefficients, which can be determined by a thermodynamic model that relates solute partition coefficients with phases solvent features, can be used as the solute descriptors in QSAR analysis [73].

2.1.2.4. Diagnostic tests

ATPSs were already used for diagnosis with studies showing that patients with different prognostic tumors exhibit distinct histograms (patterns or profiles) of total plasma proteins or single-specific biomarkers partition coefficients. Using partition coefficients values is possible to differentiate healthy from patient subjects [53], [74].

Recently, in 2015, Shin *et al.*, demonstrated the potential of a PEG–DEX ATPS to isolate and concentrate exosomes and microvesicles (a novel class of biomarkers for blood-based diagnostics) from mixtures of extracellular vesicles and proteins. The robustness of the system to isolate the

vesicles was proved by the coherency of the partitioning results using different samples sources (mouse tumor interstitial plasma and artificial vesicle-proteins mixture) [75].

Nevertheless, Zaslavsky research group is the one who has given the greatest input in this field [53], [74]. Using a DEX-Ficoll system, protein partition behavior of plasma samples from patients with ovarian cancer stage I and healthy donors (without any preliminary fractionation) was assessed. An "apparent partition coefficient" was obtained by dividing the correspondent top and bottom phases mass spectra. By correlating the "apparent partition coefficients" with the m/z values of the samples they obtained a pattern plot with several ranges matching with the health status of the patients [76].

In another work, the examination of sera pools and plasma from patients with diverse types of cancer (including patients with the corresponding benign condition) and healthy donors using multiplexed immunoassays for different antigens was performed as an attempt of identify new biomarkers. Partitioning of more than 120 different antigens in a set of 24 different ATPSs was evaluated and even though in many cases, the partition coefficients were not distinguishable between donors' conditions, promising results were obtained in ovarian cancer stage I antigens screening [66].

In a comparative study with the conventional diabetes biomarkers measuring methods, Zaslavsky *et al.*, tried to use ATPS partitioning to determine the ratio of glycated hemoglobin, HbA1c. Using a set of different ATPSs (DEX–PVP, PEG–DEX and PEG–NaPB systems) the ratio of glycosylated hemoglobin-total hemoglobin was estimated. Two hemoglobin mixtures were used as samples, each one containing different amounts of glycosylated and total hemoglobin, each one representing the "normal" and the "diabetes" status samples. In both mixtures, the glycosylated hemoglobin represented a mix of various isoforms of the protein with different glycosylation degrees. Once the ratios were determined, it was observed that ATPS partitioning allowed to detect small changes in the chemical structure of hemoglobin [77].

Aiming to overcome the limitation of the standard clinical test for alcohol abuse (pre-separation of carbohydrate-deficient transferrin, CDT, i.e., combination of transferrin isoforms with reduced content of sialic residues, from transferrin in plasma) ATPS partitioning was used. Based on previous studies, showing that overall partition coefficient of artificial mixtures of transferrin and CDT changes with the content of CDT in the mixtures, an ATPS was selected to be utilized to determine the overall partition coefficients of total transferrin in plasma from alcohol abuse patients, non-drinking and social drinking individuals. Total transferrin in plasma partition coefficients were found to be

significantly different from those determined for individual transferrin and CDT. Plus, different ranges of total transferrin K-values were found for plasma samples of non-drinking and heavily drinking individuals (in these last ones, the range of K-values depends on the CDT content in the sample) [64].

Zaslavsky and coworkers focused on finding an alternative to improve the most used method to diagnose prostate cancer; not considering significant labor and time savings. Initially, they screened a set of ATPSs seeking for variations in the partition behavior of prostate specific antigen (PSA) in urine from patients with cancer and patients with benign prostate disorders. This antigen was found to be differently glycosylated in men with prostate cancer and other benign prostate conditions. An ATPS presenting significant differences between partition coefficients was selected and this alternative diagnostic test presented a performance higher than the standard tests of total serum PSA (tPSA) level or percent of free PSA (fPSA) in serum. This new method was already evaluated in a clinical trial with 222 patients [78]. Although, due to some issues concerning storage of the urine samples, there was a need to update this methodology making it suitable to be applied to serum samples. So, a fine-tuned ATPS is being used already in the commercial IsoPSA™ test, which gives the patient diagnosis based on the partition coefficient of one PSA isoform occurring in human serum/plasma (PSA-α-1-antichymotrypsin complex (PSA-ACT)) [79], [80].

2.1.2.5. Cell biopatterning and microtissue engineering

Advances in tissue engineering and the development of new restoring strategies for damaged tissues and/or organs have increased the need for a better understanding of interactions such as homo- and heterotypic cell-cell, cells-extracellular matrix (ECM) and cells-environment. Nowadays, there are a variety of techniques offering the possibility to explore and analyze these intricate interactions in greater depth. But recently, ATPSs-based cell patterning methodologies were reported and have shown to be suitable to perform noncontact patterning of cells.

Analyses of cell growth and differentiation have been performed by ATPSs-based biopatterning techniques. Generally, these analyses take advantage of the optimal range of interfacial tensions in the interface between the two phases in ATPSs formed by two nonionic polymers, such as DEX and PEG. Briefly, small (nano or micro) droplets of DEX solutions previously inoculated with cells are dispensed onto a substrate which is coated with a PEG solution. Another procedure consists in

depositing a cell-laden PEG solution on top of patterned cell-free dextran droplets, to create cell exclusion zones. Coating the substrate with PEG avoids the pretreatment of the substrate and allows to precisely deposit the cell directly on it. Plus, these approaches have the advantage of allowing the easy removal of the polymer just by washing with fresh culture medium [1], [55].

Biopatterning of keratinocytes in colonies to enhance cell-cell contact and compare cell viability with a nonpatterned cell group was reported by Agarwal *et al.* aiming to contribute to future applications in wound healing and skin tissue regeneration [81]. The delivery of genetic material, enzymes and buoyant particles (microbubbles) used for sonoporation of cell monolayers surfaces was also patterned by means of these novel approaches. Evaluation of cell migration, cell proliferation rates and wound capacity has been made throughout cell exclusion zones by patterning studies [82], [83].

It is also possible to create coculture systems with complex heterocellular interactions. Frampton and coworkers created hepatocytes and fibroblasts cocultures to study the function of liver cells [84]. In another study, cocultures of MDA MB 231 breast cancer cells, confined in DEX droplets, and HEK 293 cells, inoculated in PEG, were used to inspect a cancer metastasis mediated-signaling pathway and evaluate cell proliferation and migration [85].

Likewise, in mammalian cells cocultures, some work has been done with microbial cells aiming to get a better insight about microbial colonization and study biofilm formation and quorum sensing phenomena. In 2012, Yaguchi *et al.* used PEG–DEX systems to pattern adjacent colonies of multiple bacterial species without intermixing [86]. In a work regarding antibiotic resistance transfer between bacterial colonies, patterned cultures of different strains of *Echerichia coli* (ampicillin-sensitive and -resistant strains) were created to show the commensal benefits provided by one species to another in complex multispecies communities [86], [87]. Dwidar *et al.* (2013) used suspensions of invasive bacterial strains confined in DEX and cocultured them with PEG coated mammalian epithelial monolayers to study the use of a virus as a bacteria predator [88].

Recently, ATPSs have been used to produce uniform aggregate cell cultures without the need of specialized equipment. ATPSs were found to have exceptional effectiveness in 3D microtissue engineering. A good example is the work of Tavana and coworkers (2016), who have created a high-throughput aggregate culture model, based on the deposition of microdroplets of bottom phase-cell rich in a large volume of top phase. The authors have been using this model to advance high-throughput oncological drugs screening using 3D tumor microtissues models [89].

2.1.2.6. Synthetic biology

The properties of phase separation in ATPSs have been exploited to mimic intracellular compartments, such as liquid-like organelles and nucleoli and to study the influence of compartmentalization within both membrane-enclosed and membrane-free structures.

One of the first studies intended to model the behavior of two sequential enzymes involved in purine biosynthesis by enzyme compartmentalization in a PEG–DEX system [90]. In another work, the increasing of the RNA cleavage rate was reported by means of promoting the partition of ribozyme to a DEX-rich phase, of PEG–DEX system, and by changing the phases volume ratio (decreasing the compartment size, DEX-rich phase) [91].

•

Successful application of partitioning in ATPSs requires the understanding of the mechanisms of solute distribution and properties of the systems at the molecular level. Understanding these mechanisms is important for both downstream processing and bioanalytical applications, although for slightly different reasons.

In the downstream processing sector, for the effective use of ATPSs it is important to predict optimal extraction conditions for the target product from diverse sources and/or to design optimized strategies for it. In this case, understanding the role of different factors affecting distribution of the target product and the remaining contaminants is much more important than understanding why the different solutes steer into a certain phase. In view of bioanalytical applications, the comprehension of the partitioning phenomenon is crucial since it is used as a measurement of the changes in the analytes' properties or as an indicator of the response of changes in the analytes partition behavior under wide-ranging partition conditions [92].

Lack of knowledge underlying the mechanisms of solute partitioning in ATPSs and in understanding the properties of the systems at the molecular level are the main reasons why ATPSs are still not utilized on a routine basis in laboratory and in the industry. However, a great input in this regard has been given by Zaslavsky since the beginning of the 90s.

2.2. Phase separation in aqueous media

2.2.1. Classic theoretical approaches

Two (or more) phases formation after mixing aqueous solutions of two incompatible polymers or polymers and salts has been well-known for several decades. This phenomenon has been intensively studied and documented carefully through the times [93], [94].

At equilibrium, two-phases formation and the equilibrium state, can be both predicted from the equality of the chemical potential of each component and system mass balances. These calculations only hold true if it is assumed that: (i) the phase-forming polymers are represented by a single component with an average molecular mass (not considering polymer polydispersity) and (ii) all the components added to the system are distributed by either the top or the bottom phases. However, for more accurate calculations, the effect of polymer polydispersity can be introduced in the determinations by using the pseudo-component method, i.e., use a continuous statistical distribution of molecular masses instead of the individual average molecular mass of each component [95].

Based on all studies carried out so far it is possible to identify 4 classical schools of thought, each one of them attempting to understand and modeling phase separation: (i) osmotic viral expansion based-models descended from Edmond and Ogston work, (ii) models based on extensions of lattice theories, (iii) models incorporating integral equation theory and (iv) group contribution schemes and excluded volume approximation models (that do not fall into the above categories) [95]. These conventional approaches are all based on the same general theoretic line of reasoning that considers the molecular nature of the phase forming components as the main factor for phase separation and secondarily the role of the water as a solvent [4].

The osmotic viral expansion models, which have gained more acceptance due to the work of Edmond and Ogston, include two different versions: the McMillan-Mayer theory and the Hill theory [96]–[101]. These theories generate modeling expressions with coefficients representing the molecular interactions between small groups of components, differing in the way the role of the solvent is considered. In the McMillan-Mayer theory the solvent is treated as a featureless background continuum and in the Hill theory interactions between solutes and solvent molecules are counted in.

However, once these models are expansions in composition, they are strictly applicable only to very low solutes concentrations and, generally, changes in molecular interactions due to solution regime (diluted or concentrated solutions) are ignored to keep the models simple.

Modeling macromolecular liquid mixtures in terms of a crystal lattice approaches, such as the Flory-Huggins theory [102]–[105] or the UNIQUAC lattice model [106]–[108], is attractive since it is easy to estimate the total number of possible configurations for the system and obtain a combinatorial entropy for the mixture. But, particularly for mixtures of aqueous nature, the enthalpic effects usually displays the more significant role than the entropic one. Another drawback of this approach is that the combinatorial entropy obtained should not be compared with the real liquid entropy, because of the differences in order degree. In contrast with the first type of models described before, the lattice models are originated assuming concentrated solutions.

The third type of models combines integral equation theory, a hard sphere equation of state, perturbation theory, the McMillan-Mayer osmotic virial expansion and, sometimes, other elements. The overall expressions of these models are very complex, they present several parameters such as solutes size indicators and osmotic viral coefficients, but they are the ones that can describe complex aqueous systems with high accuracy. Plus, these models can represent both polymer–polymer and polymer–salt ATPSs [109].

Other models, like group contribution model or the excluded volume theory, do not fit in the complete description of the previous categories [110]–[113]. Though, these models share some aspects and have similar underlying concepts with the osmotic viral expansions approaches and the lattice theories, respectively.

Another class of models capable of accounting the presence and the behavior of solutions containing electrostatically charged species (ions from phase-forming salts, ions from buffers, proteins and/or phase-forming polyelectrolytes) were also created. This class includes for example: the Debye-Hückel theory and its modifications and mean spherical approximation (MSA) [114]–[116]. In principle, these models seem to be very promising, however, as in the McMillan-Mayer osmotic viral expansion theory, the solvent is not treated like a fluid but as a background continuum in which the charged species exist, which limits their application.

2.2.2. The approach based on water primary role

The advantages and downsides associated to each one of the previous theoretical lines of reasoning have been widely discussed over the years by many authors [4], [111], [117]–[119].

Considering their main downside, in early 90s, Zaslavsky suggested a different approach to describe two-phases formation. According to what was advanced by him, a special attention should be given to the water and the main role in phase separation should be attributed to its unique character as a medium. In his understanding, the phase forming components of the ATPSs must be considered as water structure perturbing agents. The presence of the phase forming components engaged in phase formation modify the water structure of each phase which make both phases immiscible. Therefore, it can be assumed that ATPSs are the result of mixing two immiscible solutions, both of aqueous nature [4].

This way to look into ATPSs is based on the well-known fact that water molecules strongly interact with each other in a very characteristic manner [120]. Moreover, some X-ray diffraction experiments carried out for mixtures of aqueous solutions of PVA (Polyvinyl alcohol) and DEX have shown the incompatibility of these two polymers dissolved in water due to strong repulsive interactions. The same experiments for dry mixtures of the same polymers have shown that in the absence of water they are extremely compatible [4]. Likewise, Treffry and coworkers, in their model have advised that polymers are surrounded by water shells formed by steric arrangements between the polymer bonding sites and the structure of the bulk solvent. Similar shells can combine resulting in micro-aggregates or microspheres. When distinct shells are forced to coexist, structural differences lead to turbidity and, consequently, to phase separation [121].

2.3. Solute partitioning in ATPSs

2.3.1. From the conventional theoretical approaches

When a solute is added to an ATPS it will distribute between the two phases and its partition behavior can be characterized by the partition coefficient, K, which represents the top/bottom solute concentration ratio.

There are several factors that are well-known to affect solute partition in ATPSs (and consequently, K-values). Among others, type, molecular weight and concentration of phase-forming polymers, type and concentration of additives, pH and temperature are the most studied and discussed in the literature [1], [2], [4]. Nonetheless, it should be noted that the effects of some of

the aforementioned factors on solute partitioning are mutually dependent (e.g. pH and salt additives; temperature and phase-forming components equilibrium concentrations).

So far, the theories regarding the mechanisms underlying solute partitioning can be divided in two distinct lines of reasoning. The first one relies on the concept that solute partition is mainly due to the solute interactions with the phase-forming components and secondarily due to the specific features of the water as solvent in both coexisting phases. This vision comprises the models based on the Flory-Huggins theory and/or on Osmotic Virial Expansion methods. All these models take in account the Lewis-Randall standard state used to define the differences of the properties of the water as a solvent from ideal solutions [2], [4], [6], [98]–[100], [102], [122]–[126].

2.3.2. Approach based on primary role of solvent

ATPSs can be compared qualitatively to organic solvent–water biphasic systems. All the attempts to understand ATPSs follow the standard approaches used to characterize these last ones. There are some important differences, however that should be emphasized. In organic solvent–water biphasic systems only one equilibrium composition exists. However, in ATPSs it is known that different quantitative compositions may exist, meaning that the solvent features of the aqueous media in the coexisting phases at equilibrium are also different [4]. It was demonstrated that ATPSs physicochemical properties, namely dielectric, solvatochromic and potentiometric as well as partition coefficients of series of homologous monofunctional aliphatic compounds are dependent of the phases composition in equilibrium [127]–[131].

The conventional theoretical approaches do not consider these two aspects described before [4]. So, based on this, the second line of reasoning was built around the idea that the phase-forming components must be engaged only in ATPSs' formation and be neutral to the solute being partitioned. Their main role is the effect caused on changing the solvent properties of the aqueous media of the coexisting phases [4]. There are experimental evidences supporting this approach, it was proved that the solvent properties of the aqueous media in coexisting phases of different ATPSs are distinct. It was also shown that solute partitioning in ATPSs has some similarities with the partition in organic solvent–water biphasic systems [4], [5], [132]–[135].

If the second theory holds true, it means that empirical and semi-empirical approaches commonly used to describe solute partitioning in organic solvent–water biphasic systems can be applied to ATPSs.

2.3.2.1. The Collander equation

Solute partitioning in a set of different organic solvent–water biphasic systems can be correlated according to the universally known Collander Equation, also called Solvent Regression Equation, presented below [136]:

$$\log P_i = a_i \log P_0 + b_i$$

(Equation 2.3)

where *P* represents the partition coefficients in organic solvent–water biphasic systems for a given solute and subscripts *i* and *0* denote the systems under comparison; *a* and *b* are constants.

During his work, Collander has realized that parameters *a* and *b* could vary, being dependent of the type of the solutes partitioned as well as of the solvent system [136]. Some years later, it was shown for similar organic solvent–water biphasic systems under comparison that a single equation could fit all the experimental partitioning data [137].

In the last decades, some studies aiming the application of this relationship as an attempt to describe solute partitioning in ATPSs have been reported. In 1994, Zaslavsky showed that the relationship described by Collander could be applied to relate the partition of a group of randomly selected biomolecules in a limited set of ATPSs formed by Dextran and Ficoll or PEG [4]. Later, other authors also verified the applicability of this relationship after analyzing the partition of small organic neutral compounds in ATPSs composed by PEG and different inorganic salts [5], [132]. In the past years several relationships of this type were also found when the partition behavior of nonionic and ionic low molecular compounds, and proteins was analyzed. The partition of these compounds was studied in different ATPSs, PEG–salt and PEG–DEX systems for example, and the effect of the presence of several additives was under investigation too [73], [138]–[149].

An important aspect that was found out during the initial studies around the application of the Collander Equation to ATPSs was that, for systems composed by two polymers, parameters *a* and *b*

(i.e., the slope and the intercept terms of the equation) could be independently described by partitioning of a series of dinitrophenylated-amino acids (DNP-AA) [150].

2.3.2.2. The Abraham's solvation equation

The most widely used semi-empirical approach for the analysis of solute partition in organic solvent–water biphasic systems is the LSER, based on the generalized Abraham's Solvation Equation [151]–[153]:

$$\log P = vV + rR_2 + sS + aA + bB + z$$

(Equation 2.4)

where, *P* represents the partition behavior of a given solute between two phases of interest; *v*, *r*, *s*, *a*, *b* and *z* are representative coefficients of a specific two-phases system (determined by multiple linear regression analysis); *V*, R_2, *S*, *A* and *B* are the so-called solute descriptors and denote for: *V* the group-contributable solute volume comprising the solvent cavitation energy and part of the solute-solvent London dispersion interaction (it can be calculated based on solute structure as a sum of atom and bond contributions); R_2 the "excess molar refraction" of a solute, representing solute-solvent interactions involving induce dipole on the solute, supplementing the term *vV* in the equation (it can be obtained from the refractive index of the solute or by summing up fragments values); *S* the polarity/polarizability descriptor characterize interactions related to induced as well as stable polarity of the solute; A and B the total hydrogen bond donating (overall solute hydrogen bond acidity) and accepting (overall solute hydrogen bond basicity) capacities of the solute, respectively; *z* is a fitted regression constant term which depends on the standard states of *P*. While solute descriptors V and R_2 can be calculated from the solute structure, the other ones can be experimentally obtained and/or calculated. Regarding the specific two-phase systems coefficients, *v*, *r*, *s*, *a*, *b* and *z*, each one stand for the following meanings: *v* is a combination of an exoergic dispersion and endoergic cavity terms; *r* designates the difference between the tendency of the coexisting phases to interact through π- and *n*-electronic pairs with the solutes; *s* corresponds to the difference between the tendency of the coexisting phases to interact with dipolar/polarizable solutes; *a* and *b* denote for the differences between the hydrogen bond acidity and basicity of the phases [151]–[155].

The LSER has also been applied to describe partition of solutes in ATPSs and to analyze the solvent features of PEG–salt and PEG–DEX ATPSs [131], [132], [156], [157]. However, despite the successful implementation of this approach to ATPSs there are three important aspects that should be highlighted (i) if the range of K-values under analysis is too limited, the accuracy of the multiple linear regression analysis may be doubtful, (ii) the analysis of the partitioning of biomolecules in ATPSs is limited due to the fact that Abraham's approach does not take into account ion-ion and ion-dipole interactions and (iii) to obtain Abraham's descriptors for biomolecules is very difficult and may be impossible [158].

Nevertheless, the original Abraham's Solvation Equation may be modified (by adding or replacing some equation terms) to overcome some of the aforementioned conditions. A good example of Abraham's Solvation Equation update is the alternative approach suggested by Madeira *et al.*, which was already used for the effective determination of solute descriptors of nonionic and ionizable organic compounds, free amino acids and proteins [73], [139], [140].

2.3.3. Analysis of solute-solvent interactions

It is well recognized that interactions of any substance with the aqueous environment are fundamentally important for their functions *in vivo* [159]–[161]. These interactions play an important role in many biological processes *in vivo* like in the formation of active sites of oligomeric enzymes and maintenance of their effective conformation, in regulatory processes including signal transduction, electrons transport systems, DNA synthesis, antibody-antigen binding and in all kind of protein transport processes [162], [163].

Thus, the understanding of these solute-water interactions on a molecular level allows to gain better insight into fundamental mechanisms of many different biological processes and metabolic pathways. Indeed, it is perhaps the unique role of water as a solvent which mediates these interactions among proteins, and how proteins recognize their ligands and partners, being also involved with molecular complexes building and explaining how proteins interact with different aqueous environments. Besides, its understanding seems to be crucial for applications ranging from basic research in life sciences to drug discovery and design and to clinical research. Surprisingly, despite of the importance of such interactions and the concomitant rapid advances in molecular and

structural biology, the state of affairs in the scientific underpinning of aqueous solvent-solute interactions is far behind [64], [73], [78], [139], [140], [164]–[166].

So far, solute-solvent interactions studies recognize the importance of hydrophobic interactions as well as other types of interactions such as van der Waals, polar, ion-dipole and hydrogen bonding interactions. Generally, they use to be described by linear free energy relationships, particularly by the Abraham model (Eq. 2.4) as a linear combination of the so-called solute descriptors and corresponding solvent descriptors. Usually, solute descriptors for multiple compounds have been determined by separate physicochemical measurements and the solvent descriptors values are estimated as unknown coefficients in Eq. 2.4 by multiple linear regression analysis for all these compounds. This approach is generally successful for nonionic organic compounds but is hardly applicable to ionized compounds and cannot be used for analysis of biomacromolecules [64], [73], [78], [139], [140], [164]–[166].

Recently, a new analytical application based on partitioning in ATPSs suggested by Madeira *et al.*, have been developed to quantify and understand these interactions enabling to receive quantitative information about different types of small molecules and protein-water interactions. This alternative approach consists in characterize solvent descriptors values in different solvent systems using solvatochromic measurements and estimate solute descriptors as unknown coefficients in Eq. 2.4 by multiple linear regression analysis for all the solvent systems used [64], [73], [78], [139], [140], [164]–[166].

This approach was already used in the successful determination of solute descriptors of nonionic and ionizable organic compounds, free amino acids and proteins. But, its most important distinction is that ATPSs are used instead of common organic solvent–water biphasic systems and, consequently, are more adequate to simulate solvent environment *in vivo* and more suitable for analysis of biological macromolecules [64], [73], [78], [139], [140], [164]–[166].

2.4. Summary and outlook

ATPSs have been explored for the development of a variety of novel, simple and cost-effective approaches, taking advantage of their well-known features, such as low interfacial tension between the coexisting phases and the high degree of biocompatibility of phase-forming components. This

technique has provided researchers with a valuable tool set not only for innovative downstream processing procedures but mainly for bioanalytical applications.

Interestingly, despite the vast field of applications of ATPSs, solute partitioning is used in most cases, and its manipulation and/or measurement comprises the key step of most of the processes. As stated by Zaslavsky *et al.*, over the last two decades several examples of experimental data have been reported providing evidences that interactions between the phase-forming components, namely polymers, and the solutes partitioned in ATPSs do not occur. In fact, solute partitioning is governed by the differences in solute interactions with the aqueous media of each of the phases, whose solvent properties can be characterized and manipulated. Therefore, partition coefficients reflect these interactions and can be used as general numerical indexes (different changes in solutes structures may change their correspondent K-values) [53].

Thus, ATPSs partitioning based-approaches, comprising the analytical application of the method of partitioning can provide new opportunities and solutions for some of the current and future biotechnological and bioanalytical challenges.

References

[1] T. J. Peters, “Partition of cell particles and macromolecules: Separation and purification of biomolecules, cell organelles, membranes and cells in aqueous polymer two phase systems and their use in biochemical analysis and biotechnology. P-A. Albertsson. Third Edition,” Cell Biochem. Funct., vol. 5, no. 3, pp. 233–234, 1987.

[2] D. E. BROOKS, K. I. M. A. SHARP, and D. FISHER, “2 - Theoretical Aspects of Partitioning,” in Partitioning in Aqueous Two-Phase System, H. WALTER, D. E. BROOKS, and D. FISHER, Eds. Academic Press, 1985, pp. 11–84.

[3] R. Woker, J. Vernau, and M.-R. Kula, “[57] Purification of oxynitrilases from plants,” in Aqueous Two-Phase Systems, vol. Volume 228, G. J. B. T.-M. in E. Harry Walter, Ed. Academic Press, 1994, pp. 584–590.

[4] B. Y. Zaslavsky, Aqueous Two-phase Partitioning: Physical Chemistry and Bioanalytical Applications. New York, USA: Marcel Dekker, 1995.

[5] H. D. Willauer, J. G. Huddleston, and R. D. Rogers, “Solvent Properties of Aqueous Biphasic Systems Composed of Polyethylene Glycol and Salt Characterized by the Free Energy of

Transfer of a Methylene Group between the Phases and by a Linear Solvation Energy Relationship," Ind. Eng. Chem. Res., vol. 41, no. 11, pp. 2591–2601, May 2002.

[6] A. D. Diamond and J. T. Hsu, "Fundamental studies of biomolecule partitioning in aqueous two-phase systems," Biotechnol. Bioeng., vol. 34, no. 7, pp. 1000–1014, 1989.

[7] A. D. Diamond and J. T. Hsu, "Aqueous two-phase systems for biomolecule separation," in Bioseparation, G. T. Tsao, Ed. Berlin, Heidelberg: Springer Berlin Heidelberg, 1992, pp. 89–135.

[8] J. Ryden and P. Albertsson, "Interfacial tension of dextran—polyethylene glycol—water two—phase systems," J. Colloid Interface Sci., vol. 37, no. 1, pp. 219–222, 1971.

[9] D. Hagarova and A. Breier, "Distribution of proteins in aqueous two-phase systems formed by dextran and polyethylene glycol. Influence of protein hydrophobicity.," Gen. Physiol. Biophys., vol. 14, no. 4, pp. 277–291, Aug. 1995.

[10] A. Haghtalab and M. A. Asadollahi, "An excess Gibbs energy model to study the phase behavior of aqueous two-phase systems of polyethylene glycol+dextran," Fluid Phase Equilib., vol. 171, no. 1, pp. 77–90, 2000.

[11] B. Mattiasson, "Affinity Partitioning," in Chromatographic and Membrane Processes in Biotechnology, 1st ed., C. Costa and J. Cabral, Eds. Springer Netherlands, 1991, pp. 309–321.

[12] T. G. I. Ling, H. Nilsson, and B. Mattiasson, "Reppal PES — A starch derivative for aqueous two-phase systems," Carbohydr. Polym., vol. 11, no. 1, pp. 43–54, 1989.

[13] P. P. Madeira, J. A. Teixeira, E. A. Macedo, L. M. Mikheeva, and B. Y. Zaslavsky, "ΔG(CH2) as solvent descriptor in polymer/polymer aqueous two-phase systems," J. Chromatogr. A, vol. 1185, no. 1, pp. 85–92, 2008.

[14] M. Pereira, Y.-T. Wu, P. Madeira, A. Venâncio, E. Macedo, and J. Teixeira, "Liquid–Liquid Equilibrium Phase Diagrams of New Aqueous Two-Phase Systems: Ucon 50-HB5100 + Ammonium Sulfate + Water, Ucon 50-HB5100 + Poly(vinyl alcohol) + Water, Ucon 50-HB5100 + Hydroxypropyl Starch + Water, and Poly(ethylene glycol) 8000 + Poly(vinyl," J. Chem. Eng. Data, vol. 49, no. 1, pp. 43–47, Jan. 2004.

[15] K. A. Giuliano, "Aqueous two-phase protein partitioning using textile dyes as affinity ligands," Anal. Biochem., vol. 197, no. 2, pp. 333–339, 1991.

[16] L. A. Sarubbo et al., "New aqueous two-phase system based on cashew-nut tree gum and poly(ethylene glycol).," J. Chromatogr. B. Biomed. Sci. Appl., vol. 743, no. 1–2, pp. 79–84, Jun. 2000.

[17] A. S. Medin and J.-C. Janson, "Studies on aqueous polymer two-phase systems containing agarose," Carbohydr. Polym., vol. 22, no. 2, pp. 127–136, 1993.

[18] D. C. Szlag and K. A. Guiliano, "A low-cost aqueous two phase system for enzyme extraction," Biotechnol. Tech., vol. 2, no. 4, pp. 277–282, 1988.

[19] V. Gupta, S. Nath, and S. Chand, "Role of water structure on phase separation in polyelectrolyte–polyethyleneglycol based aqueous two-phase systems," Polymer (Guildf)., vol. 43, no. 11, pp. 3387–3390, 2002.

[20] U. Dissing and B. Mattiasson, "Poly(ethyleneimine) as a phase-forming polymer in aqueous two-phase systems," Biotechnol. Appl. Biochem., no. 17, pp. 15–21, 1993.

[21] A. Venâncio, C. Almeida, L. Domingues, and J. A. Teixeira, "Protein partition on a derivative guar gum based aqueous two-phase system," Bioseparation, no. 5, pp. 253–258, 1995.

[22] M. V. Miranda and O. Cascone, "Partition of horseradish in aqueous two-phase systems containing polyvinylpyrrolidone," Bioseparation, no. 7, pp. 25–30, 1998.

[23] R. Hatti-Kaul, "Aqueous two-phase systems: A general overview," Appl. Biochem. Biotechnol. - Part B Mol. Biotechnol., vol. 19, pp. 269–277, 2001.

[24] M. T. Zafarani-Moattar and A. Zaferanloo, "Measurement and correlation of phase equilibria in aqueous two-phase systems containing polyvinylpyrrolidone and di-potassium tartrate or di-potassium oxalate at different temperatures," J. Chem. Thermodyn., vol. 41, no. 7, pp. 864–871, 2009.

[25] H. Everberg, T. Leiding, A. Schiöth, F. Tjerneld, and N. Gustavsson, "Efficient and non-denaturing membrane solubilization combined with enrichment of membrane protein complexes by detergent/polymer aqueous two-phase partitioning for proteome analysis," J. Chromatogr. A, vol. 1122, no. 1, pp. 35–46, 2006.

[26] C. M. S. S. Neves, A. M. S. Silva, A. M. Fernandes, J. A. P. Coutinho, and M. G. Freire, "Toward an Understanding of the Mechanisms behind the Formation of Liquid–liquid Systems formed by Two Ionic Liquids," J. Phys. Chem. Lett., vol. 8, no. 13, pp. 3015–3019, Jul. 2017.

[27] J.-X. Xiao, U. Sivars, and F. Tjerneld, "Phase behavior and protein partitioning in aqueous two-phase systems of cationic–anionic surfactant mixtures," J. Chromatogr. B Biomed. Sci. Appl., vol. 743, no. 1, pp. 327–338, 2000.

[28] S. Oppermann, F. Stein, and U. Kragl, "Ionic liquids for two-phase systems and their application for purification, extraction and biocatalysis," Appl. Microbiol. Biotechnol., vol. 89, no. 3, pp. 493–499, 2011.

[29] F. A. Vicente et al., "Impact of Surface Active Ionic Liquids on the Cloud Points of Nonionic

Surfactants and the Formation of Aqueous Micellar Two-Phase Systems," J. Phys. Chem. B, vol. 121, no. 37, pp. 8742–8755, Sep. 2017.

[30] U. Sivars and F. Tjerneld, "Mechanisms of phase behaviour and protein partitioning in detergent/polymer aqueous two-phase systems for purification of integral membrane proteins11This work was carried out in the Swedish Center for Bioseparation.," Biochim. Biophys. Acta - Gen. Subj., vol. 1474, no. 2, pp. 133–146, 2000.

[31] Y. Liu, Z. Wu, and J. Dai, "Phase equilibrium and protein partitioning in aqueous micellar two-phase system composed of surfactant and polymer," Fluid Phase Equilib., vol. 320, pp. 60–64, 2012.

[32] M. Foroutan, N. Heidari, M. Mohammadlou, and A. J. Sojahrood, "Effect of temperature on the (liquid+liquid) equilibrium for aqueous solution of nonionic surfactant and salt: Experimental and modeling," J. Chem. Thermodyn., vol. 40, no. 7, pp. 1077–1081, 2008.

[33] M. G. Freire et al., "Aqueous biphasic systems: a boost brought about by using ionic liquids," Chem. Soc. Rev., vol. 41, no. 14, pp. 4966–4995, 2012.

[34] H.-K. Rajni, Aqueous Two-Phase Systems: Methods and Protocols, 1st ed. Totowa, NJ: Humana Press, 2000.

[35] G. Johansson, "Comparison of two aqueous biphasic systems used for the partition of biological material," J. Chromatogr. A, vol. 150, no. 1, pp. 63–71, 1978.

[36] J. G. Huddleston, H. D. Willauer, S. T. Griffin, and R. D. Rogers, "Aqueous Polymeric Solutions as Environmentally Benign Liquid/Liquid Extraction Media," Ind. Eng. Chem. Res., vol. 38, no. 7, pp. 2523–2539, Jul. 1999.

[37] J. Persson, D. C. Andersen, and P. M. Lester, "Evaluation of different primary recovery methods for E. coli-derived recombinant human growth hormone and compatibility with further down-stream purification," Biotechnol. Bioeng., vol. 90, no. 4, pp. 442–451, 2005.

[38] A. Frerix, P. Geilenkirchen, M. Müller, M.-R. Kula, and J. Hubbuch, "Separation of genomic DNA, RNA, and open circular plasmid DNA from supercoiled plasmid DNA by combining denaturation, selective renaturation and aqueous two-phase extraction," Biotechnol. Bioeng., vol. 96, no. 1, pp. 57–66, 2007.

[39] M. Dilip, G. Muthuraman, and K. Palanivelu, "Removal of textile dyes from aqueous solution using PEG based aqueous biphasic system," Toxicol. Environ. Chem., vol. 87, no. 4, pp. 499–507, Oct. 2005.

[40] P. Weschayanwiwat, O. Kunanupap, and J. F. Scamehorn, "Benzene removal from waste water using aqueous surfactant two-phase extraction with cationic and anionic surfactant mixtures," Chemosphere, vol. 72, no. 7, pp. 1043–1048, 2008.

[41] L. Bulgariu and D. Bulgariu, "Extraction of gold(III) from chloride media in aqueous polyethylene glycol-based two-phase system," Sep. Purif. Technol., vol. 80, no. 3, pp. 620–625, 2011.

[42] P. da R. Patrício, M. C. Mesquita, L. H. M. da Silva, and M. C. H. da Silva, "Application of aqueous two-phase systems for the development of a new method of cobalt(II), iron(III) and nickel(II) extraction: A green chemistry approach," J. Hazard. Mater., vol. 193, pp. 311–318, 2011.

[43] S. H. Li, C. Y. He, H. W. Liu, K. A. Li, and F. Liu, "Ionic liquid-salt aqueous two-phase system, a novel system for the extraction of abused drugs," Chinese Chem. Lett., vol. 16, no. 8, pp. 1074–1076, 2005.

[44] F. A. e Silva, T. Sintra, S. P. M. Ventura, and J. A. P. Coutinho, "Recovery of paracetamol from pharmaceutical wastes," Sep. Purif. Technol., vol. 122, pp. 315–322, 2014.

[45] J. Han et al., "(Liquid+liquid) equilibria and extraction capacity of (imidazolium ionic liquids+potassium tartrate) aqueous two-phase systems," J. Mol. Liq., vol. 193, pp. 23–28, 2014.

[46] J. Pang et al., "Partitioning Behavior of Tetracycline in Hydrophilic Ionic Liquids Two-Phase Systems," Sep. Sci. Technol., vol. 50, no. 13, pp. 1993–1998, Sep. 2015.

[47] F. A. e Silva, "Extraction and separation of drugs using alternative solvents," Universidade de Aveiro, 2018.

[48] J. Benavides and M. Rito-Palomares, "Practical experiences from the development of aqueous two-phase processes for the recovery of high value biological products," J. Chem. Technol. Biotechnol., vol. 83, no. 2, pp. 133–142, 2008.

[49] J. Benavides, O. Aguilar, B. H. Lapizco-Encinas, and M. Rito-Palomares, "Extraction and Purification of Bioproducts and Nanoparticles using Aqueous Two-Phase Systems Strategies," Chem. Eng. Technol., vol. 31, no. 6, pp. 838–845, 2008.

[50] M. Rito-Palomares and A. Lyddiatt, "Process integration using aqueous two-phase partition for the recovery of intracellular proteins," Chem. Eng. J., vol. 87, no. 3, pp. 313–319, 2002.

[51] A. L. Grilo, M. Raquel Aires-Barros, and A. M. Azevedo, "Partitioning in Aqueous Two-Phase Systems: Fundamentals, Applications and Trends," Sep. Purif. Rev., vol. 45, no. 1, pp. 68–80, Jan. 2016.

[52] J. V. D. Molino, D. de A. Viana Marques, A. P. Júnior, P. G. Mazzola, and M. S. V. Gatti, "Different types of aqueous two-phase systems for biomolecule and bioparticle extraction and purification," Biotechnol. Prog., vol. 29, no. 6, pp. 1343–1353, Nov. 2013.

[53] B. Y. Zaslavsky, V. N. Uversky, and A. Chait, "Analytical applications of partitioning in aqueous two-phase systems: Exploring protein structural changes and protein-partner interactions in vitro and in vivo by solvent interaction analysis method.," Biochim. Biophys. Acta, vol. 1864, no. 5, pp. 622–644, May 2016.

[54] M. Iqbal et al., "Aqueous two-phase system (ATPS): an overview and advances in its applications," Biol. Proced. Online, vol. 18, p. 18, Oct. 2016.

[55] A. G. Teixeira, R. Agarwal, K. R. Ko, J. Grant-Burt, B. M. Leung, and J. P. Frampton, "Emerging Biotechnology Applications of Aqueous Two-Phase Systems.," Adv. Healthc. Mater., vol. 7, no. 6, p. e1701036, Mar. 2018.

[56] J. González-Valdez, K. Mayolo-Deloisa, and M. Rito-Palomares, "Novel aspects and future trends in the use of aqueous two-phase systems as a bioengineering tool," J. Chem. Technol. Biotechnol., vol. 93, no. 7, pp. 1836–1844, 2018.

[57] J. R. SooHoo and G. M. Walker, "Microfluidic aqueous two phase system for leukocyte concentration from whole blood," Biomed. Microdevices, vol. 11, no. 2, pp. 323–329, 2009.

[58] S. Nagel, S. Gretzinger, M. Schwab, S. A. Oelmeier, F. Dismer, and J. Hubbuch, "Aqueous two-phase systems - a label-free tech-nology for cell separation," Cytotherapy, vol. 15, no. 4, p. S45, Apr. 2013.

[59] S. Zimmermann et al., "High-throughput downstream process development for cell-based products using aqueous two-phase systems," J. Chromatogr. A, vol. 1464, p. 1—11, 2016.

[60] S. Zimmermann, S. Gretzinger, P. K. Zimmermann, A. Bogsnes, M. Hansson, and J. Hubbuch, "Cell Separation in Aqueous Two-Phase Systems – Influence of Polymer Molecular Weight and Tie-Line Length on the Resolution of Five Model Cell Lines," Biotechnol. J., vol. 13, no. 2, p. 1700250, Feb. 2018.

[61] S. Hazra, K. S. Jayaprakash, K. Pandian, A. Raj, S. K. Mitra, and A. K. Sen, "Non-inertial lift induced migration for label-free sorting of cells in a co-flowing aqueous two-phase system," Analyst, vol. 144, no. 8, pp. 2574–2583, 2019.

[62] K. Berggren, A. Wolf, J. A. Asenjo, B. A. Andrews, and F. Tjerneld, "The surface exposed amino acid residues of monomeric proteins determine the partitioning in aqueous two-phase systems," Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol., vol. 1596, no. 2, pp. 253–268, Apr. 2002.

[63] J.-W. Lee and D. Forciniti, "Effect of glycosylation on the partition behavior of a human antibody in aqueous two-phase systems.," Biotechnol. Prog., vol. 29, no. 4, pp. 943–950, 2013.

[64] A. Zaslavsky, N. Gulyaeva, A. Chait, and B. Zaslavsky, "A new method for analysis of

components in a mixture without preseparation: evaluation of the concentration ratio and protein-protein interaction.," Anal. Biochem., vol. 296, no. 2, pp. 262–269, Sep. 2001.

[65] P. E. H. Jensen, T. Stigbrand, and V. P. Shanbhag, "Use of hydrophobic affinity partitioning as a method for studying various conformational states of the human α-macroglobulins," J. Chromatogr. A, vol. 668, no. 1, pp. 101–106, 1994.

[66] A. Chait and B. Y. Zaslavsky, "Systems and methods for characterization of molecules," US Patent 8,099,242, 2012.

[67] A. Chait and B. Y. Zaslavsky, "Characterization of molecules," US Patent 8,211,714, 2012.

[68] N. Gulyaeva, A. Zaslavsky, P. Lechner, A. Chait, and B. Zaslavsky, "Relative hydrophobicity of organic compounds measured by partitioning in aqueous two-phase systems," J. Chromatogr. B Biomed. Sci. Appl., vol. 743, no. 1–2, pp. 187–194, Jun. 2000.

[69] N. Gulyaeva, A. Zaslavsky, P. Lechner, A. Chait, and B. Zaslavsky, "pH dependence of the relative hydrophobicity and lipophilicity of amino acids and peptides measured by aqueous two-phase and octanol–buffer partitioning," J. Pept. Res., vol. 61, no. 2, pp. 71–79, 2003.

[70] N. Gulyaeva, A. Zaslavsky, P. Lechner, M. Chlenov, A. Chait, and B. Zaslavsky, "Relative hydrophobicity and lipophilicity of beta-blockers and related compounds as measured by aqueous two-phase partitioning, octanol-buffer partitioning, and HPLC.," Eur. J. Pharm. Sci., vol. 17, no. 1–2, pp. 81–93, Oct. 2002.

[71] N. Gulyaeva, A. Zaslavsky, A. Chait, and B. Zaslavsky, "Relative hydrophobicity of di- to hexapeptides as measured by aqueous two-phase partitioning.," J. Pept. Res., vol. 61, no. 3, pp. 129–139, Mar. 2003.

[72] N. Gulyaeva et al., "Relative hydrophobicity and lipophilicity of drugs measured by aqueous two-phase partitioning, octanol-buffer partitioning and HPLC. A simple model for predicting blood-brain distribution.," Eur. J. Med. Chem., vol. 38, no. 4, pp. 391–396, Apr. 2003.

[73] P. P. Madeira, A. Bessa, L. Alvares-Ribeiro, M. R. Aires-Barros, A. E. Rodrigues, and B. Y. Zaslavsky, "Analysis of amino acid-water interactions by partitioning in aqueous two-phase systems. I–amino acids with non-polar side-chains.," J. Chromatogr. A, vol. 1274, pp. 82–86, Jan. 2013.

[74] B. Y. Zaslavsky, "Bioanalytical Applications of Partitioning in Aqueous Polymer Two-Phase Systems," Anal. Chem., vol. 64, no. 15, pp. 765A-773A, Aug. 1992.

[75] H. Shin et al., "High-yield isolation of extracellular vesicles using aqueous two-phase system," Sci. Rep., vol. 5, p. 13103, Aug. 2015.

[76] A. Chait and B. Y. Zaslavsky, "Systems and methods involving data patterns such as spectral

biomarkers," US Patent 8,437,964, 2013.

[77] A. Chait and B. Y. Zaslavsky, "Method for evaluation of the ratio of amounts of biomolecules or their sub-populations in a mixture," US Patent 6,136,960, 2000.

[78] M. Stovsky et al., "Prostate-specific antigen/solvent interaction analysis: a preliminary evaluation of a new assay concept for detecting prostate cancer using urinary samples.," Urology, vol. 78, no. 3, pp. 601–605, Sep. 2011.

[79] E. A. Klein et al., "The Single-parameter, Structure-based IsoPSA Assay Demonstrates Improved Diagnostic Accuracy for Detection of Any Prostate Cancer and High-grade Prostate Cancer Compared to a Concentration-based Assay of Total Prostate-specific Antigen: A Preliminary Repo," Eur. Urol., vol. 72, no. 6, pp. 942–949, Dec. 2017.

[80] M. Stovsky et al., "Clinical Validation of IsoPSA, a Single Parameter, Structure Based Assay for Improved Detection of High Grade Prostate Cancer.," J. Urol., vol. 201, no. 6, pp. 1115–1120, Jun. 2019.

[81] R. Agarwal, K. R. Ko, P. F. Gratzer, and J. P. Frampton, "Biopatterning of Keratinocytes in Aqueous Two-Phase Systems as a Potential Tool for Skin Tissue Engineering," MRS Adv., vol. 2, no. 45, pp. 2443–2449, 2017.

[82] H. Tavana et al., "Nanolitre liquid patterning in aqueous environments for spatially defined reagent delivery to mammalian cells.," Nat. Mater., vol. 8, no. 9, pp. 736–741, Sep. 2009.

[83] J. P. Frampton, Z. Fan, A. Simon, D. Chen, C. X. Deng, and S. Takayama, "Aqueous Two-Phase System Patterning of Microbubbles: Localized Induction of Apoptosis in Sonoporated Cells," Adv. Funct. Mater., vol. 23, no. 27, pp. 3420–3431, Jul. 2013.

[84] J. P. Frampton, J. B. White, A. T. Abraham, and S. Takayama, "Cell co-culture patterning using aqueous two-phase systems," J. Vis. Exp., no. 73, p. 50304, Mar. 2013.

[85] Y. Fang et al., "Rapid generation of multiplexed cell cocultures using acoustic droplet ejection followed by aqueous two-phase exclusion patterning," Tissue Eng. Part C. Methods, vol. 18, no. 9, pp. 647–657, Sep. 2012.

[86] T. Yaguchi et al., "Aqueous two-phase system-derived biofilms for bacterial interaction studies.," Biomacromolecules, vol. 13, no. 9, pp. 2655–2661, Sep. 2012.

[87] A. Zipperer et al., "Human commensals producing a novel antibiotic impair pathogen colonization.," Nature, vol. 535, no. 7613, pp. 511–516, Jul. 2016.

[88] M. Dwidar, B. M. Leung, T. Yaguchi, S. Takayama, and R. J. Mitchell, "Patterning Bacterial Communities on Epithelial Cells," PLoS One, vol. 8, no. 6, p. e67165, Jun. 2013.

[89] P. Shahi Thakuri, S. L. Ham, G. D. Luker, and H. Tavana, "Multiparametric Analysis of

Oncology Drug Screening with Aqueous Two-Phase Tumor Spheroids," Mol. Pharm., vol. 13, no. 11, pp. 3724–3735, Nov. 2016.

[90] B. W. Davis, W. M. Aumiller, N. Hashemian, S. An, A. Armaou, and C. D. Keating, "Colocalization and Sequential Enzyme Activity in Aqueous Biphasic Systems: Experiments and Modeling," Biophys. J., vol. 109, no. 10, pp. 2182–2194, 2015.

[91] C. A. Strulson, R. C. Molden, C. D. Keating, and P. C. Bevilacqua, "RNA catalysis through compartmentalization.," Nat. Chem., vol. 4, no. 11, pp. 941–946, Nov. 2012.

[92] P. Madeira, "Protein Partitioning in Polymer/Polymer Aqueous Two-Phase Systems," Faculdade de Engenharia da Universidade do Porto, 2008.

[93] P. Å. Albertsson, Partition of Cell Particles and Macromolecules: Distribution and Fractionation of Cells, Viruses, Microsomes, Proteins, Nucleic Acids, and Antigen-antibody Complexes in Aqueous Polymer Two-phase Systems. J. Wiley, 1960.

[94] H. Walter, Partitioning In Aqueous Two – Phase System: Theory, Methods, Uses, and Applications To Biotechnology. Elsevier Science, 2012.

[95] H. Cabezas, "Theory of phase formation in aqueous two-phase systems," J. Chromatogr. B Biomed. Sci. Appl., vol. 680, no. 1, pp. 3–30, 1996.

[96] W. G. McMillan and J. E. Mayer, "The Statistical Thermodynamics of Multicomponent Systems," J. Chem. Phys., vol. 13, no. 7, pp. 276–305, Jul. 1945.

[97] T. Hill, An introduction to statistical thermodynamics. New York: New York : Dover Publications, 1986.

[98] E. Edmond and A. G. Ogston, "An approach to the study of phase separation in ternary aqueous systems," Biochem. J., vol. 109, no. 4, pp. 569 LP – 576, Oct. 1968.

[99] R. S. King, H. W. Blanch, and J. M. Prausnitz, "Molecular thermodynamics of aqueous two-phase systems for bioseparations," AIChE J., vol. 34, no. 10, pp. 1585–1594, 1988.

[100] D. Forciniti, C. K. Hall, and M.-R. Kula, "Influence of polymer molecular weight and temperature on phase composition in aqueous two-phase systems," Fluid Phase Equilib., vol. 61, no. 3, pp. 243–262, 1991.

[101] F. Doebert, A. Pfennig, and M. Stumpf, "Derivation of the Consistent Osmotic Virial Equation and Its Application to Aqueous Poly(ethylene glycol)-Dextran Two-Phase Systems," Macromolecules, vol. 28, no. 23, pp. 7860–7868, 1995.

[102] P. J. Flory, Principles of Polymer Chemistry. Cornell University Press, 1953.

[103] A. Sjoeberg and G. Karlstroem, "Temperature dependence of the phase equilibria for the

system poly(ethylene glycol)/dextran/water. A theoretical and experimental study," Macromolecules, vol. 22, no. 3, pp. 1325–1330, 1989.

[104] P. J. Flory, "Thermodynamics of High Polymer Solutions," J. Chem. Phys., vol. 10, no. 1, pp. 51–61, Jan. 1942.

[105] M. L. Huggins, "THERMODYNAMIC PROPERTIES OF SOLUTIONS OF LONG-CHAIN COMPOUNDS," Ann. N. Y. Acad. Sci., vol. 43, no. 1, pp. 1–32, Mar. 1942.

[106] C. H. Kang and S. I. Sandler, "Phase behavior of aqueous two-polymer systems," Fluid Phase Equilib., vol. 38, no. 3, pp. 245–272, 1987.

[107] C. H. Kang and S. I. Sandler, "A thermodynamic model for two-phase aqueous polymer systems," Biotechnol. Bioeng., vol. 32, no. 9, pp. 1158–1164, 1988.

[108] D. S. Abrams and J. M. Prausnitz, "Statistical thermodynamics of liquid mixtures: A new expression for the excess Gibbs energy of partly or completely miscible systems," AIChE J., vol. 21, no. 1, pp. 116–128, Jan. 1975.

[109] C. A. Haynes, F. J. Benitez, H. W. Blanch, and J. M. Prausnitz, "Application of integral-equation theory to aqueous two-phase partitioning systems," AIChE J., vol. 39, no. 9, pp. 1539–1557, Sep. 1993.

[110] C. Großmann, R. Tintinger, J. Zhu, and G. Maurer, "Aqueous two-phase systems of poly(ethylene glycol) and dextran — experimental results and modeling of thermodynamic properties," Fluid Phase Equilib., vol. 106, no. 1, pp. 111–138, 1995.

[111] Y. Guan, T. H. Lilley, and T. E. Treffry, "Theory of phase equilibria for multicomponent aqueous solutions: applications to aqueous polymer two-phase systems," J. Chem. Soc. Faraday Trans., vol. 89, no. 24, pp. 4283–4298, 1993.

[112] H. Reiss, "Statistical geometry in the study of fluids and porous media," J. Phys. Chem., vol. 96, no. 12, pp. 4736–4747, Jun. 1992.

[113] Y. Guan, T. H. Lilley, and T. E. Treffry, "A new excluded volume theory and its application to the coexistence curves of aqueous polymer two-phase systems," Macromolecules, vol. 26, no. 15, pp. 3971–3979, Jul. 1993.

[114] P. Debye and E. Hückel, "Zur Theorie der Elektrolyte. I. Gefrierpunktserniedrigung und verwandte Erscheinungen," Phys. Zeitschrift, vol. 24, no. 9, pp. 185–206, 1923.

[115] E. Waisman and J. L. Lebowitz, "Mean Spherical Model Integral Equation for Charged Hard Spheres I. Method of Solution," J. Chem. Phys., vol. 56, no. 6, pp. 3086–3093, Mar. 1972.

[116] E. Waisman and J. L. Lebowitz, "Exact Solution of an Integral Equation for the Structure of a Primitive Model of Electrolytes," J. Chem. Phys., vol. 52, no. 8, pp. 4307–4309, Apr. 1970.

[117] H. Walter, G. Johansson, and D. E. Brooks, "Partitioning in aqueous two-phase systems: recent results," Anal. Biochem., vol. 197, no. 1, pp. 1–18, 1991.

[118] J. N. Baskir, T. A. Hatton, and U. W. Suter, "Protein partitioning in two-phase aqueous polymer systems," Biotechnol. Bioeng., vol. 34, no. 4, pp. 541–558.

[119] N. L. Abbott, D. Blankschtein, and T. A. Hatton, "On protein partitioning in two-phase aqueous polymer systems.," Bioseparation, vol. 1, no. 3–4, pp. 191–225, 1990.

[120] S. W. Benson and E. D. Siebert, "A simple two-structure model for liquid water," J. Am. Chem. Soc., vol. 114, no. 11, pp. 4269–4276, May 1992.

[121] T. E. Treffry, T. H. Lilley, and P. J. Cheek, "Separations using aqueous phase systems : applications in cell biology and biotechnology," 1989.

[122] C. A. Haynes, H. W. Blanch, and J. M. Prausnitz, "Separation of protein mixtures by extraction: Thermodynamic properties of aqueous two-phase polymer systems containing salts and proteins," Fluid Phase Equilib., vol. 53, pp. 463–474, 1989.

[123] C. A. Haynes, R. A. Beynon, R. S. King, H. W. Blanch, and J. M. Prausnitz, "Thermodynamic properties of aqueous polymer solutions: poly(ethylene glycol)/dextran," J. Phys. Chem., vol. 93, no. 14, pp. 5612–5617, 1989.

[124] J.-F. P. Hamel, J. B. Hunter, and S. K. Sikdar, Downstream Processing and Bioseparation. Washington, DC: American Chemical Society, 1990.

[125] H. J. Cabezas, M. Kabiri-Badr, and D. C. Szlag, "Statistical thermodynamics of phase separation and ion partitioning in aqueous two-phase systems.," Bioseparation, vol. 1, no. 3–4, pp. 227–233, 1990.

[126] H. Cabezas, J. D. Evans, and D. C. Szlag, "A statistical mechanical model of aqueous two-phase systems," Fluid Phase Equilib., vol. 53, pp. 453–462, 1989.

[127] B. Y. Zaslavsky, L. M. Miheeva, M. N. Rodnikova, G. V Spivak, V. S. Harkin, and A. U. Mahmudov, "Dielectric properties of water in the coexisting phases of aqueous polymeric two-phase systems," J. Chem. Soc. Faraday Trans. 1 Phys. Chem. Condens. Phases, vol. 85, no. 9, pp. 2857–2865, 1989.

[128] B. Y. Zaslavsky, A. A. Borvskaya, N. D. Gulyaeva, and L. M. Miheeva, "Physico-chemical features of solvent media in the phases of aqueous polymer two-phase systems," Biotechnol. Bioeng., vol. 40, no. 1, pp. 1–7, Jun. 1992.

[129] B. Y. Zaslavsky, L. M. Miheeva, and S. V Rogozhin, "Possibility of analytical application of the partition in aqueous biphasic polymeric systems technique," Biochim. Biophys. Acta - Biomembr., vol. 510, no. 1, pp. 160–167, Jun. 1978.

[130] B. Y. Zaslavsky, A. A. Borovskaya, N. D. Gulaeva, and L. M. Miheeva, "Influence of ionic and polymer composition on the properties of the phases of aqueous two-phase systems formed by non-ionic polymers," J. Chem. Soc. Faraday Trans., vol. 87, no. 1, pp. 141–145, 1991.

[131] M. L. Moody, H. D. Willauer, S. T. Griffin, J. G. Huddleston, and R. D. Rogers, "Solvent Property Characterization of Poly(ethylene glycol)/Dextran Aqueous Biphasic Systems Using the Free Energy of Transfer of a Methylene Group and a Linear Solvation Energy Relationship," Ind. Eng. Chem. Res., vol. 44, no. 10, pp. 3749–3760, Apr. 2005.

[132] H. D. Willauer, J. G. Huddleston, and R. D. Rogers, "Solute Partitioning in Aqueous Biphasic Systems Composed of Polyethylene Glycol and Salt: The Partitioning of Small Neutral Organic Species," Ind. Eng. Chem. Res., vol. 41, no. 7, pp. 1892–1904, Mar. 2002.

[133] R. D. Rogers, H. D. Willauer, S. T. Griffin, and J. G. Huddleston, "Partitioning of small organic molecules in aqueous biphasic systems.," J. Chromatogr. B. Biomed. Sci. Appl., vol. 711, no. 1–2, pp. 255–263, Jun. 1998.

[134] H. D. Willauer, J. G. Huddleston, S. T. Griffin, and R. D. Rogers, "PARTITIONING OF AROMATIC MOLECULES IN AQUEOUS BIPHASIC SYSTEMS," Sep. Sci. Technol., vol. 34, no. 6–7, pp. 1069–1090, Jan. 1999.

[135] A. R. Katritzky et al., "Aqueous Biphasic Systems. Partitioning of Organic Molecules: A QSPR Treatment," J. Chem. Inf. Comput. Sci., vol. 44, no. 1, pp. 136–142, Dec. 2003.

[136] R. COLLANDER, "On lipoid solubility.," Acta Physiol. Scand., vol. 13, no. 4, pp. 363–381, Jun. 1947.

[137] A. Leo, C. Hansch, and D. Elkins, "Partition coefficients and their uses," Chem. Rev., vol. 71, no. 6, pp. 525–616, Dec. 1971.

[138] L. A. Ferreira, O. Fedotoff, V. N. Uversky, and B. Y. Zaslavsky, "Effects of osmolytes on protein–solvent interactions in crowded environments: study of sucrose and trehalose effects on different proteins by solvent interaction analysis," RSC Adv., vol. 5, no. 34, pp. 27154–27162, 2015.

[139] P. P. Madeira et al., "Solvatochromic relationship: prediction of distribution of ionic solutes in aqueous two-phase systems," J. Chromatogr. A, vol. 1271, no. 1, pp. 10–16, 2013.

[140] P. P. Madeira, C. A. Reis, A. E. Rodrigues, L. M. Mikheeva, A. Chait, and B. Y. Zaslavsky, "Solvent properties governing protein partitioning in polymer/polymer aqueous two-phase systems," J. Chromatogr. A, vol. 1218, no. 10, p. 1379—1384, 2011.

[141] P. P. Madeira, J. A. Teixeira, E. A. Macedo, L. M. Mikheeva, and B. Y. Zaslavsky, "'On the Collander equation': Protein partitioning in polymer/polymer aqueous two-phase systems," J. Chromatogr. A, vol. 1190, no. 1–2, pp. 39–43, May 2008.

[142] L. A. Ferreira, J. A. Teixeira, L. M. Mikheeva, A. Chait, and B. Y. Zaslavsky, "Effect of salt additives on partition of nonionic solutes in aqueous PEG–sodium sulfate two-phase system," J. Chromatogr. A, vol. 1218, no. 31, pp. 5031–5039, Aug. 2011.

[143] L. A. Ferreira, P. Parpot, J. A. Teixeira, L. M. Mikheeva, and B. Y. Zaslavsky, "Effect of NaCl additive on properties of aqueous PEG–sodium sulfate two-phase system," J. Chromatogr. A, vol. 1220, pp. 14–20, 2012.

[144] L. Ferreira, P. P. Madeira, L. Mikheeva, V. N. Uversky, and B. Zaslavsky, "Effect of salt additives on protein partition in polyethylene glycol–sodium sulfate aqueous two-phase systems," Biochim. Biophys. Acta - Proteins Proteomics, vol. 1834, no. 12, pp. 2859–2866, Dec. 2013.

[145] L. Ferreira et al., "Structural features important for differences in protein partitioning in aqueous dextran-polyethylene glycol two-phase systems of different ionic compositions," Biochim. Biophys. Acta, vol. 1844, no. 3, p. 694—704, 2014.

[146] L. A. Ferreira et al., "Effects of low urea concentrations on protein-water interactions.," J. Biomol. Struct. Dyn., vol. 35, no. 1, pp. 207–218, Jan. 2017.

[147] L. A. Ferreira, L. Breydo, C. Reichardt, V. N. Uversky, and B. Y. Zaslavsky, "Effects of osmolytes on solvent features of water in aqueous solutions," J. Biomol. Struct. Dyn., vol. 35, no. 5, pp. 1055–1068, Apr. 2017.

[148] L. A. Ferreira, Z. Wu, L. Kurgan, V. N. Uversky, and B. Y. Zaslavsky, "How to manipulate partition behavior of proteins in aqueous two-phase systems: Effect of trimethylamine N-oxide (TMAO)," Fluid Phase Equilib., vol. 449, pp. 217–224, 2017.

[149] L. A. Ferreira, V. N. Uversky, and B. Y. Zaslavsky, "Phase equilibria, solvent properties, and protein partitioning in aqueous polyethylene glycol-600-trimethylamine N-oxide and polyethylene glycol-600-choline chloride two-phase systems," J. Chromatogr. A, vol. 1535, pp. 154–161, 2018.

[150] B. Y. Zaslavsky, L. M. Miheeva, and S. V Rogozhin, "Comparison of conventional partitioning systems used for studying the hydrophobicity of polar organic compounds," J. Chromatogr. A, vol. 216, no. 0, pp. 103–113, Oct. 1981.

[151] M. H. Abraham, "Scales of solute hydrogen-bonding: their construction and application to physicochemical and biochemical processes," Chem. Soc. Rev., vol. 22, no. 2, pp. 73–83, 1993.

[152] J. A. Platts, D. Butina, M. H. Abraham, and A. Hersey, "Estimation of Molecular Linear Free Energy Relation Descriptors Using a Group Contribution Approach," J. Chem. Inf. Comput. Sci., vol. 39, no. 5, pp. 835–845, Sep. 1999.

[153] M. H. Abraham, A. Ibrahim, and A. M. Zissimos, "Determination of sets of solute descriptors from chromatographic measurements.," J. Chromatogr. A, vol. 1037, no. 1–2, pp. 29–47, May 2004.

[154] M. H. Abraham and J. C. McGowan, "The use of characteristic volumes to measure cavity terms in reversed phase liquid chromatography," Chromatographia, vol. 23, no. 4, pp. 243–246, 1987.

[155] J. S. Arey, W. H. J. Green, and P. M. Gschwend, "The electrostatic origin of Abraham's solute polarity parameter.," J. Phys. Chem. B, vol. 109, no. 15, pp. 7564–7573, Apr. 2005.

[156] M. H. Abraham, A. M. Zissimos, J. G. Huddleston, H. D. Willauer, R. D. Rogers, and W. E. Acree, "Some Novel Liquid Partitioning Systems: Water–Ionic Liquids and Aqueous Biphasic Systems," Ind. Eng. Chem. Res., vol. 42, no. 3, pp. 413–418, Feb. 2003.

[157] J. G. Huddleston, H. D. Willauer, M. T. Burney, L. J. Tate, A. D. Carruth, and R. D. Rogers, "Comparison of an empirical and a theoretical linear solvation energy relationship applied to the characterization of solute distribution in a poly(ethylene) glycol-salt aqueous biphasic system.," J. Chem. Inf. Comput. Sci., vol. 44, no. 2, pp. 549–558, 2004.

[158] M. Plass, K. Valko, and M. H. Abraham, "Determination of solute descriptors of tripeptide derivatives based on high-throughput gradient high-performance liquid chromatography retention data1Dedicated to Professor A. Kolbe on the occasion of his 65th birthday.1," J. Chromatogr. A, vol. 803, no. 1, pp. 51–60, 1998.

[159] P. Ball, "Water as a Biomolecule," ChemPhysChem, vol. 9, no. 18, pp. 2677–2685, 2008.

[160] P. Ball, "Biophysics: More than a bystander," Nature, vol. 478, no. 7370, pp. 467–468, Oct. 2011.

[161] P. Ball, "Concluding remarks: Cum grano salis," Faraday Discuss., vol. 160, no. 0, pp. 405–414, 2013.

[162] S. S. Negi and W. Braun, "Statistical analysis of physical-chemical properties and prediction of protein-protein interfaces," J. Mol. Model., vol. 13, no. 11, pp. 1157–1167, Nov. 2007.

[163] S. A. K. Ong, H. H. Lin, Y. Z. Chen, Z. R. Li, and Z. Cao, "Efficacy of different protein descriptors in predicting protein functional families," BMC Bioinformatics, vol. 8, no. 1, p. 300, 2007.

[164] A. Zaslavsky, P. Madeira, L. Breydo, V. N. Uversky, A. Chait, and B. Zaslavsky, "High throughput characterization of structural differences between closely related proteins in solution," Biochim. Biophys. Acta - Proteins Proteomics, vol. 1834, no. 2, pp. 583–592, Feb. 2013.

[165] P. P. Madeira, C. A. Reis, A. E. Rodrigues, L. M. Mikheeva, and B. Y. Zaslavsky, "Solvent Properties Governing Solute Partitioning in Polymer/Polymer Aqueous Two-Phase Systems: Nonionic Compounds," J. Phys. Chem. B, vol. 114, no. 1, pp. 457–462, Jan. 2010.

[166] P. P. Madeira et al., "Study of organic compounds–water interactions by partition in aqueous two-phase systems," J. Chromatogr. A, vol. 1322, pp. 97–104, 2013.

This chapter is based on the following paper

Nuno R. da Silva[a], Luisa A. Ferreira[a,b], Larissa M. Mikheeva[b], José A. Teixeira[a] and Boris Y. Zaslavsky[b] (2014). Origin of salt additive effect on solute partitioning in aqueous polyethylene glycol-8000-sodium sulfate two-phase system. *Journal of Chromatography A*, 1337, 3-8.

[a] Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga (Portugal)

[b] Analiza Inc./Cleveland Diagnostics, Superior Ave, Cleveland, OH (USA)

Highlights

- Relative hydrophobicity and electrostatic properties of the two phases in PEG8K–Na_2SO_4 ATPS with 0–0.54 M NaCl, NaSCN, $NaClO_4$, and NaH_2PO_4 additives are characterized;
- Partition coefficients for multiple organic compounds are analyzed in terms of linear solvent regression relationship;
- Effects of the salts additives are found to be related to the salts' influence on the water structure.

3.1. Introduction

ATPSs are formed in mixtures of two (or more) water-soluble polymers or a single polymer and a specific salt in an aqueous solution above certain critical concentrations or temperature. Two

immiscible aqueous phases are formed in such mixtures. The uniqueness of these systems is that each of the phases contains over 80% water on a molal basis, but the phases are immiscible and differ in their solvent properties [1]–[4]. Therefore, they can be used for the discriminating distribution of added solutes. ATPSs have been used for separation of biological macromolecules and particles for over 50 years [5]–[7] and recently found use in a variety of analytical applications as well [8], [9].

The most studied polymer–salt ATPSs are formed by PEG and inorganic salts, such as sodium sulfate, phosphate, carbonate or citrate. Salt effects on polymers and biopolymers, such as proteins, in water generally follow the well-known Hofmeister series [10] with qualitative order of the anions being:

$$\text{citrate}^{3-} > CO_3^{2-} > SO_4^{2-} > H_2PO_4^{-} > F^{-} > OH^{-} > Cl^{-} > NO_3^{-} > Br^{-} > I^{-} > ClO_4^{-} > SCN^{-}$$

Anions to the left of chloride are often called kosmotropes and they typically stabilize proteins and salt them out of solution. Anions to the right of chloride are called chaotropes and they commonly destabilize proteins and salt them into solution. The positions of some anions in the series, such as ClO_4^{-} and SCN^{-} or SO_4^{2-} and $H_2PO_4^{-}$ may interchange depending on the particular effect in question and the nature of cation. The Hofmeister effect is commonly explained by the ability of various ions to “make” or “break” water structure in aqueous solution [11], [12]. This explanation was put in doubt by many recent experimental and theoretical [13]–[16] studies showing that the properties of bulk water are not noticeably perturbed by ions in solution. It should be mentioned, however, that some recent experimental data [11], [12], [17] contradict this conclusion. The mechanism of the ions’ effects on proteins in aqueous solutions remains debatable.

The anions of sodium salts capable of forming ATPSs with PEG (citrate, carbonate, sulfate, phosphate, fluoride, and hydroxide) all belong to the kosmotropes. Attempts to investigate mechanism of phase separation in aqueous polymer–salt mixtures based on thermodynamic analysis of experimental data [18], [19] can hardly be viewed as successful and any attempt at considering phase separation in aqueous solution based on deviation from ideality is doomed. This issue, however, is beyond the scope of the present work.

One of the most interesting aspects of solutes behavior in PEG–salt ATPSs is that relatively small amounts of salt additives may significantly affect the solute partitioning [20]–[22]. The most widely used salt additive in this ATPS is NaCl [20]–[22]. The data obtained in the studies of protein partition behavior, however, typically leave too much room for interpretation being assigned to the protein-ion

specific interactions, protein conformational changes and other often hard to prove (and disprove) possibilities. The data obtained for structurally simple organic compounds generally cannot be interpreted as ambiguously and hence may provide better insight into mechanism of the effect under study.

Recently, effects of different salt additives (NaCl, NaSCN, $NaClO_4$, NaH_2PO_4) over a wide concentration range up to ca. 2 M on partition behavior of small organic compounds in PEG8K–Na_2SO_4 were reported [23]. Later we examined the effect of NaCl in the concentration range of 0-1.9 M on the phase diagram of this ATPS and characterized solvent properties of the phases [24].

We extended this study here and examined the differences between the relative hydrophobicities and electrostatic properties of the phases in PEG8K–Na_2SO_4 ATPS with additives of NaSCN, $NaClO_4$, and NaH_2PO_4. As reported previously [23], adenosine and guanosine displayed complicated partition behavior in PEG8K–Na_2SO_4 ATPS with increasing concentration of NaSCN, $NaClO_4$, and NaH_2PO_4 additives. Their partition coefficients increased with salt additive concentration increasing up to ~0.2 M and decreased with further increasing salt concentration. Therefore, we also examined here partitioning of adenine and adenosine mono-, di-, and tri-phosphates in all the ATPSs, including those with NaCl additive. We attempted to answer the question of how relatively small amounts of the above salt additives may affect solute partitioning in ATPS formed by exceeding amount of sodium sulfate and PEG.

3.2. Experimental

3.2.1. Materials

Polyethylene glycol 8000 (Lot 048K00241) with an average molecular weight (Mw) of 8000 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Adenine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP) were purchased from Sigma-Aldrich. Dinitrophenylated (DNP) amino acids – DNP-alanine, DNP-norvaline, DNP-norleucine, and DNP-α-amino-*n*-octanoic acid, were purchased from Sigma-Aldrich. The sodium salts of the DNP-amino acids were prepared by titration. All salts and other chemicals used were of analytical-reagent grade.

3.2.2. Methods

3.2.2.1. Aqueous two-phase systems

Stock solutions of PEG8K (50 wt.%) and Na_2SO_4 (20.3 wt.%) were prepared in water. Sodium phosphate buffer (NaPB; 0.5 M, pH 6.8) was prepared by mixing 3.45 g of $NaH_2PO_4 \cdot H_2O$ and 6.70 g $Na_2HPO_4 \cdot 7H_2O$ in 100 mL aqueous solution. A mixture of PEG and salts was prepared by dispensing appropriate amounts of the aqueous stock PEG8K Na_2SO_4 and NaPB solutions into a 1.2 mL microtube using a Hamilton (Reno, NV, USA) ML-4000 four-probe liquid-handling workstation. Appropriate amounts of water and/or stock salt additives solutions were added to give the required ionic and polymer composition of the final system with total weight of 0.5 g (after addition of the solute sample, see below). All ATPSs had a fixed composition of 11.10 wt.% PEG8K, 6.33 wt.% Na_2SO_4 and 0.01 M NaPB, pH 6.8, with different salt additive concentrations as indicated below. Stock solutions of each salt additive (NaCl, $NaClO_4$, NaSCN or NaH_2PO_4) of 0.5 or 5.0 M concentration were prepared and appropriated amounts were added to the two-phase systems to provide the required concentrations from 0.027 M up to ca. 0.5 M.

3.2.2.2. Partitioning experiments

The aqueous two-phase partitioning experiments were performed using an automated instrument, Automated Signature Workstation, ASW (Analiza, Cleveland, OH, USA). The ASW system is based on the ML-4000 liquid-handling workstation (Hamilton Company) integrated with a UV–VIS microplate spectrophotometer (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA, USA). Solutions of all compounds were prepared in water at concentrations of 2-100 mM, depending on the compound solubility. Varied amounts (0, 20, 40, 60, 80, and 100 µl) of a given compound solution and the corresponding amounts (100, 80, 60, 40, 20, and 0 µl) of water were added to a set of the same polymer–salts mixtures. The systems were vortexed in a multi-pulse vortexer and centrifuged for 60 min at 3000 × g at 23 °C temperature in a refrigerated centrifuge (Jouan, BR4i) to accelerate phase settling. The upper phase in each system was partially removed, the interface discarded, and aliquots of 15-75 µl from the upper and lower phases were withdrawn in duplicate for analysis. The aliquots were transferred into microplate wells and diluted up to 300 µl. In the cases

of considerable difference between the concentrations of a given compound in one phase relative to the other phase, different dilution factors were used for the upper and lower phases. Water was used as a diluent for all compounds. The microplate was sealed and following moderate shaking for 30 min in an incubator (Vortemp 56EVC, Labnet International, Edison, NJ, USA) at room temperature and short centrifugation (3 min at 1500 rpm), optical absorbance was measured at the wavelength of maximum absorption with the UV–VIS plate reader. The maximum absorption wavelength for each compound was determined in separate experiments by analysis of the absorption spectrum over the 250-500 nm range. In all measurements the correspondingly diluted pure phases were used as blank solutions.

The partition coefficient, K, defined as the ratio of the sample concentration in the upper phase to the sample concentration in the lower phase was determined as the slope of the compound concentration in the upper phase plotted as a function of the concentration in the lower phase averaged over the results obtained from two to four partition experiments carried out at the specified polymer and salt composition of the system, taking into consideration the corresponding dilution factors used in the experiment. Deviation from the average K-value was consistently below 3% and, in most cases, lower than 2%.

3.3. Results and discussion

In order to characterize the differences between the relative hydrophobicities and electrostatic properties of the phases in the ATPSs with varied concentrations of salt additives, the partition coefficients of the homologous series of sodium salts of DNP-amino acids with aliphatic side-chains (alanine, norvaline, norleucine, and α-amino-n-caprylic acid) in each ATPS were analyzed as follows. Typical experimental data obtained for sodium salts of the DNP-amino acids are plotted in Fig. 3.1, and the linear curves observed may be described as:

$$\ln K_{DNP-AA}^{(i)} = C^{(i)} + E^{(i)} N_C$$

(Equation 3.1)

where $K_{DNP\text{-}AA}$ is the partition coefficient of a DNP-amino acid with aliphatic side-chain; superscript *(i)* denotes the particular i-th ATPS used for the partition experiments; N_c is equivalent number of CH_2 groups in the aliphatic side-chain of a given DNP-amino acid; E is an average *ln K* increment per CH_2 group; C represents the total contribution of the non-alkyl part of the structure of a DNP-amino acid into $ln\ K_{DNP\text{-}AA}$.

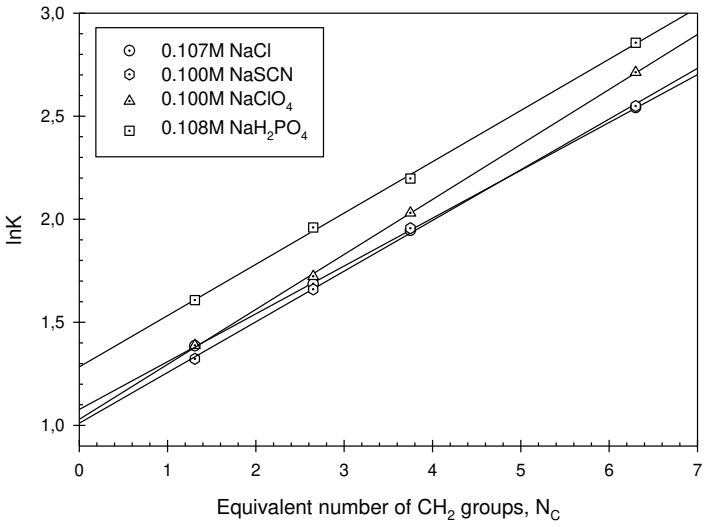


Figure 3.1. Partition coefficients of sodium salts of DNP-AA with aliphatic side-chains as functions of side-chain lengths in PEG8K–Na_2SO_4 system with indicated concentration of salt additive (pH 6.8; temperature 23 °C).

The coefficients $E^{(i)}$ and $C^{(i)}$ values determined for all the ATPSs examined are listed in Table 3.1. As the standard free energy of transfer of a solute between the coexisting phases is described as:

$$\Delta G^0 = -RT\ \ln K$$

(Equation 3.2)

where R is the universal gas constant and T is the absolute temperature in Kelvin, it follows that:

$$\Delta G^0(CH_2) = -RTE$$

(Equation 3.3)

where $\Delta G^0(CH_2)$ is the standard free energy of transfer of a methylene group from one phase to another. The $\Delta G^0(CH_2)$ values calculated from the experimental data with Eqs. 3.1-3.3 are listed in Table 3.1.

Table 3.1. Coefficients *E* and *C* (see Eq. 3.1) and the free energy of transfer of a CH_2 group between the coexisting phases of PEG8K–Na_2SO_4 ATPS of a fixed composition with indicated concentration of salt additives.

NaCl, M [24]*	E [24]*	C [24]*	$-\Delta G^o(CH_2)$, cal/mol [24]*
0	0.207±0.005	1.120±0.020	122±2.9
0.027	0.214±0.006	1.040±0.020	126±3.5
0.053	0.223±0.005	1.020±0.020	131±2.9
0.107	0.230±0.002	1.086±0.008	135±1.2
0.215	0.259±0.001	1.002±0.005	125±0.6
0.543	0.284±0.002	0.983±0.008	167±1.2
1.109	0.341±0.004	1.020±0.020	200±2.4
1.936	0.438±0.006	1.150±0.020	258±3.5

NaSCN, M	E	C	$-\Delta G^o(CH_2)$, cal/mol
0	0.207±0.005	1.120±0.020	122±2.9
0.025	0.209±0.004	1.100±0.020	123±2.4
0.050	0.230±0.011	1.050±0.040	135±6.5
0.100	0.246±0.005	1.010±0.020	145±2.9
0.215	0.260±0.010	0.810±0.040	153±5.9
0.543	0.263±0.003	0.590±0.010	155±1.8
1.109	0.275±0.003	0.440±0.010	162±1.8
1.936	0.311±0.005	0.220±0.020	183±2.9

Table 3.1. (cont.) Coefficients *E* and *C* (see Eq. 3.1) and the free energy of transfer of a CH_2 group between the coexisting phases of PEG8K–Na_2SO_4 ATPS of a fixed composition with indicated concentration of salt additives.

$NaClO_4$, M	E	C	$-\Delta G^0(CH_2)$, cal/mol
0	0.207±0.005	1.120±0.020	122±2.9
0.027	0.219±0.002	1.175±0.007	129±1.2
0.054	0.228±0.005	1.190±0.020	134±2.9
0.108	0.250±0.010	1.030±0.030	147±5.9
0.216	0.263±0.005	0.910±0.020	155±2.9
0.549	0.300±0.001	0.706±0.005	177±0.6
1.157	0.363±0.001	0.449±0.001	214±0.6
1.759	0.443±0.005	0.170±0.020	261±2.9

NaH_2PO_4, M	E	C	$-\Delta G^0(CH_2)$, cal/mol
0	0.207±0.005	1.120±0.020	122±2.9
0.027	0.220±0.010	1.060±0.040	131±5.9
0.054	0.230±0.008	1.170±0.030	135±4.7
0.108	0.249±0.005	1.280±0.020	147±2.9
0.217	0.276±0.002	1.562±0.009	162±1.2
0.556	0.420±0.010	1.930±0.050	247±5.9
1.160	0.580±0.010	2.820±0.030	341±5.9
1.751	-	-	-

* Data for the NaCl additive were previously reported [24] and are presented here for comparison.

Examination of the parameter E or $\Delta G^{\circ}(CH_2)$ values listed in Table 3.1, and plotted in Fig. 3.2, indicates that the difference between the relative hydrophobicity of the phases increases with increasing concentration of each salt additive.

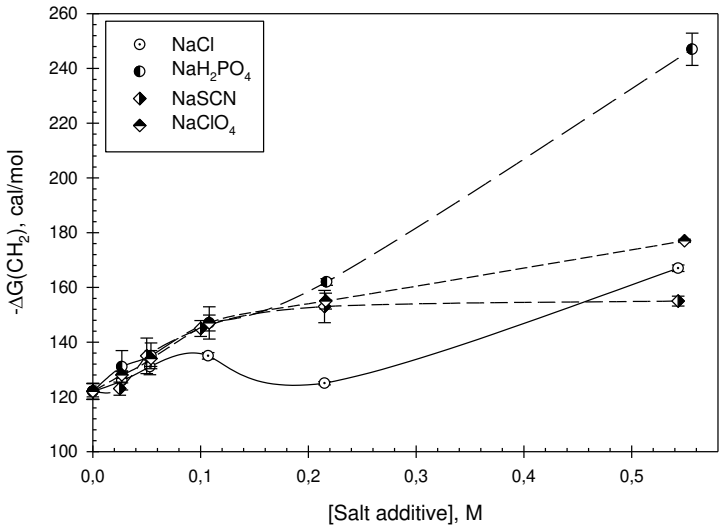


Figure 3.2. The standard free energy of transfer of a CH_2 group between the coexisting phases, $\Delta G^{\circ}(CH_2)$, as function of the salt additive concentration. Lines are added for eye-guidance only.

The difference between the electrostatic properties of the phases represented by parameter C value in Eq. 3.1 (see in Table 3.1) also changes with increasing concentration of each salt additive in a salt-specific manner as shown in Fig. 3.3.

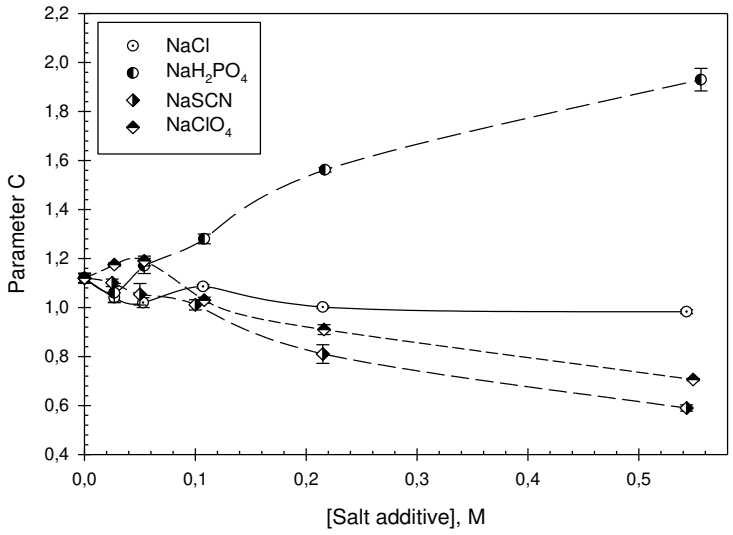


Figure 3.3. The difference between the electrostatic properties of the phases, parameter *C*, as function of the salt additive concentration. Lines are added for eye-guidance only.

It was suggested by Zaslavsky [1], pp. 208-216, to use the contribution of an ionic group into the solute partition coefficient as an empirical measure of the difference in question. The experimental results here were obtained with sodium salts of *p*-dinitrophenyl-amino acids, i.e., compounds possessing a DNP-NH-CH-COO^--Na^+ group. This moiety is bulky and contains a substituted aromatic ring. Use of this particular group as a probe for electrostatic ion-ion, ion-dipole and dipole-dipole interactions obviously has some limitations. Only to a first approximation the free energy of transfer of this group between the coexisting phases of an ATPS may be viewed as a measure of the ability of aqueous media to participate in a particular kind of intermolecular interactions. The difference between the electrostatic properties of the two phases characterized by parameter *C* value is governed mostly by the difference between the ionic composition of the phases.

As shown in Fig. 3.3, in the presence of NaCl additive the parameter *C* value decreases gradually with increasing NaCl concentration except at the NaCl concentration of ~0.1 M where parameter *C*-value increases. It might be attributed to the fact that the phase diagrams for the ATPSs with 0-0.05 M NaCl additive are extremely close [23], their phase compositions are very similar, and that the first noticeable shift of the phase diagram is observed at NaCl concentration of ~0.1 M. The data presented in Fig. 3.3 show that the difference between the electrostatic properties of the phases increases significantly with increasing concentration of NaH_2PO_4 additive likely because this salt is immiscible with PEG and in the PEG–sulfate ATPS it concentrates in the bottom sulfate-rich phase. NaSCN and $NaClO_4$ additives in contrast to NaH_2PO_4 are likely to distribute predominantly in the

upper PEG-rich phase [1] and that is why the parameter *C* value gradually decreases with increasing NaSCN and $NaClO_4$ concentrations. It seems that the salt additive effect changes according to the anion position in Hofmeister series: $H_2PO_4^-$ > Cl^- > ClO_4^- ≈ SCN^-.

Partition coefficients for adenine, AMP, ADP, and ATP, in all ATPSs are listed in Table 3.2. It was reported recently [25] that partition coefficients of solutes in PEG–salt ATPSs with and without salt additives may be compared based on the so-called Collander linear solvent regression relationship [26]–[30]:

$$\ln K^i_{PEG-Sulfate-Salt} = a_{i0} + b_{i0} \ln K^i_{PEG-Sulfate}$$

(Equation 3.4)

where $K_{PEG-Sulfate-Salt}$ is the partition coefficient of the i-th compound in PEG–sulfate ATPS with a given salt additive; $K_{PEG-Sulfate}$ is the partition coefficient of the same i-th compound in PEG–sulfate ATPS without salt additive; and a_{i0} and b_{i0} are constants depending on the ATPSs under comparison.

Table 3.2. Partition coefficients of compounds in ATPSs 11.10 wt.% PEG8K – 6.33 wt.% Na_2SO_4 – 0.01 M NaPB, pH 6.8 with indicated concentration of salt additives.

NaCl, M	Adenine	AMP	ADP	ATP
0	3.40±0.12	1.01±0.05	0.75±0.02	0.62±0.04
0.027	3.20±0.12	0.97±0.06	0.70±0.02	0.57±0.03
0.053	2.95±0.08	0.95±0.06	0.57±0.02	0.52±0.02
0.107	2.80±0.11	0.88±0.04	0.60±0.01	0.46±0.02
0.215	3.70±0.16	0.83±0.04	0.54±0.03	0.40±0.02
0.543	4.10±0.15	0.72±0.02	0.43±0.03	0.30±0.02

Table 3.2. (cont.) Partition coefficients of compounds in ATPSs 11.10 wt.% PEG8K – 6.33 wt.% Na_2SO_4 – 0.01 M NaPB, pH 6.8 with indicated concentration of salt additives.

$NaClO_4$, M	Adenine	AMP	ADP	ATP
0.027	3.29±0.05	0.91±0.01	0.63±0.01	0.46±0.01
0.054	3.57±0.07	0.80±0.03	0.53±0.01	0.37±0.02
0.108	3.78±0.07	0.65±0.02	0.40±0.01	0.26±0.02
0.216	4.10±0.20	0.50±0.01	0.30±0.01	0.18±0.01
0.549	4.10±0.10	0.34±0.01	0.19±0.01	0.11±0.01

NaSCN, M	Adenine	AMP	ADP	ATP
0.025	3.27±0.04	0.90±0.01	0.64±0.01	0.47±0.01
0.050	3.52±0.01	0.80±0.01	0.540±0.003	0.39±0.01
0.100	3.70±0.20	0.68±0.01	0.431±0.004	0.29±0.02
0.215	3.75±0.07	0.52±0.01	0.32±0.01	0.21±0.01
0.543	3.51±0.05	0.41±0.01	0.253±0.003	0.16±0.01

NaH_2PO_4, M	Adenine	AMP	ADP	ATP
0.027	2.90±0.02	1.28±0.02	0.89±0.02	0.64±0.01
0.054	2.80±0.03	1.38±0.02	0.89±0.01	0.63±0.01
0.108	2.61±0.04	1.41±0.03	0.86±0.01	0.57±0.01
0.217	2.18±0.09	1.26±0.02	0.74±0.01	0.41±0.01

The Collander equation [26]–[30] describes a linear correlation between distribution coefficients of solutes of the similar chemical nature in different organic solvent–water biphasic systems. The coefficients of the relationship (slope and intercept) depend on the particular systems under

comparison as well as on the type of the solutes being examined. It was suggested that these coefficients represent the distinctive features of the interactions of the solute functional moieties with the solvents being compared [1]. It has been shown that the Collander relationship may be used for comparison of partition coefficients for solutes in PEG–Na_2SO_4 ATPSs with NaCl additives at different concentrations [24].

Typical examples of the observed relationships described by Eq. 3.4 are presented in Fig. 3.4.

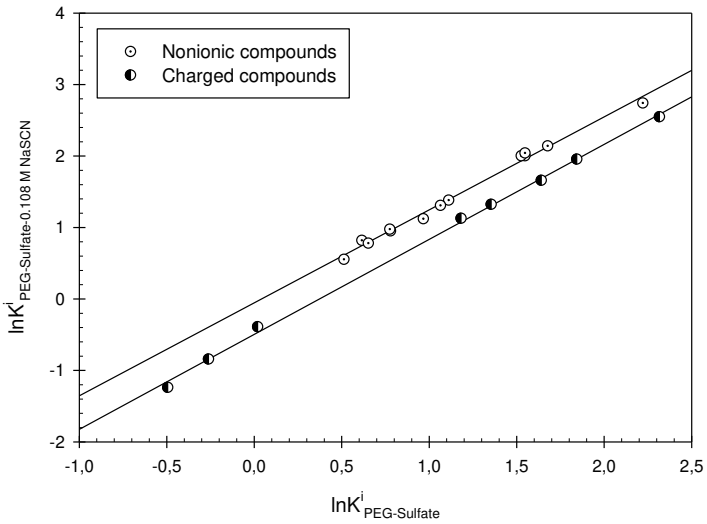


Figure 3.4. Logarithms of partition coefficients for ionic and nonionic compounds in PEG8K–Na_2SO_4 systems with 0.108 M NaSCN versus logarithms of partition coefficients for same compounds in PEG8K–Na_2SO_4 systems without salt additive (reference system).

The a_{i0} and b_{i0} coefficients values and the corresponding correlation coefficients for compounds examined here and previously reported [23], [24] are listed in Table 3.3. These data indicate that: (i) the relationships for nonionic and charged compounds are shifted relatively to each other but are essentially parallel (have the same b_{i0} coefficients), and (ii) the data scatter increases (correlation coefficients decrease) with increasing salt additive concentration.

Table 3.3. Coefficients a_{io} and b_{io} in solvent regression equation (Eq. 3.4) with PEG8K–Na_2SO_4 ATPSs without salt additive used as a reference system.

[Salt], M	COMPOUND	a_{io}	b_{io}	r^2*
NaCl, 0.027	Nonionic	-0.02±0.02	1.05±0.01	0.9981
	Charged	-0.06±0.01	1.04±0.01	0.9991
NaCl, 0.053	Nonionic	-0.04±0.01	1.09±0.01	0.9989
	Charged	-0.15±0.03	1.11±0.02	0.9966
NaCl, 0.107	Nonionic	-0.07±0.03	1.17±0.03	0.9942
	Charged	-0.18±0.02	1.17±0.01	0.9991
NaCl, 0.215	Nonionic	-0.04±0.02	1.25±0.01	0.9987
	Charged	-0.27±0.02	1.24±0.02	0.9983
NaCl, 0.543	Nonionic	-0.03±0.02	1.38±0.02	0.9979
	Charged	-0.46±0.03	1.38±0.02	0.9982
$NaClO_4$, 0.027	Nonionic	-0.02±0.02	1.14±0.01	0.9984
	Charged	-0.15±0.01	1.18±0.01	0.9990
$NaClO_4$, 0.054	Nonionic	-0.04±0.01	1.09±0.01	0.9956
	Charged	-0.07±0.03	1.16±0.02	0.9984
$NaClO_4$, 0.108	Nonionic	-0.12±0.05	1.41±0.04	0.9888
	Charged	-0.55±0.03	1.42±0.02	0.9983
$NaClO_4$, 0.215	Nonionic	-0.14±0.07	1.52±0.06	0.9784
	Charged	-0.83±0.04	1.50±0.03	0.9968
$NaClO_4$, 0.549	Nonionic	-0.30±0.10	1.73±0.09	0.9609
	Charged	-1.23±0.04	1.68±0.03	0.9973

Table 3.3. (cont.) Coefficients a_{io} and b_{io} in solvent regression equation (Eq. 3.4) with PEG8K–Na_2SO_4 ATPSs without salt additive used as a reference system.

[Salt], M	COMPOUND	a_{io}	b_{io}	r^2
NaSCN, 0.025	Nonionic	-0.04±0.02	1.14±0.01	0.9984
	Charged	-0.16±0.02	1.12±0.01	0.9990
NaSCN, 0.050	Nonionic	-0.05±0.03	1.22±0.02	0.9968
	Charged	-0.29±0.02	1.22±0.02	0.9984
NaSCN, 0.100	Nonionic	-0.05±0.04	1.30±0.03	0.9926
	Charged	-0.50±0.02	1.33±0.02	0.9987
NaSCN, 0.215	Nonionic	-0.12±0.05	1.43±0.04	0.9991
	Charged	-0.77±0.03	1.40±0.03	0.9971
NaSCN, 0.543	Nonionic	-0.17±0.08	1.50±0.07	0.9698
	Charged	-1.01±0.03	1.42±0.02	0.9983
NaH_2PO_4, 0.027	Nonionic	-0.02±0.03	1.07±0.03	0.9938
	Charged	0.11±0.04	0.97±0.03	0.9941
NaH_2PO_4, 0.054	Nonionic	-0.04±0.02	1.13±0.02	0.9839
	Charged	-0.12±0.02	1.17±0.01	0.9952
NaH_2PO_4, 0.108	Nonionic	-0.04±0.09	1.20±0.07	0.9458
	Charged	0.07±0.03	1.17±0.02	0.9978
NaH_2PO_4, 0.217	Nonionic	0.06±0.04	1.35±0.03	0.9942
	Charged	0.01±0.05	1.42±0.04	0.9947
NaH_2PO_4, 0.556	Nonionic	-0.07±0.05	2.00±0.04	0.9920
	Charged	-0.16±0.01	1.97±0.01	0.9997

The coefficients b_{i0} obtained for salt additives studied are plotted in Fig. 3.5 as functions of the salt additives concentrations. It should be noted particularly that the NaH_2PO_4 additive affects the coefficient b_{i0} much more significantly (and linearly) than the other salts additives examined.

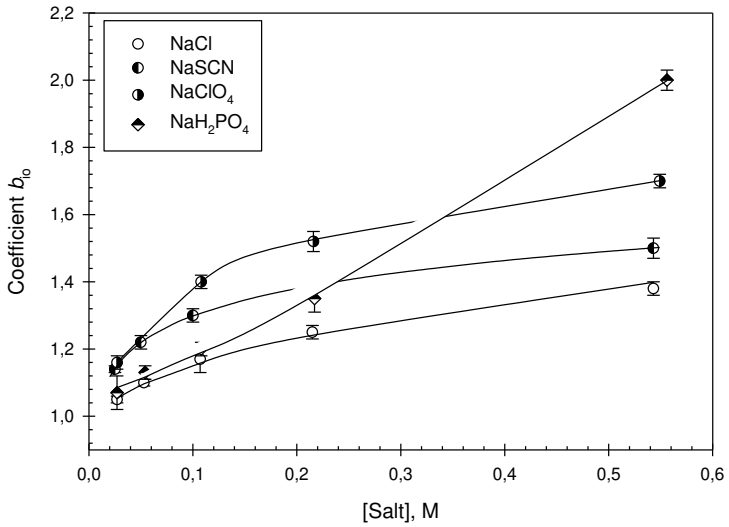


Figure 3.5. Coefficients b_{i0} in solvent regression equation (Eq. 3.4, PEG8K–Na_2SO_4 ATPS without salt additive used as a reference system) as functions of the salt additive concentration. Lines are added for eye-guidance only.

The reason seems to be that this salt is the only one among those studied that is immiscible with PEG and concentrates completely in the sulfate-rich phase. Other salts distribute predominantly into PEG-rich phase (NaSCN, $NaClO_4$) or in both phases (NaCl) depending on the salt additive concentration. The salt additives concentrations dependences of the b_{i0} coefficients shown in Fig. 3.5 may be described as:

$$\log b_{i0}^{salt} = \alpha^{salt} + \beta^{salt} \log[salt]$$

(Equation 3.5)

where *[salt]* is the salt additive concentration in PEG–sulfate ATPS, expressed in mol/kg; b_{i0}^{salt} is the b_{i0} coefficient for a given salt additive concentration; α^{salt} and β^{salt} are constants. The α^{salt} and β^{salt} coefficients values are listed in Table 3.4.

Table 3.4. Coefficients α^{salt} and β^{salt} values (Eq. 3.5) and the structural entropy of hydration [31] of anions of Na salts additives.

Anion	a^{salt}	b^{salt}	$D_{struc}S$, J K^{-1} mol^{-1}
ClO_4^-	0.263±0.005	0.121±0.005	107
$NaSCN^-$	0.222±0.006	0.105±0.005	83
Cl^-	0.158±0.004	0.090±0.003	58
$H_2PO_4^-$	0.130±0.010	0.064±0.008	-4

The relationships under discussion are presented in Fig. 3.6. It should be mentioned that the concentration range for each salt additive used was up to ~0.5 M. For NaH_2PO_4 additive the b_{i0} coefficients determined for nonionic solutes differ significantly from those for charged compounds, and only the former were used, as partition behavior of charged compounds in the ATPS PEG–Na_2SO_4–NaH_2PO_4 with both salts in the bottom phase only may have more complicated mechanism of partitioning. Averaged b_{i0} coefficients determined for nonionic and charged compounds were used for ATPS with other salts additives.

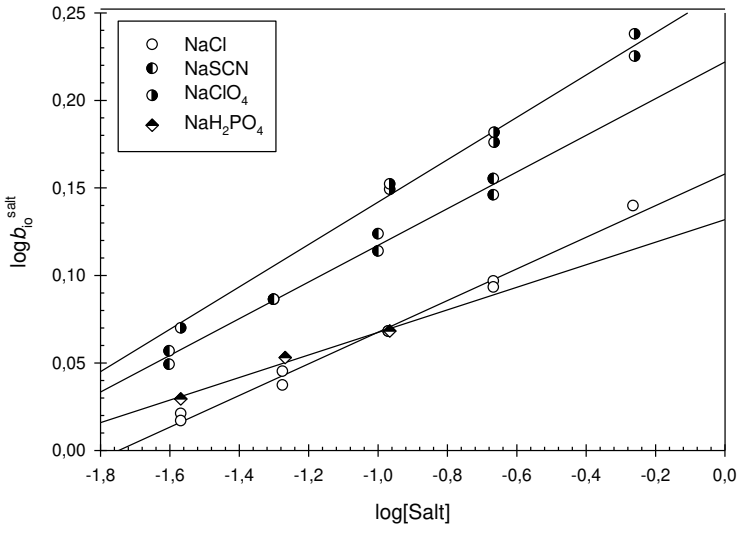


Figure 3.6. Salt additive concentration dependence of the b_{io}^{salt} coefficients (Eq. 3.5).

It was suggested previously [1] that phase separation and partitioning of solutes in ATPS is governed by different effects of phase-forming components on the water structure. Hence, it seems reasonable to compare the effects of salts additives (or their anions) on the solute partitioning observed here and represented by the α^{salt} and β^{salt} coefficients with the effects of these anions on the water structure. If we define the structure of water according to Marcus [11], as the extent of hydrogen bonding, the effect of ions on the structure means the enhancement or diminution of this extent. These effects may be quantified by the structural entropies of hydration of ions as discussed by Marcus [11], [31]. These entropies values, $\Delta_{struc}S$, taken from [31] are listed in Table 3.4 for the anions of the Na salts additives explored. The plots of α^{salt} and β^{salt} coefficients versus $\Delta_{struc}S$ are presented in Fig. 3.7.a and 3.7.b, respectively. The relationship observed for coefficient β^{salt} in Fig. 3.7.b may be described as:

$$\beta^{salt} = -0.073_{\pm 0.004} + 0.011_{\pm 0.003} \times exp^{\left(\frac{\Delta_{struc}S}{71_{\pm 11}}\right)}$$

N = 4; r^2 = 0.9988; SD = 0.001; F = 406

(Equation 3.6)

where β^{salt} and $\Delta_{struc}S$ are defined above; N is the number of salt additives examined; r^2 is the correlation coefficient; SD is the standard deviation; and F is the ratio of variance. The relationship observed for α^{salt} coefficient may be partially fitted by power function as shown in Fig. 3.7.a.

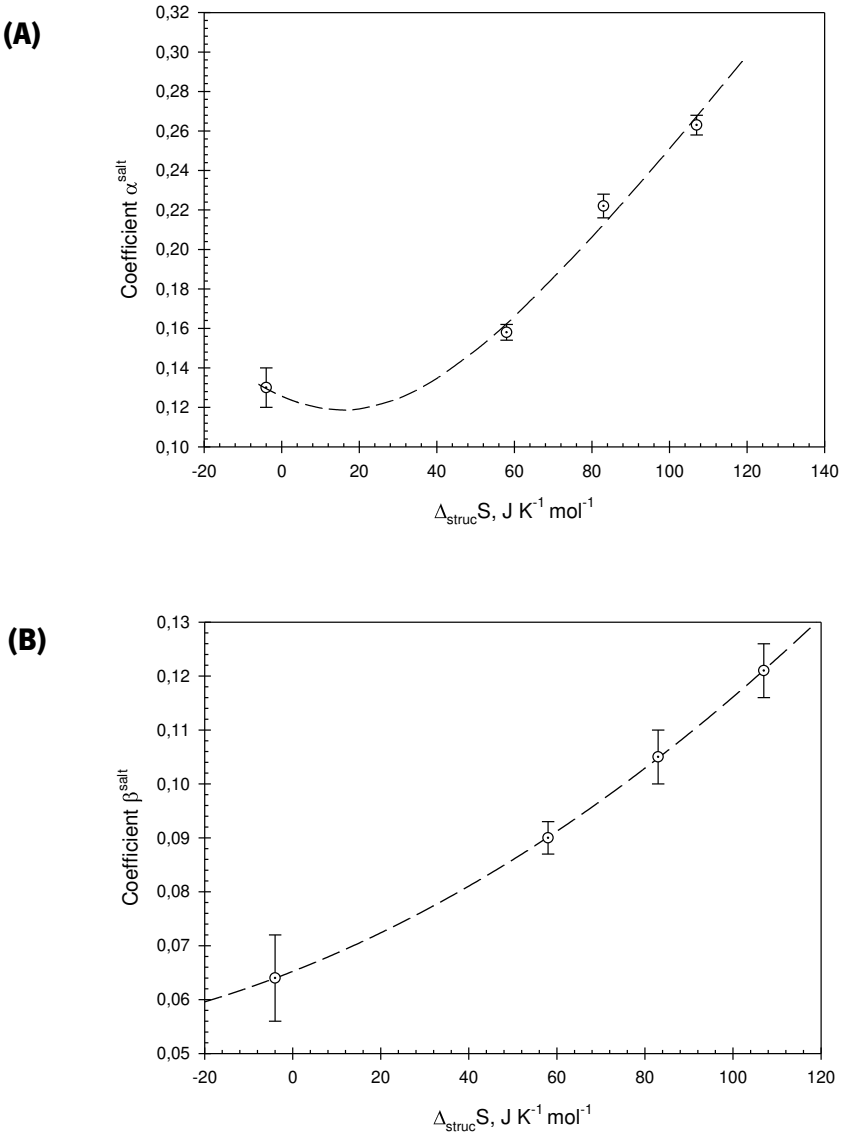


Figure 3.7. Coefficients **(A)** α^{salt} and **(B)** β^{salt} values (Eq. 3.6) as a function of the structural entropy of hydration [31] of anions of Na salts additives. In **(A)** line is for eye-guidance only.

The α^{salt} and β^{salt} coefficients in Eq. 3.5 characterize the effect of salt additive concentration on partition coefficients of different small organic compounds in the PEG–Na_2SO_4–salt additive ATPSs. The observed relationships for both α^{salt} and β^{salt} coefficients suggest that the effects of the salt additives on solute partitioning in the PEG–Na_2SO_4 ATPS originate from their effects on the water structure in the phases of ATPS. This finding agrees with the data reported previously [32] about effects of different salt additives on the solvent properties of aqueous media in different ATPSs formed by two nonionic polymers. It was shown in [32] that the difference between the electrostatic

properties of the coexisting phases may be described only taking into account the effect of a given salt additive on the hydrogen bonds in water. This effect was represented by a parameter ($K_{b/l}$), characterizing the equilibrium between populations of hydrogen bonds with a bent hydrogen bond conformation and with linear hydrogen bond conformation affected by a given salt additive influence on the hydrogen bonds network in water [17]. The fact that similar effects are displayed with the salt additive concentration of ca. 0.025-0.1 M on the background of ~1 M Na_2SO_4 in the lower phase is rather surprising. The limited number of salts additives examined here prevents any general conclusion. Further work is necessary and is currently in progress in our laboratories.

References

[1] B. Y. Zaslavsky, *Aqueous Two-Phase Partitioning: Physical Chemistry and Bioanalytical Applications*. Taylor & Francis, 1994.

[2] H. D. Willauer, J. G. Huddleston, and R. D. Rogers, "Solvent Properties of Aqueous Biphasic Systems Composed of Polyethylene Glycol and Salt Characterized by the Free Energy of Transfer of a Methylene Group between the Phases and by a Linear Solvation Energy Relationship," *Ind. Eng. Chem. Res.*, vol. 41, no. 11, pp. 2591–2601, May 2002.

[3] M. L. Moody, H. D. Willauer, S. T. Griffin, J. G. Huddleston, and R. D. Rogers, "Solvent Property Characterization of Poly(ethylene glycol)/Dextran Aqueous Biphasic Systems Using the Free Energy of Transfer of a Methylene Group and a Linear Solvation Energy Relationship," *Ind. Eng. Chem. Res.*, vol. 44, no. 10, pp. 3749–3760, Apr. 2005.

[4] P. P. Madeira, C. A. Reis, A. E. Rodrigues, L. M. Mikheeva, and B. Y. Zaslavsky, "Solvent Properties Governing Solute Partitioning in Polymer/Polymer Aqueous Two-Phase Systems: Nonionic Compounds," *J. Phys. Chem. B*, vol. 114, no. 1, pp. 457–462, Jan. 2010.

[5] P. Å. Albertsson, *Partition of Cell Particles and Macromolecules: Distribution and Fractionation of Cells, Viruses, Microsomes, Proteins, Nucleic Acids, and Antigen-antibody Complexes in Aqueous Polymer Two-phase Systems*. J. Wiley, 1960.

[6] D. E. BROOKS, K. I. M. A. SHARP, and D. FISHER, "2 - Theoretical Aspects of Partitioning," in *Partitioning in Aqueous Two-Phase System*, H. WALTER, D. E. BROOKS, and D. FISHER, Eds. Academic Press, 1985, pp. 11–84.

[7] H.-K. Rajni, *Aqueous Two-Phase Systems: Methods and Protocols*, 1st ed. Totowa, NJ:

Humana Press, 2000.

[8] M. Stovsky *et al.*, "Prostate-specific antigen/solvent interaction analysis: a preliminary evaluation of a new assay concept for detecting prostate cancer using urinary samples.," *Urology*, vol. 78, no. 3, pp. 601–605, Sep. 2011.

[9] A. Zaslavsky, P. Madeira, L. Breydo, V. N. Uversky, A. Chait, and B. Zaslavsky, "High throughput characterization of structural differences between closely related proteins in solution," *Biochim. Biophys. Acta - Proteins Proteomics*, vol. 1834, no. 2, pp. 583–592, Feb. 2013.

[10] W. Kuntz, P. Lo Nostro, and B. W. Ninham, "No Title," *Curr. Opin. Colloid Interface Sci.*, vol. 9, p. 1, 2004.

[11] Y. Marcus, "Effect of ions on the structure of water," *Pure Appl. Chem.*, vol. 82, no. 10, pp. 1889–1899, 2010.

[12] L. M. Pegram and M. T. Record, "Thermodynamic Origin of Hofmeister Ion Effects," *J. Phys. Chem. B*, vol. 112, no. 31, pp. 9428–9436, Jul. 2008.

[13] A. W. Omta, M. F. Kropman, S. Woutersen, and H. J. Bakker, "Negligible Effect of Ions on the Hydrogen-Bond Structure in Liquid Water," *Science (80-.).*, vol. 301, no. 5631, pp. 347–349, 2003.

[14] J. Kherb, S. C. Flores, and P. S. Cremer, "Role of Carboxylate Side Chains in the Cation Hofmeister Series," *J. Phys. Chem. B*, vol. 116, no. 25, pp. 7389–7397, Jun. 2012.

[15] Y. Cho, Y. Zhang, T. Christensen, L. B. Sagle, A. Chilkoti, and P. S. Cremer, "Effects of Hofmeister Anions on the Phase Transition Temperature of Elastin-like Polypeptides," *J. Phys. Chem. B*, vol. 112, no. 44, pp. 13765–13771, Oct. 2008.

[16] J. Paterová *et al.*, "Reversal of the Hofmeister Series: Specific Ion Effects on Peptides," *J. Phys. Chem. B*, vol. 117, no. 27, pp. 8150–8158, Jun. 2013.

[17] N. V Nucci and J. M. Vanderkooi, "Effects of salts of the Hofmeister series on the hydrogen bond network of water," *J. Mol. Liq.*, vol. 143, no. 2–3, pp. 160–170, Oct. 2008.

[18] R. Sadeghi and F. Jahani, "Salting-In and Salting-Out of Water-Soluble Polymers in Aqueous Salt Solutions," *J. Phys. Chem. B*, vol. 116, no. 17, pp. 5234–5241, Apr. 2012.

[19] L. H. M. da Silva and W. Loh, "Calorimetric Investigation of the Formation of Aqueous Two-Phase Systems in Ternary Mixtures of Water, Poly(ethylene oxide) and Electrolytes (Or Dextran)," *J. Phys. Chem. B*, vol. 104, no. 43, pp. 10069–10073, Oct. 2000.

[20] B. A. Andrews, S. Nielsen, and J. A. Asenjo, "Partitioning and purification of monoclonal antibodies in aqueous two-phase systems.," *Bioseparation*, vol. 6, no. 5, pp. 303–313, 1996.

[21] P. A. J. Rosa, A. M. Azevedo, S. Sommerfeld, M. Mutter, M. R. Aires-Barros, and W. Bäcker, "Application of aqueous two-phase systems to antibody purification: A multi-stage approach," *J. Biotechnol.*, vol. 139, no. 4, pp. 306–313, Feb. 2009.

[22] S. A. Oelmeier, F. Dismer, and J. Hubbuch, "Application of an aqueous two-phase systems high-throughput screening method to evaluate mAb HCP separation," *Biotechnol. Bioeng.*, vol. 108, no. 1, pp. 69–81, 2011.

[23] L. A. Ferreira, J. A. Teixeira, L. M. Mikheeva, A. Chait, and B. Y. Zaslavsky, "Effect of salt additives on partition of nonionic solutes in aqueous PEG–sodium sulfate two-phase system," *J. Chromatogr. A*, vol. 1218, no. 31, pp. 5031–5039, Aug. 2011.

[24] L. A. Ferreira, P. Parpot, J. A. Teixeira, L. M. Mikheeva, and B. Y. Zaslavsky, "Effect of NaCl additive on properties of aqueous PEG–sodium sulfate two-phase system," *J. Chromatogr. A*, vol. 1220, pp. 14–20, 2012.

[25] L. Ferreira, P. P. Madeira, L. Mikheeva, V. N. Uversky, and B. Zaslavsky, "Effect of salt additives on protein partition in polyethylene glycol–sodium sulfate aqueous two-phase systems," *Biochim. Biophys. Acta - Proteins Proteomics*, vol. 1834, no. 12, pp. 2859–2866, Dec. 2013.

[26] R. COLLANDER, "On lipoid solubility.," *Acta Physiol. Scand.*, vol. 13, no. 4, pp. 363–381, Jun. 1947.

[27] A. Leo, C. Hansch, and D. Elkins, "Partition coefficients and their uses," *Chem. Rev.*, vol. 71, no. 6, pp. 525–616, Dec. 1971.

[28] A. J. Leo and C. Hansch, "Linear free energy relations between partitioning solvent systems," *J. Org. Chem.*, vol. 36, no. 11, pp. 1539–1544, Jun. 1971.

[29] C. Hansch and W. J. Dunn, "Linear relationships between lipophilic character and biological activity of drugs," *J. Pharm. Sci.*, vol. 61, no. 1, pp. 1–19, 1972.

[30] B. Y. Zaslavsky, L. M. Miheeva, and S. V Rogozhin, "Comparison of conventional partitioning systems used for studying the hydrophobicity of polar organic compounds," *J. Chromatogr. A*, vol. 216, no. 0, pp. 103–113, Oct. 1981.

[31] Y. Marcus, "ViscosityB-coefficients, structural entropies and heat capacities, and the effects of ions on the structure of water," *J. Solution Chem.*, vol. 23, no. 7, pp. 831–848, 1994.

[32] P. P. Madeira *et al.*, "Salt effects on solvent features of coexisting phases in aqueous polymer/polymer two-phase systems," *J. Chromatogr. A*, vol. 1229, no. 0, pp. 38–47, Mar. 2012.

CHAPTER 4 | The osmolytes effect

This chapter is based on the following paper

Nuno R. da Silva[a], Luisa A. Ferreira[b], Pedro P. Madeira[c], José A. Teixeira[a], Vladimir N. Uversky[d] and Boris Y. Zaslavsky[b] (2015). Analysis of partitioning of organic compounds and proteins in aqueous polyethylene glycol-sodium sulfate aqueous two-phase systems in terms of solute-solvent interactions. *Journal of Chromatography A*, 1415, 1-10.

[a] Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga (Portugal)

[b] Analiza Inc./Cleveland Diagnostics, Superior Ave, Cleveland, OH (USA)

[c] Laboratory of Separation and Reaction Engineering, Department of Chemical Engineering, Faculty of Engineering of the University of Porto, Porto (Portugal)

[d] Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, Tampa, FL (USA)

Highlights

- Solvent properties of PEG–salt ATPS with osmolytes additives are characterized;
- Partition of organic compounds and proteins varies with solvent properties of ATPS;
- Partition of solutes is described in terms of solute-solvent interactions;
- Solute-solvent interactions in PEG–sulfate ATPS differ from those in two polymer ATPS;
- Solute size is unimportant for solute partitioning.

4.1. Introduction

ATPS are formed in mixtures of two (or more) water-soluble polymers, such as DEX and Ficoll, or a single polymer and specific salt, e.g., PEG and sodium sulfate, in water above certain critical concentrations. Two immiscible aqueous phases are formed in the mixtures. Solutes from small organic compounds to proteins and nucleic acids distribute unevenly between the phases of an ATPS and may be separated. ATPS formed by PEG and inorganic salt, such as sodium sulfate, phosphate or citrate, are commonly used for separation of proteins and nucleic acids due to their low cost, good operational characteristics (low viscosity of the phases, high settling speed) and easy scale-up [1]–[18]. Extraction in ATPS has been demonstrated as an efficient method for large scale recovery and purification of proteins [1], [2], [16]–[18], [3]–[5], [11]–[15] and nucleic acids [6], [7] as well as various other materials. Design of optimal extraction conditions for any target product remains currently an empirical process, and high throughput methods for screening different separation conditions have been developed [8]–[10]. For rational design of the optimal separation conditions it is important to understand the mechanisms of solute distribution in polymer–salt ATPS at the molecular level.

We reported [19]–[21] previously that different salt additives (NaCl, NaH_2PO_4, $NaClO_4$, NaSCN) at the concentrations from 0.027 M up to ca. 1.9 M affect partition behavior of small organic compounds in PEG–Na_2SO_4 ATPS according to the salt effects on the water structure. Despite broad biotechnological and pharmacological applications of this approach, the molecular mechanism of solute partitioning in PEG–salt ATPS remains unclear, however.

It has been established [22]–[29] that solute partitioning in two-polymer ATPS is governed by the solute-solvent interactions in the coexisting phases. Partition coefficient of a solute in an ATPS is defined as the ratio of the solute concentration in the top phase to the solute concentration in the bottom phase and therefore maybe described as [23]–[28]:

$$\log K = S_S\,\Delta\pi^* + B_S\,\Delta\alpha + A_S\,\Delta\beta + C_S\,c$$

(Equation 4.1)

where K is the solute partition coefficient; $\Delta\pi^*$, $\Delta\alpha$, $\Delta\beta$ and c are the differences between the solvent properties of the top and bottom phases (solvent dipolarity/polarizability, hydrogen-bond donor acidity, hydrogen-bond acceptor basicity, and electrostatic interactions, respectively; S_s, B_s, A_s, and C_s are

constants (solute-specific coefficients) that describe the complementary interactions of the solute with the solvent media in the coexisting phases; the subscript 'S' designates the solute.

The differences between the solvent dipolarity/polarizability, $\Delta\pi^*$, hydrogen-bond donor acidity, $\Delta\alpha$, hydrogen-bond acceptor basicity, $\Delta\beta$, may be quantified using a set of solvatochromic dyes [23]–[29] (see below). The difference between the electrostatic properties of the phases may be determined from the analysis of the partition coefficients of a homologous series of sodium salts of dinitrophenylated (DNP-) amino acids with aliphatic alkyl side-chains [22]–[28] (see below). It has been shown that for a given compound (including proteins) the solute-specific coefficients may be determined by multiple linear regression analysis of the partition coefficients of the compound in multiple two polymer ATPSs formed by different polymers but with the same ionic composition [23]–[28]. It was also shown [23], [26] that the partition coefficients of compounds with pre-determined solute specific coefficients in new ATPS with established solvent properties of the phases could be predicted with the 90-95% accuracy.

Huddleston *et al.* [30], [31] examined the solvent properties of the coexisting phases in PEG2000–K_3PO_4 and PEG2000–$(NH_4)_2SO_4$ ATPSs and found negligible differences between the solvent dipolarity/polarizability, $\Delta\pi^*$, and hydrogen bond acceptor basicity, $\Delta\beta$, of the phases. The authors [30], [31] also reported on the challenges regarding accurate determination of the solvent hydrogen-bond donor acidity, $\Delta\alpha$, in such ATPSs due to effects of high salt concentrations on the solvatochromic probe used. We assumed that the challenges encountered by Huddleston *et al.* [30], [31] might be related to the low molecular weight of PEG used in the studies requiring using high total salt concentration (9-10 wt.% K_3PO_4) for ATPS formation. Hence in this work we used PEG8K and PEG10K enabling us to decrease the salt concentration necessary for phase separation down to 6.3 wt.% Na_2SO_4.

It has been shown previously [32]–[35] that the osmolytes additives change the solvent properties of the phases under fixed salt composition of the system, while not being engaged in direct interactions with compounds (including proteins) being partitioned. Therefore, these additives enable one to vary solvent properties of the polymer–salt ATPS without changing overall polymer and salt composition of the particular system.

The purpose of the present work was to explore if partitioning of different solutes in PEG–Na_2SO_4 ATPS is governed by the factors similar to those established in the ATPSs formed by two polymers. To this end, partitioning of several different organic compounds and proteins was examined in several PEG–Na_2SO_4 ATPSs in the presence of different nonionic additives.

4.2. Experimental

4.2.1. Materials

Polyethylene glycol-8000 (Lot 091M01372 V) with an average molecular weight (Mw) of 8000 and polyethylene glycol-10000 (Lot 043K2522) with an average molecular weight (Mw) of 10000 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The solvatochromic probes 4-nitrophenol (reagent grade, >98%) was purchased from Aldrich (Milwaukee, WI, USA) and 4-nitroanisole (>97%, GC) was received from Acros Organics. Reichardt's carboxylated betaine dye, 2,6-diphenyl-4-[2,6-diphenyl-4-(4-carboxyphenyl)-1-pyridino]phenolate, sodium salt was kindly provided by Professor C. Reichardt (Philipps University, Marburg, Germany).

Sorbitol, TMAO, and trehalose were purchased from Sigma-Aldrich, and sucrose was received from USB (Cleveland, OH, USA). 4-aminophenol, benzyl alcohol, caffeine, coumarin, methylanthranilate, 4-nitrophenyl-α-D-glucopyranoside, phenol, 2-phenylethanol, vanillin, and *o*-phthaldialdehyde (OPA) reagent (complete) were purchased from Sigma-Aldrich. All compounds were of 98–99% purity and used as received without further purification. All salts and other chemicals used were of analytical-reagent grade.

4.2.1.1. Dinitrophenylated amino acids

Dinitrophenylated (DNP) amino acids – DNP-glycine, DNP-alanine, DNP-norvaline, DNP-norleucine, and DNP-α-amino-*n*-octanoic acid, were purchased from Sigma-Aldrich. The sodium salts of the DNP-amino acids were prepared by titration.

4.2.1.2. Proteins

α-chymotrypsin from bovine pancreas, α-chymotrypsinogen A from bovine pancreas, concanavalin A from *Canavalia ensiformis* (jack beans), lysozyme from chicken egg white, and papain from papaya latex were purchased from Sigma-Aldrich. Porcine pancreatic lipase was purchased from USB Corp. (Solon, OH, USA). All protein samples were characterized by SDS-PAGE electrophoresis in a microfluidic chip

using Experion automated electrophoresis station (Bio-Rad, USA) under non-reduced conditions. All proteins were observed as single bands in the electrophoregrams.

4.2.2. Methods

4.2.2.1. Aqueous two-phase systems

Stock solutions of PEG8K (50 wt.%), PEG10K (50 wt.%) and Na_2SO_4 (20.3 wt.%) were prepared in water. Sodium phosphate buffer (NaPB; 0.5 M, pH 6.8) was prepared by mixing 3.45 g of $NaH_2PO_4 \cdot H_2O$ and 3.55 g Na_2HPO_4 in 100 mL aqueous solution. Stock solutions of osmolytes: sorbitol (2 M), sucrose (1.8 M), trehalose (1.4 M), and TMAO (1.8 M) were prepared in water. A mixture of PEG8K or PEG10K and buffer was prepared by dispensing appropriate amounts of the aqueous stock PEG8K, Na_2SO_4 and NaPB solutions into a 1.2 mL microtube using a Hamilton (Reno, NV, USA) ML-4000 four-probe liquid-handling workstation. Appropriate amounts of water and/or stock osmolytes solutions were added to give the required ionic, polymer, and osmolyte composition of the final system with total weight of 0.5 g (after addition of the solute sample, see below). All aqueous PEG8K–Na_2SO_4 two-phase systems had a fixed composition of 11.10 wt.% PEG8K, 6.33 wt.% Na_2SO_4 and 0.01 M NaPB, pH 6.8, with different 0.5 M osmolyte additive. The aqueous PEG10K–Na_2SO_4 two-phase system had the same composition of 11.10 wt.% PEG10K, 6.33 wt.% Na_2SO_4 and 0.01 M NaPB, pH 6.8.

4.2.2.2. Partitioning experiments

The aqueous two-phase partitioning experiments were performed using an Automated Signature Workstation, ASW (Analiza, Cleveland, OH, USA). The ASW system is based on the ML-4000 liquid-handling workstation (Hamilton Company) integrated with a FL600 fluorescence microplate reader (Bio-Tek Instruments, Winooski, VT, USA) and a UV–VIS microplate spectrophotometer (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA, USA). Solutions of all organic compounds were prepared in water at concentrations of 2-5 mg/mL depending on the compound solubility. Solutions of all proteins were prepared in water at concentrations of 1-5 mg/mL. Varied amounts (0, 15, 30, 45, 60, and 75 μL) of a

given compound solution and the corresponding amounts (75, 60, 45, 30, 15, and 0 μL) of water were added to a set of the same polymer–buffer mixtures with and without osmolyte additives. The systems were then vortexed in a Multi-pulse Vortexer and centrifuged for 60 min at 3500 × g at 23 °C in a refrigerated centrifuge (Hettich Universal 320R, Germany) to accelerate phase settling. The upper phase in each system was partially removed, the interface discarded, and aliquots from the upper and lower phases were withdrawn in duplicate for analysis.

For the analysis of organic compounds partitioning, aliquots of 50-120 μL from both phases were diluted up to 600 μL in 1.2 mL microtubes. Water was used as diluent for all except phenol, and vanillin. 20 mM universal buffer with pH 12.4 was used as diluent (Universal buffer is composed of 0.01 M each of phosphoric, boric, and acetic acids adjusted to pH 12.4 with NaOH). Following vortexing and a short centrifugation (12 min), aliquots of 250-300 μL were transferred into microplate wells, and the UV–VIS plate reader was used to measure optical absorbance at wavelengths previously determined to correspond to maximum absorption. The maximum absorption wavelength for each compound was determined in separate experiments by analysis of the absorption spectrum over the 240-500 nm range. In the case of the four aforementioned compounds the maximum absorption was found to be more concentration sensitive in the presence of the universal buffer at pH 12.4. In all measurements the same dilution factor was used for the upper and lower phases and correspondingly diluted pure phases were used as blank solutions.

For the analysis of the partitioning of proteins aliquots of 30 μL from both phases were transferred and diluted with water up to 70 μL into microplate wells. Then, the microplate was sealed, shortly centrifuged (2 min at 1500 rpm) and following the moderate shaking for 45 min in an incubator at 37°C, 250 μL of *o*-phthaldialdehyde reagent was added. After moderate shaking for 4 min at room temperature, fluorescence was determined using a fluorescence plate reader with a 360 nm excitation filter and a 460 nm emission filter, with a sensitivity setting of 100-125.

The partition coefficient, K, defined as the ratio of the sample concentration in the upper phase to the sample concentration in the lower phase was determined as the slope of the compound concentration in the upper phase plotted as a function of the concentration in the lower phase averaged over the results obtained from two to four partition experiments carried out at the specified polymer, buffer, and osmolyte composition of the system, taking into consideration the corresponding dilution factors used in the experiment. The UV absorption measured in a given phase was used as a measure of a given organic compound concentration, and fluorescence intensity was used as a measure of the protein concentration. Deviation from the average K-value was consistently below 3% and, in most cases, lower than 2%.

4.2.2.3. Solvatochromic studies

All ATPSs were prepared as described above with the total weight of 4 g. Systems were centrifuged as described above and the phases were separated. The solvatochromic probes 4-nitroanisole, 4-nitrophenol and Reichardt's carboxylated betaine dye were used to measure the dipolarity/polarizability π^*, HBA basicity β, and HBD acidity α, of the media in the separated phases of ATPS. Aqueous solutions (ca. 10 mM) of each solvatochromic dye were prepared, and aliquots of 5-15 μL of each dye were added separately to a total volume of 500 μL of a given phase of each ATPS. A strong base was added to the samples (~5 μL of 1 M NaOH to 500 μL of a given phase) containing Reichardt's carboxylated betaine dye to ensure a basic pH. A strong acid (~10 μL of 1 M HCl to 500 μL of the solution) was added to the phase containing 4-nitrophenol in order to eliminate charge-transfer bands of the phenolate anion that were observed in some solutions. The samples were mixed thoroughly in a vortex mixer and the absorption spectra of each solution were acquired. To check the reproducibility, possible aggregation and specific interactions effects, the position of the band maximum in each sample was measured in six separate aliquots. A UV–VIS microplate reader spectrophotometer SpectraMax Plus384 (Molecular Devices, Sunnyvale, CA, USA) with a bandwidth of 2.0 nm, data interval of 1 nm, and high-resolution scan (~0.5 nm/s) was used for acquisition of the UV–Vis molecular absorbance data. The absorption spectra of the probes were determined over the spectral range from 240 to 600 nm in each solution. Pure osmolyte solutions or phase of ATPS containing no dye (blank) were scanned first to establish a baseline. The wavelength of maximum absorbance in each solution was determined as described by Huddleston *et al.* [31] using PeakFit software package (Systat Software Inc., San Jose, CA, USA) and aver-aged. Standard deviation for the measured maximum absorption wavelength was ≤0.4 nm for all dyes in all solutions examined.

The behavior of the probes (4-nitrophenol, and Reichardt's carboxylated betaine dye) in several solvents (water, n-hexane, methanol) was tested in the presence and absence of HCl (for 4-nitrophenol) and NaOH (for the betaine dye) at different concentrations of the probes, acid or base, and the maximum shifts of the probes were compared to reference values found in the literature and were within the experimental errors in all cases (data not shown).

The results of the solvatochromic studies were used to calculate π^*, α and β as described by Marcus [36].

4.2.2.3.1. Determination of the solvent dipolarity/polarizability π*

The π^* values were determined from the wave number ($\nu 1$) of the longest wavelength absorption band of the 4-nitroanisole dye using the relationship:

$$\pi^* = 0.427(34.12 - \nu 1)$$

(Equation 4.2)

4.2.2.3.2. Determination of the solvent hydrogen-bond acceptor basicity β

The β values were determined from the wave number ($\nu 2$) of the longest wavelength absorption band of the 4-nitrophenol dye using the relationship:

$$\beta = 0.346(35.045 - \nu 2) - 0.57\pi^*$$

(Equation 4.3)

4.2.2.3.3. Determination of the solvent hydrogen-bond donor acidity α

The α values were determined from the longest wavelength absorption band of Reichardt's betaine dye using the relationship:

$$\alpha = 0.0649E_T(30) - 2.03 - 0.72\pi^*$$

(Equation 4.4)

the $E_T(30)$ values are based on the solvatochromic pyridinium N-phenolate betaine dye (Reichardt's dye) as probe, and are obtained directly from the wavelength (λ, nm) of the absorption band of the carboxylated form, as:

$$E_T(30) = \left(\frac{1}{0{,}932}\right) \times \left[\left(\frac{28591}{\lambda}\right) - 3.335\right]$$

(Equation 4.5)

4.3. Results

Differences between the solvent properties of the coexisting phases (solvent dipolarity/polarizability, $\Delta\pi^*$, hydrogen-bond donor acidity, $\Delta\alpha$, and hydrogen-bond acceptor basicity, $\Delta\beta$) were determined using solvatochromic dyes as described above. The values of these differences calculated as the difference between the measured value of a given solvent feature in the top phase and that of the same feature in the bottom phase are listed in Table 4.1.

The difference between the solvent properties in the PEG8K–Na_2SO_4 ATPS clearly increases significantly in the presence of osmolyte additives. Osmolytes effects on the difference between the solvent hydrogen-bond donor acidity, $\Delta\alpha$, between the two phases increases as follows:

Sorbitol ≈ Trehalose > Sucrose > TMAO

Osmolyte effects on the difference between the solvent hydrogen bond acceptor basicity, $\Delta\beta$, between the two phases are very similar. The difference between the solvent dipolarity/polarizability, $\Delta\pi^*$, changes in the sequence:

Sucrose > Trehalose > Sorbitol > Osmolyte free ATPS > TMAO

The difference between the hydrophobic and electrostatic properties of the coexisting phases was determined in each ATPS by partitioning of a homologous series of sodium salts of dinitrophenylated (DNP-) amino acids with the aliphatic alkyl side-chains of the increasing length (alanine, norvaline, norleucine, and α-amino-*n*-octanoic acid).

Table 4.1. Differences between the solvent properties of the phases and partition coefficients for simple organic compounds and free amino acids in PEG(8K or 10K)–Na_2SO_4–0.01 M NaPB, pH 6.8 and PEG8K–Na_2SO_4–0.5 M osmolyte–0.01 M NaPB, pH 6.8 ATPSs.

Solvent Properties	0.01M NaPB[a]	0.01M NaPB[b]	0.5M Sorbitol	0.5M Sucrose	0.5M Trehalose	0.5M TMAO
$\Delta G(CH_2)$, cal/mole	-122±1.8	-137±5.8	-144±2.4	-178±3.5	-181±3.5	-146±2.4
E	0.048±0.002	0.100±0.004	0.102±0.003	0.123±0.006	0.125±0.006	0.108±0.002
C	0.067±0.007	0.44±0.017	0.67±0.011	0.67±0.02	0.71±0.021	0.625±0.007
$\Delta\pi^*$	-0.029±0.003	-0.020±0.003	-0.046±0.004	-0.077±0.005	-0.067±0.004	-0.010±0.003
$\Delta\alpha$	-0.128±0.004	-0.075±0.004	-0.248±0.005	-0.228±0.007	-0.247±0.004	-0.208±0.004
$\Delta\beta$	0.015±0.004	0.013±0.004	0.021±0.008	0.028±0.008	0.022±0.007	0.021±0.009

a – Data for osmolyte-free PEG8K–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPS

b – Data for osmolyte-free PEG10K–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPS

Table 4.1. (cont.) Differences between the solvent properties of the phases and partition coefficients for simple organic compounds and free amino acids in PEG(8K or 10K)–Na_2SO_4–0.01 M NaPB, pH 6.8 and PEG8K–Na_2SO_4–0.5 M osmolyte–0.01 M NaPB, pH 6.8 ATPSs.

	Partition coefficients					
COMPOUND	**0.01M NaPB[a]**	**0.01M NaPB[b]**	**0.5M Sorbitol**	**0.5M Sucrose**	**0.5M Trehalose**	**0.5M TMAO**
4-aminophenol	3.04±0.049	3.17±0.007	4.45±0.013	4.55±0.014	4.66±0.014	4.11±0.012
Benzyl alcohol	3.50±0.007	3.55±0.031	5.36±0.017	6.11±0.035	5.69±0.068	3.86±0.019
Caffeine	1.85±0.039	1.98±0.005	2.48±0.010	2.49±0.013	2.44±0.012	2.45±0.012
Coumarin	4.86±0.014	4.55±0.023	8.23±0.024	8.46±0.065	7.97±0.039	6.27±0.024
Glucoside	2.18±0.023	2.41±0.008	3.48±0.011	3.75±0.019	3.59±0.016	2.87±0.010
Methyl anthranilate	7.28±0.035	7.29±0.040	13.48±0.091	14.21±0.057	14.90±0.220	9.06±0.051
Phenol	4.60±0.084	4.80±0.020	8.84±0.039	10.43±0.078	9.86±0.046	5.70±0.020
2-Phenylethanol	4.16±0.020	3.80±0.029	6.67±0.026	7.70±0.032	6.87±0.047	4.71±0.020
Vanillin	6.78±0.027	5.79±0.018	11.11±0.047	11.38±0.046	11.00±0.072	6.91±0.021
DNP-Ala Na	3.70±0.070	3.86±0.025	6.21±0.080	6.30±0.290	6.88±0.033	5.90±0.180
DNP-NVal Na	4.73±0.057	5.15±0.022	8.72±0.024	9.80±0.250	10.70±0.110	8.00±0.180
DNP-NLeu Na	6.00±0.110	6.40±0.023	11.10±0.130	13.20±0.390	15.00±0.120	10.70±0.190
DNP-AO Na	10.30±0.190	12.34±0.040	21.20±0.980	28.70±0.890	32.10±0.280	20.20±0.450

Partition coefficients of these compounds are listed in Table 4.1, and are presented graphically in Fig. 4.1, where the logarithms of their partition coefficients are plotted against the length of the side-chain expressed in equivalent number of methylene groups, N_c.

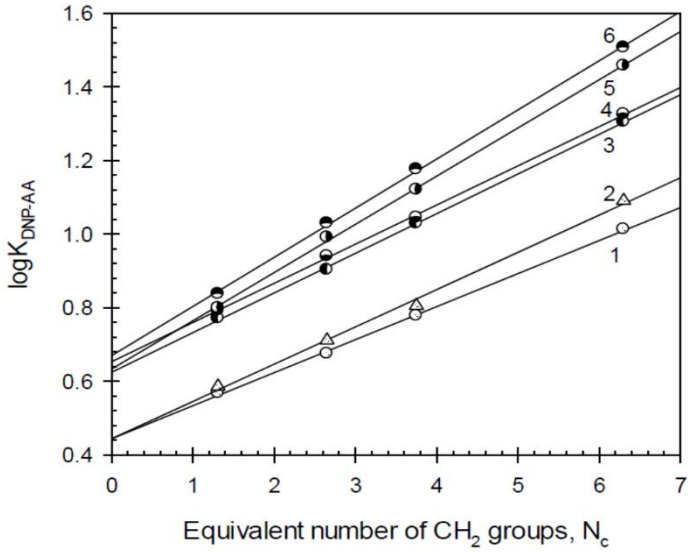


Figure 4.1. Logarithm of the partition coefficient value, $lnK_{DNP\text{-}AA}$, for sodium salts of DNP-AA in PEG–Na_2SO_4 ATPSs as a function of equivalent length of the side-chain, N_C: **1)** PEG8K–Na_2SO_4–0.01 M NaPB, pH 6.8; **2)** PEG10K–Na_2SO_4–0.01 M NaPB, pH 6.8; **3)** PEG8K–Na_2SO_4–0.5 M TMAO–0.01 M NaPB, pH 6.8; **4)** PEG8K–Na_2SO_4–0.5 M sorbitol–0.01 M NaPB, pH 6.8; **5)** PEG8K–Na_2SO_4–0.5 M sucrose–0.01 M NaPB, pH 6.8; **6)** PEG8K–Na_2SO_4–0.5 M trehalose–0.01 M NaPB pH 6.8.

It can be seen in Fig. 4.1 that the data in each ATPS maybe described as [22]–[28]:

$$\ln K_{DNP-AA}^{(i)} = C^{(i)} + E^{(i)} N_C$$

(Equation 4.6)

where $K_{DNP\text{-}AA}$ is the partition coefficient of a DNP-amino acids Na-salt; N_C is the equivalent number of CH_2 groups in the side-chain [19]–[28], E and C are constants for a given i-th ATPS characterizing the difference between the relative hydrophobicity and electrostatic properties of the phases correspondingly.

The values of the $E^{(l)}$ and $C^{(l)}$ coefficients determined for the ATPSs examined are listed in Table 4.1. As the standard free energy of transfer of a solute from the bottom phase to the top phase is described as:

$$\Delta G^0 = -RT \ln K$$

(Equation 4.7)

where R is the universal gas constant and T is the absolute temperature in Kelvin, it follows that:

$$\Delta G^0(CH_2) = -RTE^*$$

(Equation 4.8)

where E^* is parameter E expressed in natural logarithm units; $\Delta G°(CH_2)$ is the standard free energy of transfer of a methylene group from one phase to another. The $\Delta G°(CH_2)$ values calculated from the experimental data with Eqs. 4.7 and 4.8 are listed in Table 4.1. The data in Table 4.1 show that the difference between the relative hydrophobicity of the phases increases in the sequence:

Osmolyte-free ATPS < Sorbitol ≤ TMAO < Sucrose ≤ Trehalose

The essentially similar sequence is observed for the difference between the electrostatic properties of the phases:

Osmolyte-free ATPS < TMAO < Sorbitol ≈ Sucrose ≤ Trehalose

In order to check if the molecular weight of PEG is important for the solute partitioning in terms of solute-solvent interactions (see below) we attempted to use ATPSs based on PEG600–Na_2SO_4, PEG4000–Na_2SO_4, and PEG10K–Na_2SO_4. We found out that the solvatochromic measurements could not be reliably performed in the salt-rich phase of ATPS when Na_2SO_4 concentration exceeds 11-12 wt.%. Beyond 12 wt.% Na_2SO_4 it is not possible to determine the peak maximum for the solvatochromic absorption peak using the method employed. According to Huddleston *et al.* [30], [31] who observed similar phenomena in the presence of potassium phosphate, the aforementioned challenges may be explained by aggregation of the

dye due to high salt concentration. Therefore, we omitted measurements in PEG600–Na_2SO_4 and PEG4000–Na_2SO_4 ATPSs where the salt concentrations necessary for the formation of two-phase systems were too high and used only PEG10K–Na_2SO_4 ATPS with the overall composition identical to that used in PEG8K–Na_2SO_4 ATPS. Differences between the solvent properties of the two phases in this ATPS are presented in Table 4.1 together with partition coefficients for nine organic compounds examined here.

Partitioning of six proteins was examined in all aforementioned ATPSs and the corresponding partition coefficients are listed in Table 4.2.

Table 4.2. Partition coefficients for proteins in PEG(8K or 10K)–Na_2SO_4–0.01 M NaPB, pH 6.8 and PEG8K–Na_2SO_4–0.5 M osmolyte–0.01 M NaPB, pH 6.8 ATPSs.

	Partition coefficients					
PROTEIN	**0.01M NaPB[a]**	**0.01M NaPB[b]**	**0.5M Sorbitol**	**0.5M Sucrose**	**0.5M Trehalose**	**0.5M TMAO**
α-chymotrypsinogen A	0.429±0.003	0.379±0.001	0.204±0.001	0.157±0.001	0.119±0.001	0.456±0.002
α-chymotrypsin	0.117±0.001	0.092±0.0003	0.058±0.0002	0.047±0.001	0.027±0.0007	0.112±0.001
Concanavalin A	0.192±0.001	0.195±0.002	0.159±0.0007	0.144±0.001	0.116±0.0006	0.182±0.001
Lipase	0.618±0.001	0.640±0.002	0.569±0.002	0.537±0.002	0.516±0.003	0.595±0.002
Lysozyme	0.406±0.003	0.411±0.003	0.145±0.002	0.055±0.0003	0.045±0.0004	0.297±0.002
Papain	2.220±0.011	1.913±0.008	2.210±0.011	2.220±0.006	1.374±0.008	2.290±0.010

a – Data for osmolyte-free PEG8K–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPS

b – Data for osmolyte-free PEG10K–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPS

4.4. Discussion

Analysis of partition coefficients determined in this study for various organic compounds and proteins listed in Tables 4.1 and 4.2 shows that the so-called Collander solvent regression equation (see, e.g., in [22], [32]–[35]) holds for all compounds (including proteins) in the PEG8K–Na_2SO_4 and PEG10K–Na_2SO_4 ATPSs as shown in Fig. 4.2.

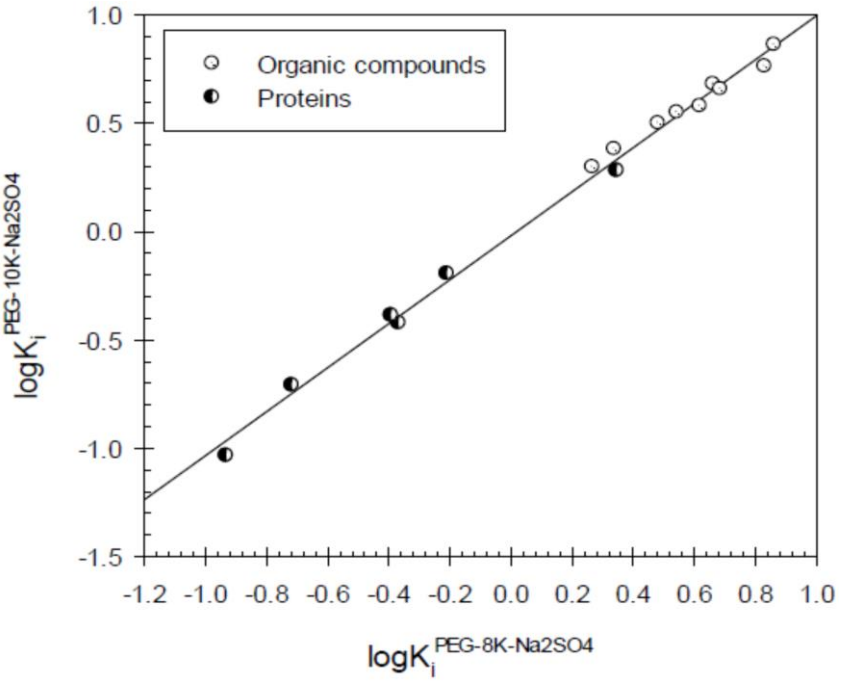


Figure 4.2. Logarithms of partition coefficients for organic compounds and proteins in PEG8K–Na_2SO_4–0.01 M NaPB, pH 6.8 versus those for the same compounds and proteins in PEG10K–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPSs.

The linear relationship plotted in Fig. 4.2 may be described as:

$$\log K_i^{PEG10K-Na_2SO_4} = -0.02_{\pm 0.01} + 1.02_{\pm 0.02} \times \log K_i^{PEG8K-Na_2SO_4}$$

N = 15; r^2 = 0.9950; SD = 0.043; F = 2587

(Equation 4.9)

where $K_i^{PEG8K-Na2SO4}$ and $K_i^{PEG10K-Na2SO4}$ are partition coefficients for the i-th compound in PEG8K–Na_2SO_4 and PEG10K–Na_2SO_4 ATPS correspondingly.

Similar relationship is observed between partition coefficients for all compounds examined in the PEG8K–Na_2SO_4 ATPS and same ATPS containing 0.5 M TMAO. The relationship is plotted in Fig. 4.3 and it may be described as:

$$\log K_i^{PEG8K-Na_2SO_4-TMAO} = 0.03_{\pm 0.02} + 1.08_{\pm 0.03} \times \log K_i^{PEG8K-Na_2SO_4}$$

N = 15; r^2 = 0.9918; SD = 0.059; F = 1582

(Equation 4.10)

where $K_i^{PEG8K-Na2SO4-TMAO}$ is partition coefficient for the i-th compound in PEG8K–Na_2SO_4 ATPS containing 0.5 M TMAO; all the other parameters are as defined above.

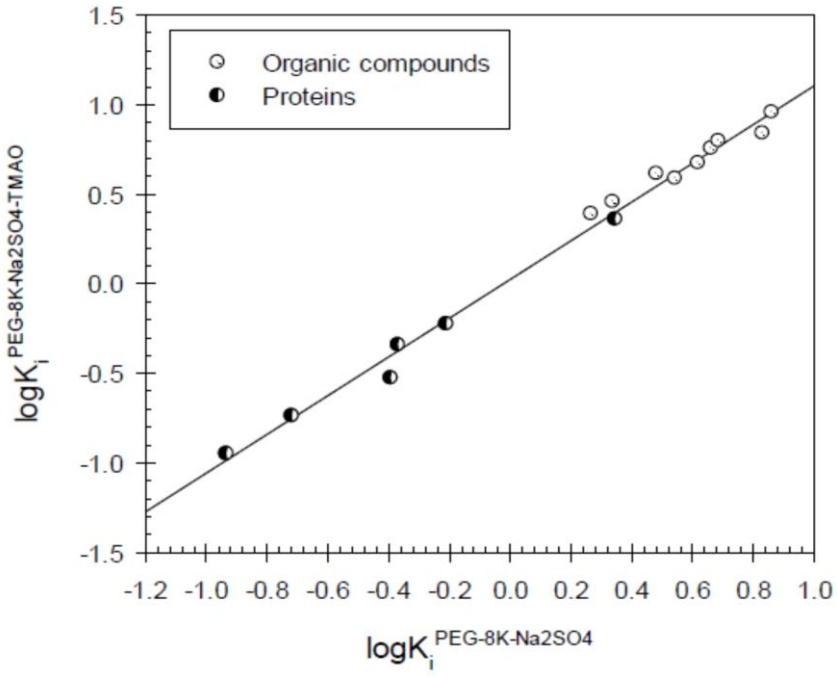


Figure 4.3. Logarithms of partition coefficients for organic compounds and proteins in PEG8K–Na_2SO_4–0.5 M TMAO–0.01 M NaPB, pH 6.8 versus those for the same compounds and proteins in PEG8K–Na_2SO_4–0.01 M sodium NaPB, pH 6.8 ATPSs.

Partition coefficients for all compounds in ATPS containing 0.5 M trehalose and 0.5 M sucrose fit the Collander relationship very well (see Fig. 4.4) and may be described as:

$$\log K_i^{PEG8K-Na_2SO_4-Trehalose} = -0.07_{\pm 0.02} + 1.06_{\pm 0.02} \times \log K_i^{PEG8K-Na_2SO_4-Sucrose}$$

N = 15; r^2 = 0.9961; SD = 0.060; F = 3325

(Equation 4.11)

where $K_i^{PEG8K-Na2SO4-trehalose}$ and $K_i^{PEG8K-Na2SO4-sucrose}$ are partition coefficients for the i-th compound in PEG8K–Na_2SO_4 ATPS containing 0.5 M trehalose and 0.5 M sucrose correspondingly; all the other parameters are as defined above. Similar relationship was established [33] previously in PEG–DEX ATPS with the same osmolytes additives.

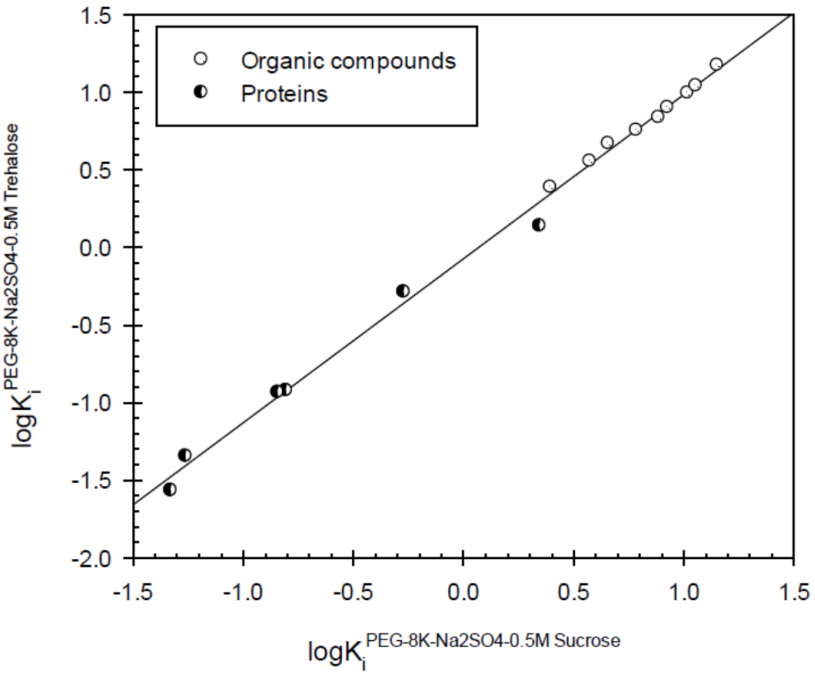


Figure 4.4. Logarithms of partition coefficients for organic compounds and proteins in PEG8K–Na_2SO_4–0.5 M trehalose–0.01 M NaPB, pH 6.8 versus those for the same compounds and proteins in PEG8K–Na_2SO_4–0.5 M sucrose–0.01 M NaPB, pH 6.8 ATPSs.

Analysis of the partition coefficients for all compounds in other PEG8K–Na_2SO_4 ATPSs containing 0.5 M osmolyte and in osmolyte-free PEG8K–Na_2SO_4 ATPS shows that the Collander relationship holds for small organic compounds but not for proteins. Typical relationship is shown in Fig. 4.5 for trehalose.

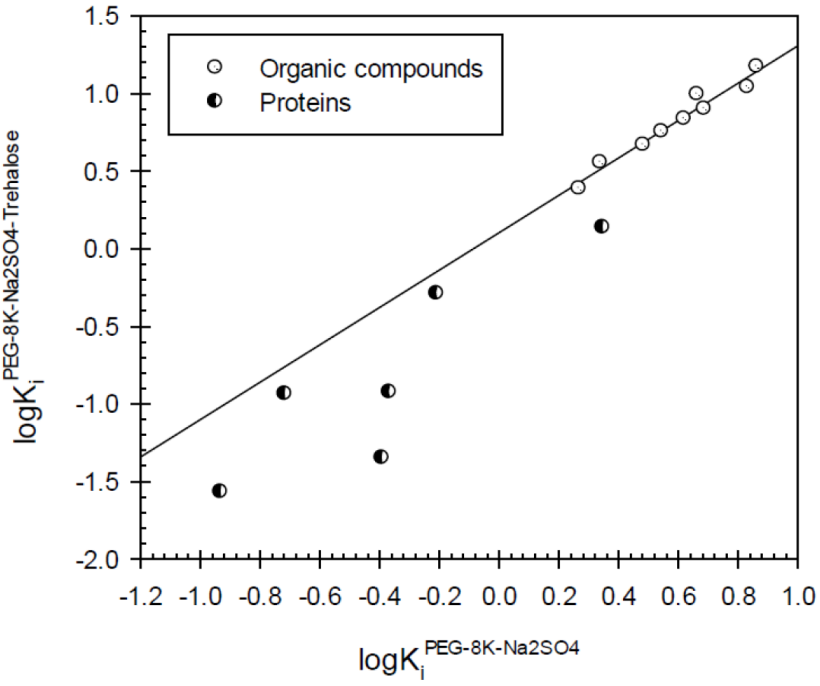


Figure 4.5. Logarithms of partition coefficients for organic compounds and proteins in PEG8K–Na_2SO_4–0.5 M trehalose (or sucrose)–0.01 M NaPB, pH 6.8 ATPSs versus those for the same compounds and proteins in PEG8K–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPS (line describes the linear relationship observed for organic compounds).

The data for partition coefficient for the same compounds and proteins in the presence of 0.5 M sucrose are presented for comparison. The relationship obtained for trehalose may be described as:

$$\log K_i^{PEG8K-Na_2SO_4-Trehalose} = -0.16_{\pm 0.06} + 1.60_{\pm 0.11} \times \log K_i^{PEG8K-Na_2SO_4}$$

N = 15; r^2 = 0.9447; SD = 0.230; F = 222

(Equation 4.12)

where all the parameters are as defined above. The reduced r^2 and *F* values as well as the increased *SD* value indicate that the relationship in question describes a trend rather than reliable correlation, and it is readily seen from Fig. 4.5 that proteins fit the relationship rather poorly. It should be noted that the data for partition coefficients of organic compounds in the presence of 0.5 M sucrose fit the above relationship perfectly, while those for proteins clearly deviate from it.

It has been shown [33], [35] previously that logarithms of partition coefficients of proteins in PEG–DEX–0.5 M osmolyte ATPS, all containing 0.01 M K/NaPB, pH 7.4 are linearly interrelated in a three-dimensional space. Analysis of the partition coefficients listed in Tables 4.1 and 4.2 show that similar relationships exists for all compounds examined in PEG–Na_2SO_4–0.5 M osmolyte ATPS as well. The relationship illustrated graphically in Fig. 4.6 is observed between logarithms of partition coefficients of compounds (including proteins) in PEG–Na_2SO_4, PEG–Na_2SO_4–0.5 M sucrose, and PEG–Na_2SO_4–0.5 M sorbitol ATPSs.

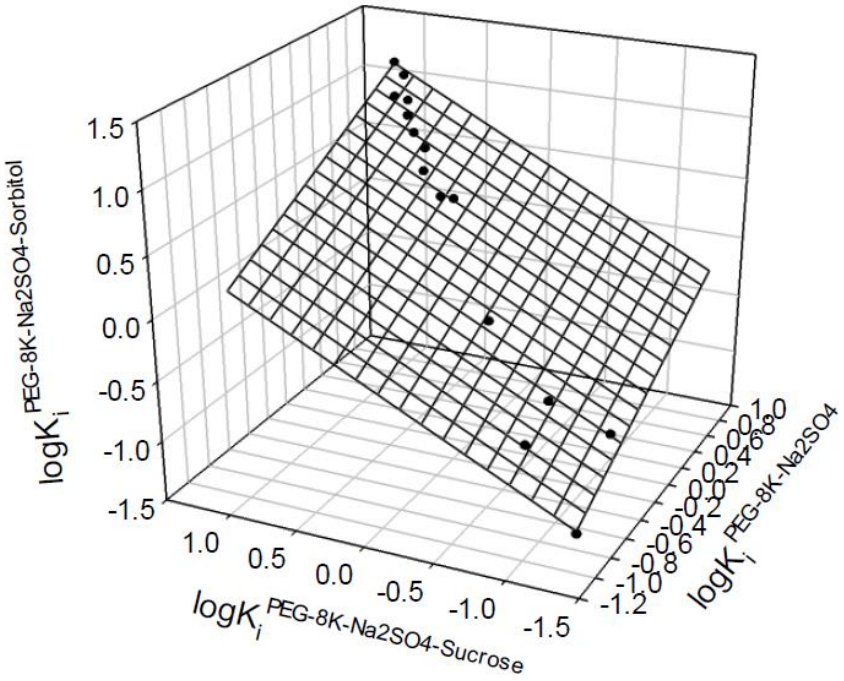


Figure 4.6. Logarithms of partition coefficients for organic compounds and proteins in PEG8K–Na_2SO_4–0.5 M sorbitol–0.01 M NaPB, pH 6.8 versus those for the same compounds and proteins in PEG8K–Na_2SO_4–0.01 M NaPB, pH 6.8 and in PEG8K–Na_2SO_4–0.5 M sucrose–0.01 M NaPB, pH 6.8 ATPSs.

This relationship may be described as:

$$\log K_i^{PEG8K-Na_2SO_4-Sorbitol} = 0.02_{\pm 0.01} + 0.55_{\pm 0.07} \times \log K_i^{PEG8K-Na_2SO_4} + 0.55_{\pm 0.04} \times \log K_i^{PEG8K-Na_2SO_4-Sucrose}$$

N = 15; r^2 = 0.9985; SD = 0.033; F = 4087

(Equation 4.13)

where all the parameters are as defined above.

Similar relationship for the PEG–Na_2SO_4–TMAO, PEG–Na_2SO_4–0.5 M sorbitol, and PEG–Na_2SO_4–0.5 M trehalose ATPSs is illustrated graphically in Fig. 4.7 and may be described as:

$$\log K_i^{PEG8K-Na_2SO_4-TMAO} = -0.01_{\pm 0.03} + 1.30_{\pm 0.20} \times \log K_i^{PEG8K-Na_2SO_4-Sorbitol} - 0.40_{\pm 0.19} \times \log K_i^{PEG8K-Na_2SO_4-Trehalose}$$

N = 15; r^2 = 0.9906; SD = 0.066; F = 635

(Equation 4.14)

where all the parameters are as defined above.

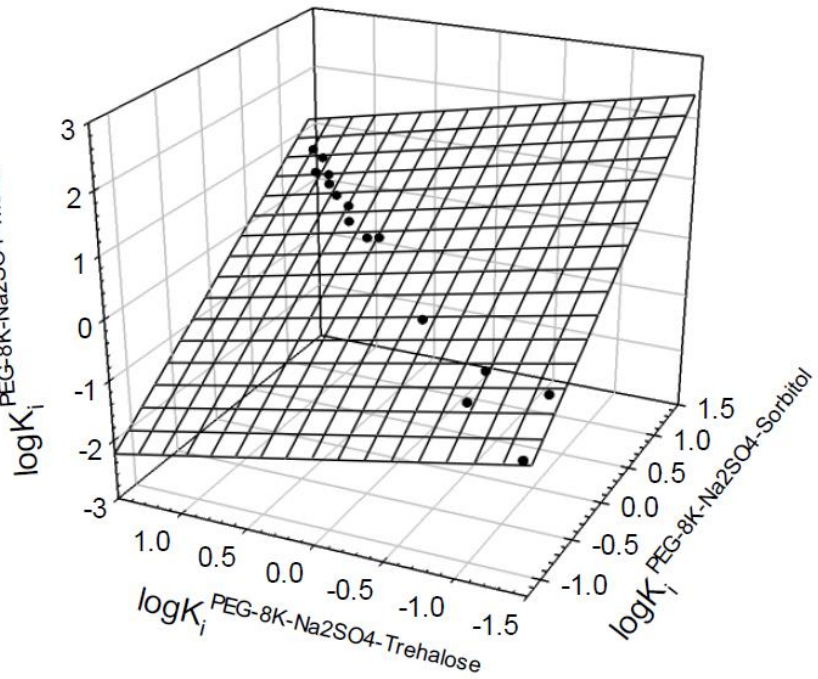


Figure 4.7. Logarithms of partition coefficients for organic compounds and proteins in PEG8K–Na_2SO_4–0.5 M TMAO–0.01 M NaPB, pH 6.8 versus those for the same compounds and proteins in PEG8K–Na_2SO_4–0.5 M sorbitol–0.01 M NaPB, pH 6.8 and in PEG8K–Na_2SO_4–0.5 M trehalose–0.01 M NaPB, pH 6.8 ATPSs.

It was suggested previously [37], [38] that the relationships of the type represented by Eqs. 4.13 and 4.14 imply that the compounds respond to their environment in aqueous solutions in the compound structure- and environment-specific manner, and also that the responses are governed by changes in the compound-water interactions possibly originating from the compound dipole-ion interactions.

4.4.1. Organic compound-water interactions in PEG–Na_2SO_4 ATPS

The partition coefficients for organic compounds listed in Table 4.1 were examined with Eq. 4.1. The solute-specific coefficients were determined by multiple linear regression analysis using the procedure described by Ab Rani *et al.* [39]. According to this procedure [39], the p-value was used for a given compound as a test for significance for each solute-specific coefficient in Eq. 4.1. In view of the small number of six ATPSs employed (and additional condition of partition coefficient K-value = 1 for the

compound in the theoretical critical point in an ATPS, when both phases have identical composition; i.e., zero difference between each of the solvent properties of the phases [22]), we have chosen to use the maximum statistical significance value of $p \leq 0.1$. If all four coefficients (S_s, A_s, B_s, and C_s) proved statistically significant ($p \leq 0.1$), the correlation was accepted. If one or more values reveal a p-value > 0.1, then equations contained different combinations of coefficients were examined. The equation with a set of coefficients providing p-values for all parameters below or equal to 0.1 was accepted.

The solute-specific coefficients determined for each compound are presented in Table 4.3 together with the corresponding p-values (except the cases when $p < 0.001$).

It has been reported [40] recently that there seems to be a cooperativity between the different types of solute-water interactions due to which the solute-specific coefficients are linearly interrelated. The interrelationship between the solute-specific coefficients presented in Table 4.3 is illustrated graphically in Fig. 4.8, and it may be described as:

$$B_S^i = -0.50_{\pm 0.30} + 0.80_{\pm 0.16} \times C_S^i - 0.11_{\pm 0.04} \times S_S^i$$

N = 9; r^2 = 0.8785; SD = 0.200; F = 21.7

(Equation 4.15)

where B_s, C_s, and S_s are solute-specific coefficients for the i-th compound; all the other parameters are as defined above.

Table 4.3. Solute-specific coefficients (see Eq. 4.1) for organic compounds in PEG–Na_2SO_4–0.01 M NaPB (calculated by multiple linear regression analysis from data in Table 4.1.).

COMPOUND	S_s	A_s	B_s	C_s	N; SD; F
4-aminophenol p-value	-0.7±0.21 0.040	-	0.8±0.13 0.004	1.2±0.05	7; 0.01; 7030
Benzyl alcohol p-value	-2.9±0.55 0.006	-	1.1±0.33 0.03	1.3±0.10 0.0002	7; 0.03; 1304
Caffeine p-value	-	-	0.3±0.10 0.08	0.7±0.05	7; 0.01; 2638
Coumarin p-value	-	-	-	1.4±0.03	7; 0.05; 1673
Glucoside p-value	-1.4±0.40 0.020	-	-	0.7±0.03	7; 0.02; 1637
Methyl anthranilate p-value	-3.0±0.82 0.0200	-	1.7±0.49 0.02	2.1±0.16 0.0002	7; 0.04; 1366
Phenol p-value	-3.8±0.48 0.001	-	1.0±0.99 0.025	1.5±0.09	7; 0.02; 2839
2-Phenylethanol p-value	-2.4 ±0.87 0.040	-	-	1.1±0.07	7; 0.05; 818
Vanillin p-value	-3.0±1.40 0.100	-	1.5±0.85 0.100	1.9±0.27 0.002	7; 0.07; 382
DNP-Ala Na p-value	-0.5±0.14 0.030	-	0.5±0.08 0.004	1.4±0.03	7; 0.007; 23785
DNP-NVal Na p-value	1.5±0.31 0.009	-	0.8±0.18 0.010	1.7±0.06	7; 0.01; 7372
DNP-NLeu Na p-value	-1.7±0.56 0.040	-	1.0±0.30 0.040	1.9±0.10	7; 0.03; 2876
DNP-AO Na p-value	-2.8±0.73 0.020	-	2.1±0.44 0.009	2.7±0.14	7; 0.03; 2774

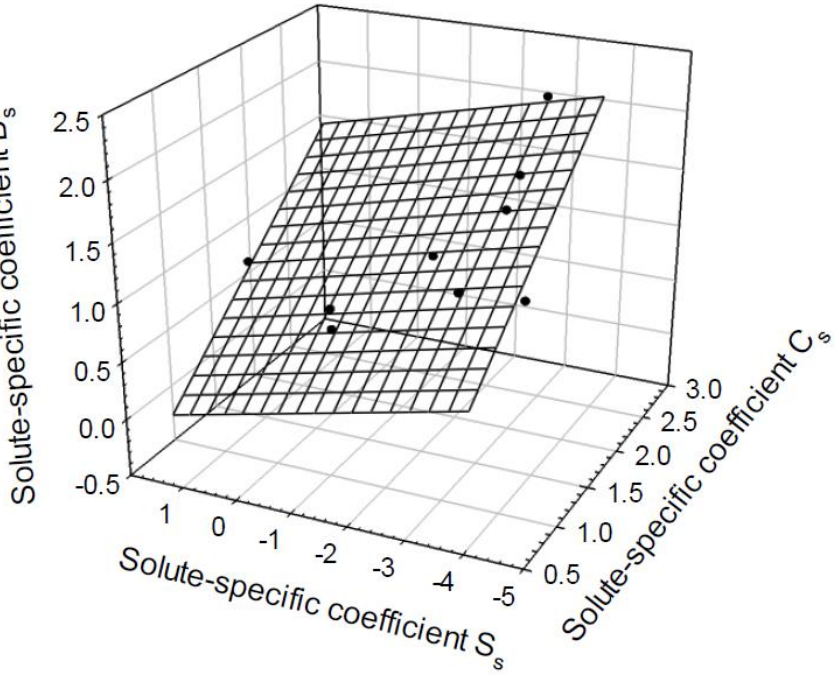


Figure 4.8. Interrelationship between solute-specific coefficients B_s for organic compounds and solute-specific coefficients C_s and S_s for the same compounds.

Analysis of solute-specific coefficients presented in Table 4.3 for compounds with solute-specific coefficients determined [40] in different multiple polymer–polymer ATPS containing 0.15 M Na_2SO_4 in 0.01 M NaPB, pH 7.4 shows the linear relationship for solute-specific coefficients S_s representing contributions of dipole-dipole and induced dipole-dipole solute-water interactions in the partition coefficients of compounds in the corresponding ATPS. The relationship illustrated graphically in Fig. 4.9 may be described as:

$$S_S^{i\ PEG-Na_2SO_4} = -0.30_{\pm 0.16} + 0.74_{\pm 0.05} \times S_S^{i\ pol-pol-0.15M\ Na_2SO_4}$$

N = 10; r^2 = 0.9623; SD = 0.200; F = 204

(Equation 4.16)

where $S_S^{i\ PEG-Na2SO4}$ and $S_S^{i\ polymer-polymer-0.15\ M\ Na2SO4-0.01\ M\ NaPB}$ are the solute specific coefficients S_s for i-th organic compound determined in PEG–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPS (Table 4.3) and in polymer–polymer ATPS containing 0.15 M Na_2SO_4 in 0.01 M NaPB, pH 7.4 [40]. It should be mentioned that only compounds with determined S_s values in both types of ATPS were considered.

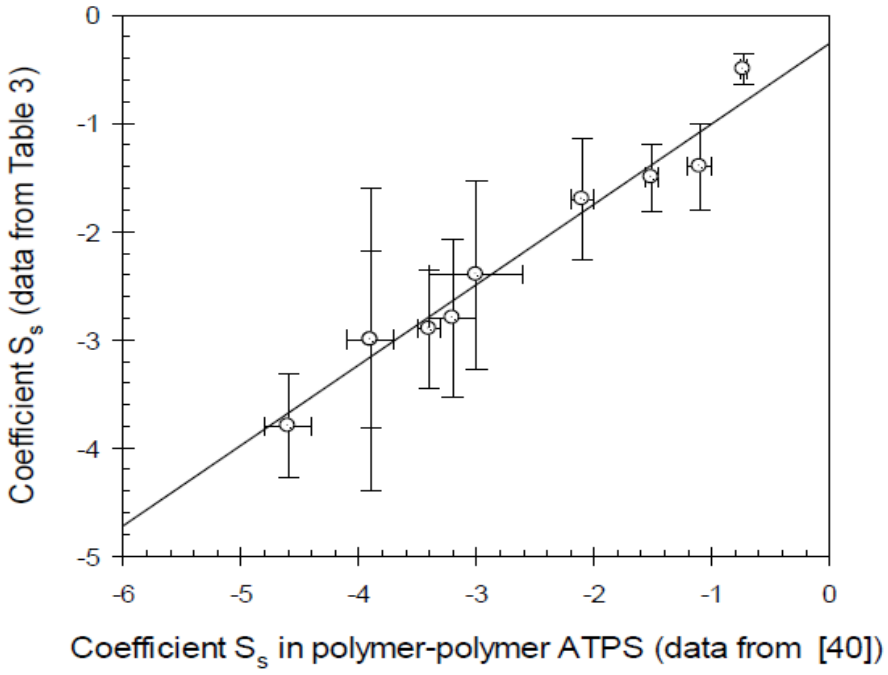


Figure 4.9. Relationship between solute specific coefficients S_s for organic compounds determined in PEG–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPSs and solute specific coefficients S_s for the same compounds determined in polymer–polymer ATPS containing 0.15 M Na_2SO_4 in 0.01 M NaPB, pH 7.4 (data from [40]).

Comparison of the solute-specific coefficient C_s values determined for the same compounds in the PEG–Na_2SO_4 ATPS and in polymer–polymer ATPS containing 0.15 M Na_2SO_4 in 0.01 M NaPB, pH 7.4 [40] shows that those determined in the former (see Table 4.3) exceed the C_s values determined in the latter from 1.2-fold to 5.6-fold. It seems possible to explain these observations assuming that the differences between the electrostatic properties of the phases in the PEG–Na_2SO_4 ATPSs used in this study varied from 0.067 to 0.71, while in polymer–polymer ATPSs containing 0.15 M Na_2SO_4 in 0.01 M NaPB, pH 7.4 [40] it varied from -0.011 to 0.456. The sodium sulfate concentrations in the top and bottom phases of the PEG8K–Na_2SO_4 ATPS used was reported [20] to be ~0.69 M and 0.24 M correspondingly (with the difference between the phases of ca.0.45 M Na_2SO_4), while in polymer–polymer ATPS used in [40] the difference between the Na_2SO_4 concentrations in the phases is less than 0.15 M Na_2SO_4. Therefore, it seems reasonable that the contribution of electrostatic ion-dipole solute-solvent interactions in the partition coefficients of solutes in PEG–Na_2SO_4 ATPS exceed those in the polymer–polymer ATPS containing 0.15 M Na_2SO_4.

It should be mentioned that Willauer *et al.* in the study [41] of partitioning of a set of 29 organic compounds in PEG2000–K_3PO_4 ATPS described partition behavior of compounds by the so-called LFER model by Abraham [42]–[45]. It was concluded [41] that partitioning of organic compounds in PEG–salt ATPS is governed by the solute size, basicity, and aromaticity or halogenicity. The results obtained here and discussed below show that the molecular size of the solute is not the factor governing the solute partition behavior in PEG–Na_2SO_4 ATPS.

4.4.2. Protein-water interactions in PEG-Na_2SO_4 ATPS

It should be noted that essentially all the proteins examined (except for papain) distribute into lower salt-rich phase in clear contradiction with the aforementioned conclusion by Willauer *et al.* [41] that the increasing molecular size of a solute drives the solute partitioning into PEG-rich phase. Partition coefficients of papain (K = 2.22; molecular weight of ca. 23,400 Da) in PEG8K–Na_2SO_4 ATPS is very close to that of *p*-nitrophenyl-D-glucopyranoside (K = 2.18; molecular weight ~301 Da) may serve as additional illustration of the contradiction.

The solute-specific coefficients for proteins were determined similarly to those for organic compounds from the solvent properties of ATPSs (Table 4.1) and from proteins partition coefficients (Table 4.2) and are listed in Table 4.4.

Table 4.4. Solute-specific coefficients (see Eq. 4.1) for proteins in PEG–Na_2SO_4–0.01 M NaPB (calculated by multiple linear regression analysis from data in Table 4.1.).

COMPOUND	S_s	A_s	B_s	C_s	N; SD; F
α-chymotrypsinogen A p-value	9.6±0.96 0.040	24.0±7.10 0.030	-	-1.2±0.21 0.006	7; 0.04; 518
α-chymotrypsin p-value	9.9±1.10 0.003	33.2±7.70 0.020	-2.3±0.50 0.020	-3.2 ±0.30 0.001	7; 0.04; 1221
Concanavalin A p-value	2.4±1.10 0.100	-	-2.2±0.60 0.030	-1.9±0.20 0.0007	7; 0.05; 469.8
Lipase p-value	0.8±0.28 0.040	-	-0.4±0.17 0.070	-0.5±0.05 0.0008	7; 0.01; 644.5
Lysozyme p-value	12.0±2.40 0.005	-	1.7±0.58 0.040	-	7; 0.11; 174.8
Papain p-value	4.0±2.00 0.100	23.0 ±4.60	-	-	7; 0.09; 36.2

It should be noted that in contrast to small organic compounds, the solute-specific coefficients A_s are quite significant for three out of six proteins studied. Contributions of dipole-dipole interactions (solute-specific coefficient S_s) into the partition coefficients are large for three proteins (α-chymotrypsinogen A, α-chymotrypsin and lysozyme), while the contributions of electrostatic interactions in the PEG–Na_2SO_4 ATPS containing 0.01 M NaPB, pH 6.8 are less significant than those determined for the same proteins in PEG–DEX ATPS containing 0.01 M K/NaPB, pH 7.4 [35]. As an example, for α-chymotrypsinogen A, the coefficient C_s = 7.6 ± 0.02 in PEG–DEX ATPS [35] and −1.2 ± 0.21 in PEG–Na_2SO_4 ATPS (Table 4.4), for concanavalin A, C_s = 5.1 ± 0.2 in PEG–DEX ATPS [35] and −1.9 ± 0.2 in PEG–Na_2SO_4 ATPS (Table 4.4). The decreasing electrostatic interactions of proteins with the solvent appear to agree with the suggested by Ninham *et al.* [46], [47] changes in the nature of protein-ion interactions in the presence of high salt concentration exceeding 0.2 M.

Analysis of the data in Table 4.4 confirms that the linear relationship similar to the one found for organic compounds exists for proteins as well. This relationship illustrated graphically in Fig. 4.10 may be described as:

$$B_S^i = -0.10_{\pm 0.20} + 1.20_{\pm 0.08} \times C_S^i - 0.16_{\pm 0.02} \times S_S^i$$

N = 5; r^2 = 0.9931; SD = 0.200; F = 144.5

(Equation 4.17)

where B_s, C_s, and S_s are solute-specific coefficients for the i-th protein; all the other parameters are as defined above. (Papain was not considered for the above relationship because its solute-specific coefficients B_s and C_s could not be determined).

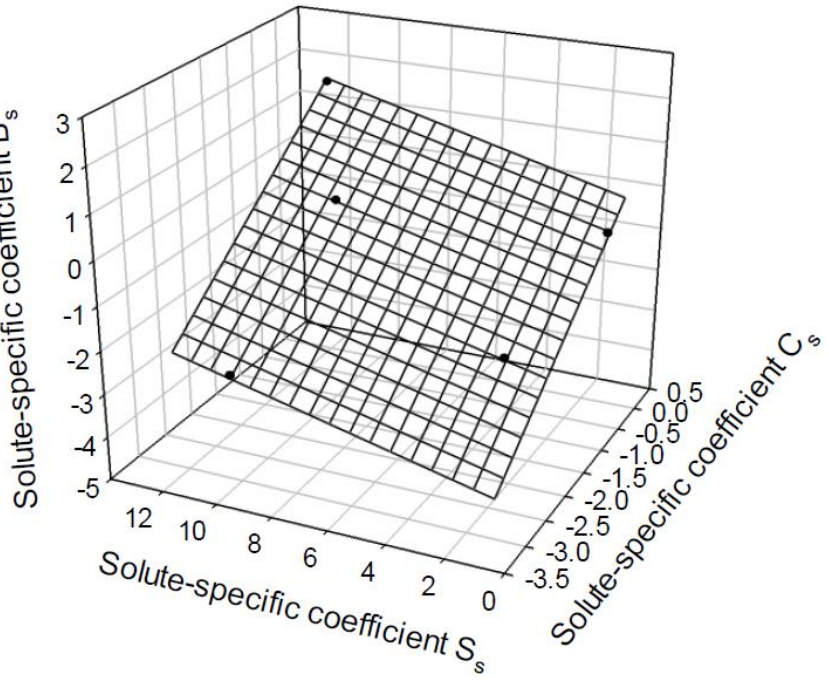


Figure 4.10. Interrelationship between solute-specific coefficients B_s for proteins and solute-specific coefficients C_s and S_s for the same proteins.

The limited number of proteins examined here prevents any general conclusion. It should be emphasized, however, that the partition behavior of both small organic compounds and proteins in PEG–Na_2SO_4 ATPS can be described in terms of solute-solvent interactions.

It seems also important that the results obtained in our study indicate that the solute-specific coefficients representing contributions of different types of solute-solvent interactions under the conditions explored differ from those determined at relatively low salt concentrations [35], supporting the previously suggested hypothesis that the solute-solvent interactions depend upon the solvent environment as well as

upon the solute structure. Further studies are clearly necessary for gaining better insight into mechanisms of these interactions, and these studies are currently in progress in our laboratories.

References

[1] H. HUSTEDT, K. H. KRONER, and M.-R. KULA, "15 - Applications of Phase Partitioning in Biotechnology," H. WALTER, D. E. BROOKS, and D. B. T.-P. in A. T.-P. S. FISHER, Eds. Academic Press, 1985, pp. 529–587.

[2] J. A. Asenjo and B. A. Andrews, "Aqueous two-phase systems for protein separation: Phase separation and applications," *J. Chromatogr. A*, vol. 1238, pp. 1–10, 2012.

[3] J. Muendges, A. Zalesko, A. Górak, and T. Zeiner, "Multistage aqueous two-phase extraction of a monoclonal antibody from cell supernatant," *Biotechnol. Prog.*, vol. 31, no. 4, pp. 925–936, Jul. 2015.

[4] J. Goll, G. Audo, and M. Minceva, "Comparison of twin-cell centrifugal partition chromatographic columns with different cell volume," *J. Chromatogr. A*, vol. 1406, pp. 129–135, 2015.

[5] M. A. Torres-Acosta, J. M. Aguilar-Yañez, M. Rito-Palomares, and N. J. Titchener-Hooker, "Economic analysis of royalactin production under uncertainty: Evaluating the effect of parameter optimization," *Biotechnol. Prog.*, vol. 31, no. 3, pp. 744–749, May 2015.

[6] F. Luechau, T. C. Ling, and A. Lyddiatt, "Two-step process for initial capture of plasmid DNA and partial removal of RNA using aqueous two-phase systems," *Process Biochem.*, vol. 45, no. 8, pp. 1432–1436, 2010.

[7] F. Luechau, T. C. Ling, and A. Lyddiatt, "Selective partition of plasmid DNA and RNA from crude Escherichia coli cell lysate by aqueous two-phase systems," *Biochem. Eng. J.*, vol. 55, no. 3, pp. 230–232, 2011.

[8] S. A. Oelmeier, F. Dismer, and J. Hubbuch, "Application of an aqueous two-phase systems high-throughput screening method to evaluate mAb HCP separation," *Biotechnol. Bioeng.*, vol. 108, no. 1, pp. 69–81, 2011.

[9] S. Amrhein, M.-L. Schwab, M. Hoffmann, and J. Hubbuch, "Characterization of aqueous two phase systems by combining lab-on-a-chip technology with robotic liquid handling stations," *J. Chromatogr. A*, vol. 1367, pp. 68–77, 2014.

[10] P. Diederich, M. Hoffmann, and J. Hubbuch, "High-throughput process development of purification

alternatives for the protein avidin," *Biotechnol. Prog.*, vol. 31, no. 4, pp. 957–973, Jul. 2015.

[11] P. A. J. Rosa, A. M. Azevedo, S. Sommerfeld, W. Bäcker, and M. R. Aires-Barros, "Aqueous two-phase extraction as a platform in the biomanufacturing industry: Economical and environmental sustainability," *Biotechnol. Adv.*, vol. 29, no. 6, pp. 559–567, 2011.

[12] A. Glyk, T. Scheper, and S. Beutel, "PEG–salt aqueous two-phase systems: an attractive and versatile liquid–liquid extraction technology for the downstream processing of proteins and enzymes," *Appl. Microbiol. Biotechnol.*, vol. 99, no. 16, pp. 6599–6616, 2015.

[13] J. F. Buyel, R. M. Twyman, and R. Fischer, "Extraction and downstream processing of plant-derived recombinant proteins," *Biotechnol. Adv.*, vol. 33, no. 6, Part 1, pp. 902–913, 2015.

[14] J. V. D. Molino, D. de A. Viana Marques, A. P. Júnior, P. G. Mazzola, and M. S. V. Gatti, "Different types of aqueous two-phase systems for biomolecule and bioparticle extraction and purification," *Biotechnol. Prog.*, vol. 29, no. 6, pp. 1343–1353, Nov. 2013.

[15] Y. Yuzugullu and Y. A. Duman, "Aqueous Two-Phase (PEG4000/Na2SO4) Extraction and Characterization of an Acid Invertase from Potato Tuber (Solanum tuberosum)," *Prep. Biochem. Biotechnol.*, vol. 45, no. 7, pp. 696–711, Oct. 2015.

[16] R. L. Pérez, D. B. Loureiro, B. B. Nerli, and G. Tubio, "Optimization of pancreatic trypsin extraction in PEG/citrate aqueous two-phase systems," *Protein Expr. Purif.*, vol. 106, pp. 66–71, 2015.

[17] V. H. Nagaraja and R. Iyyaswami, "Aqueous two phase partitioning of fish proteins: partitioning studies and ATPS evaluation," *J. Food Sci. Technol.*, vol. 52, no. 6, pp. 3539–3548, Jun. 2015.

[18] Y. H. Chow *et al.*, "Characterization of bovine serum albumin partitioning behaviors in polymer-salt aqueous two-phase systems," *J. Biosci. Bioeng.*, vol. 120, no. 1, pp. 85–90, 2015.

[19] L. A. Ferreira, J. A. Teixeira, L. M. Mikheeva, A. Chait, and B. Y. Zaslavsky, "Effect of salt additives on partition of nonionic solutes in aqueous PEG–sodium sulfate two-phase system," *J. Chromatogr. A*, vol. 1218, no. 31, pp. 5031–5039, Aug. 2011.

[20] L. A. Ferreira, P. Parpot, J. A. Teixeira, L. M. Mikheeva, and B. Y. Zaslavsky, "Effect of NaCl additive on properties of aqueous PEG–sodium sulfate two-phase system," *J. Chromatogr. A*, vol. 1220, pp. 14–20, 2012.

[21] N. R. da Silva, L. A. Ferreira, L. M. Mikheeva, J. A. Teixeira, and B. Y. Zaslavsky, "Origin of salt additive effect on solute partitioning in aqueous polyethylene glycol-8000–sodium sulfate two-phase system," *J. Chromatogr. A*, vol. 1337, no. 0, pp. 3–8, Apr. 2014.

[22] B. Y. Zaslavsky, *Aqueous Two-Phase Partitioning: Physical Chemistry and Bioanalytical Applications*. Taylor & Francis, 1994.

[23] P. P. Madeira, C. A. Reis, A. E. Rodrigues, L. M. Mikheeva, and B. Y. Zaslavsky, "Solvent Properties

Governing Solute Partitioning in Polymer/Polymer Aqueous Two-Phase Systems: Nonionic Compounds," *J. Phys. Chem. B*, vol. 114, no. 1, pp. 457–462, Jan. 2010.

[24] P. P. Madeira, C. A. Reis, A. E. Rodrigues, L. M. Mikheeva, A. Chait, and B. Y. Zaslavsky, "Solvent properties governing protein partitioning in polymer/polymer aqueous two-phase systems," *J. Chromatogr. A*, vol. 1218, no. 10, p. 1379—1384, 2011.

[25] P. P. Madeira *et al.*, "Solvatochromic relationship: prediction of distribution of ionic solutes in aqueous two-phase systems," *J. Chromatogr. A*, vol. 1271, no. 1, pp. 10–16, 2013.

[26] P. P. Madeira *et al.*, "Study of organic compounds–water interactions by partition in aqueous two-phase systems," *J. Chromatogr. A*, vol. 1322, pp. 97–104, 2013.

[27] P. P. Madeira, A. Bessa, L. Alvares-Ribeiro, M. R. Aires-Barros, A. E. Rodrigues, and B. Y. Zaslavsky, "Analysis of amino acid-water interactions by partitioning in aqueous two-phase systems. I–amino acids with non-polar side-chains.," *J. Chromatogr. A*, vol. 1274, pp. 82–86, Jan. 2013.

[28] P. P. Madeira *et al.*, "Amino acid/water interactions study: a new amino acid scale," *J. Biomol. Struct. Dyn.*, vol. 32, no. 6, pp. 959–968, Jun. 2014.

[29] P. P. Madeira *et al.*, "Salt effects on solvent features of coexisting phases in aqueous polymer/polymer two-phase systems," *J. Chromatogr. A*, vol. 1229, no. 0, pp. 38–47, Mar. 2012.

[30] J. G. Huddleston, H. D. Willauer, and R. D. Rogers, "Solvatochromic studies in polyethylene glycol–salt aqueous biphasic systems," *J. Chromatogr. B Biomed. Sci. Appl.*, vol. 743, no. 1, pp. 137–149, 2000.

[31] J. G. Huddleston, H. D. Willauer, and R. D. Rogers, "The solvatochromic properties, α, β, and π^*, of PEG-salt aqueous biphasic systems," *Phys. Chem. Chem. Phys.*, vol. 4, no. 16, pp. 4065–4070, 2002.

[32] L. Breydo *et al.*, "Effects of osmolytes on protein-solvent interactions in crowded environment: Analyzing the effect of TMAO on proteins in crowded solutions," *Arch. Biochem. Biophys.*, vol. 570, pp. 66–74, 2015.

[33] L. A. Ferreira, O. Fedotoff, V. N. Uversky, and B. Y. Zaslavsky, "Effects of osmolytes on protein–solvent interactions in crowded environments: study of sucrose and trehalose effects on different proteins by solvent interaction analysis," *RSC Adv.*, vol. 5, no. 34, pp. 27154–27162, 2015.

[34] L. A. Ferreira, P. P. Madeira, V. N. Uversky, and B. Y. Zaslavsky, "Analyzing the effects of protecting osmolytes on solute–water interactions by solvatochromic comparison method: I. Small organic compounds," *RSC Adv.*, vol. 5, no. 74, pp. 59812–59822, 2015.

[35] L. A. Ferreira, X. Fan, P. P. Madeira, L. Kurgan, V. N. Uversky, and B. Y. Zaslavsky, "Analyzing the effects of protecting osmolytes on solute–water interactions by solvatochromic comparison

method: II. Globular proteins," *RSC Adv.*, vol. 5, no. 73, pp. 59780–59791, 2015.

[36] Y. Marcus, "The properties of organic liquids that are relevant to their use as solvating solvents," *Chem. Soc. Rev.*, vol. 22, no. 6, pp. 409–416, 1993.

[37] L. A. Ferreira, A. Chervenak, S. Placko, A. Kestranek, P. P. Madeira, and B. Y. Zaslavsky, "Responses of polar organic compounds to different ionic environments in aqueous media are interrelated," *Phys. Chem. Chem. Phys.*, vol. 16, no. 42, pp. 23347–23354, 2014.

[38] L. A. Ferreira, P. P. Madeira, A. V Uversky, V. N. Uversky, and B. Y. Zaslavsky, "Responses of proteins to different ionic environment are linearly interrelated.," *J. Chromatogr. A*, vol. 1387, pp. 32–41, Mar. 2015.

[39] M. A. Ab Rani *et al.*, "Understanding the polarity of ionic liquids," *Phys. Chem. Chem. Phys.*, vol. 13, no. 37, pp. 16831–16840, 2011.

[40] P. P. Madeira, A. Bessa, J. A. Loureiro, L. Álvares-Ribeiro, A. E. Rodrigues, and B. Y. Zaslavsky, "Cooperativity between various types of polar solute-solvent interactions in aqueous media," *J. Chromatogr. A*, vol. 1408, p. 108—117, 2015.

[41] H. D. Willauer, J. G. Huddleston, and R. D. Rogers, "Solvent Properties of Aqueous Biphasic Systems Composed of Polyethylene Glycol and Salt Characterized by the Free Energy of Transfer of a Methylene Group between the Phases and by a Linear Solvation Energy Relationship," *Ind. Eng. Chem. Res.*, vol. 41, no. 11, pp. 2591–2601, May 2002.

[42] M. H. Abraham, A. Ibrahim, and A. M. Zissimos, "Determination of sets of solute descriptors from chromatographic measurements.," *J. Chromatogr. A*, vol. 1037, no. 1–2, pp. 29–47, May 2004.

[43] J. E. Cometto-Muñiz and M. H. Abraham, "Compilation and analysis of types and concentrations of airborne chemicals measured in various indoor and outdoor human environments," *Chemosphere*, vol. 127, pp. 70–86, 2015.

[44] J.-C. Bradley, M. H. Abraham, W. E. Acree Jr, and A. S. Lang, "Predicting Abraham model solvent coefficients," *Chem. Cent. J.*, vol. 9, p. 12, Mar. 2015.

[45] M. H. Abraham, "Human Intestinal Absorption—Neutral Molecules and Ionic Species," *J. Pharm. Sci.*, vol. 103, no. 7, pp. 1956–1966, 2014.

[46] B. W. Ninham and V. Yaminsky, "Ion Binding and Ion Specificity: The Hofmeister Effect and Onsager and Lifshitz Theories," *Langmuir*, vol. 13, no. 7, pp. 2097–2108, Apr. 1997.

[47] A. Salis and B. W. Ninham, "Models and mechanisms of Hofmeister effects in electrolyte solutions, and colloid and protein systems revisited," *Chem. Soc. Rev.*, vol. 43, no. 21, pp. 7358–7377, 2014.

CHAPTER 5 | Effect of sodium chloride

This chapter is based on the following paper

Nuno R. da Silva[a], Luisa A. Ferreira[b], Pedro P. Madeira[c], José A. Teixeira[a], Vladimir N. Uversky[d] and Boris Y. Zaslavsky[b] (2015). Effect of sodium chloride on solute-solvent interactions in aqueous polyethylene glycol-sodium sulfate two-phase systems. *Journal of Chromatography A*, 1425, 51-61.

[a] Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga (Portugal)

[b] Analiza Inc./Cleveland Diagnostics, Superior Ave, Cleveland, OH (USA)

[c] Laboratory of Separation and Reaction Engineering, Department of Chemical Engineering, Faculty of Engineering of the University of Porto, Porto (Portugal)

[d] Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, Tampa, FL (USA)

Highlights

- Solvent properties of PEG8K–Na_2SO_4 ATPS containing 0.215 M NaCl and 0.5M osmolyte (sorbitol, sucrose, TMAO) and PEG10K–Na_2SO_4-0.215 M NaCl are characterized;
- Partitioning of eight organic compounds and six proteins in the systems are examined;
- Partition behavior of all solutes is considered in terms of solute-solvent interactions;
- It is established that NaCl additive interacts with the solutes in the presence of exceeding amount of Na_2SO_4.

5.1. Introduction

ATPSs formed in aqueous mixtures of a single polymer and specific salt, such as PEG and sodium sulfate, phosphate or citrate, are commonly used for separation of proteins and nucleic acids [1]. These ATPSs are inexpensive and have good operational characteristics (low viscosity of the phases, high settling speed) and are easily scaled-up. Extraction in ATPS has been demonstrated as an efficient method for large scale recovery and purification of proteins and nucleic acids [1] as well as various other materials. Design of optimal extraction conditions for any target product remains currently an empirical process, and high throughput methods for screening different separation conditions have been developed [2], [3]. For rational design of the optimal separation conditions itis important to understand the mechanisms of solute distribution in polymer–salt ATPS at the molecular level.

One of the factors commonly used for manipulating partition behavior of proteins and nucleic acids in PEG–salt ATPSs is addition of NaCl [2], [4]–[14]. The mechanism of effects of relatively small amounts of NaCl in the ATPS containing large amount of phase-forming salt remains unclear. We reported [15]–[17] previously that different salt additives (NaCl, NaH_2PO_4, $NaClO_4$, NaSCN) at the concentrations from 0.027 M up to ca. 1.9 M affect partition behavior of small organic compounds in PEG–Na_2SO_4 ATPS according to the salt effects on the water structure. It has been shown [18] recently that solute partitioning in PEG–Na_2SO_4 ATPS is governed by the solute-solvent interactions in the coexisting phases. Partition coefficient of a solute in an ATPS is defined as the ratio of the solute concentration in the top phase to the solute concentration in the bottom phase and may be described as [18]:

$$\log K = S_S\,\Delta\pi^* + B_S\,\Delta\alpha + A_S\,\Delta\beta + C_S\,c$$

(Equation 5.1)

where K is the solute partition coefficient; $\Delta\pi^*$, $\Delta\alpha$, $\Delta\beta$ and c are the differences between the solvent properties of the top and bottom phases (solvent dipolarity/polarizability, hydrogen-bond donor acidity, hydrogen-bond acceptor basicity, and electrostatic interactions, respectively; S_s, B_s, A_s, and C_s are constants (solute-specific coefficients) that describe the complementary interactions of the solute with the solvent media in the coexisting phases; the subscript 'S' designates the solute.

The differences between the solvent dipolarity/polarizability, $\Delta\pi^*$, hydrogen-bond donor acidity, $\Delta\alpha$, hydrogen-bond acceptor basicity, $\Delta\beta$, may be quantified with solvatochromic dyes [18]. The difference

between the electrostatic properties of the phases may be determined by analysis of the partition coefficients of a homologous series of sodium salts of dinitrophenylated (DNP-) amino acids with aliphatic alkyl side-chains [18], [19]. It has been shown that for a given compound (including proteins) the solute-specific coefficients may be determined by multiple linear regression analysis of the partition coefficients of the compound in multiple ATPSs with the same ionic composition.

The purpose of the present work was to explore the effect of NaCl additive on partitioning of different solutes in PEG–Na_2SO_4 ATPS in terms of solute–solvent interactions. It has been shown [18] previously that the solvatochromic dyes may be used for analysis of the solvent properties of the phases in PEG–Na_2SO_4 ATPS with NaCl additive in the concentration range from 0 to 0.54 M. We selected the NaCl concentration of 0.215 M, and examined partitioning of eight different organic compounds and six proteins in several PEG–Na_2SO_4–0.215 M NaCl ATPSs in the presence of different osmolytes (sorbitol, sucrose, and trimethylamine N-oxide) previously established [18] to affect solvent properties of the phases but not to engage in direct interactions with the solutes being partitioned.

5.2. Experimental

5.2.1. Materials

Polyethylene glycol-8000 (Lot 091M01372V) with an average molecular weight (Mw) of 8,000 and polyethylene glycol-10000 (Lot 043K2522) with an average molecular weight (Mw) of 10,000 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The solvatochromic probes 4-nitrophenol (reagent grade, >98%) was purchased from Aldrich (Milwaukee, WI, USA) and 4-nitroanisole (>97%, GC) was received from Acros Organics. Reichardt's carboxylated betaine dye, 2,6-diphenyl-4-[2,6-diphenyl-4-(4-carboxyphenyl)-1-pyridino]phenolate, sodium salt was kindly provided by Professor C. Reichardt (Philipps University, Marburg, Germany). Sorbitol and trimethylamine N-oxide (TMAO) were purchased from Sigma-Aldrich, and sucrose was received from

USB (Cleveland, OH, USA). Benzyl alcohol, caffeine; coumarin, methyl anthranilate, 4-nitrophenyl-α-D-glucopyranoside, phenol, 2-phenylethanol, vanillin, and *o*-phthaldialdehyde (OPA) reagent (complete) were purchased from Sigma-Aldrich. All compounds were of 98-9% purity and used as received without further purification. All salts and other chemicals used were of analytical-reagent grade.

5.2.1.1. Dinitrophenylated amino acids

Dinitrophenylated (DNP) amino acids: DNP-glycine, DNP-alanine, DNP-norvaline, DNP-norleucine, and DNP-α-amino-*n*-octanoic acid, were purchased from Sigma-Aldrich. The sodium salts of the DNP-amino acids were prepared by titration.

5.2.1.2. Proteins

α-chymotrypsin from bovine pancreas, α-chymotrypsinogen A from bovine pancreas, concanavalin A from *Canavalia ensiformis* (jack beans), lysozyme from chicken egg white, and papain from papaya latex were purchased from Sigma-Aldrich. Porcine pancreatic lipase was purchased from USB Corp. (Solon, OH, USA). All protein samples were characterized by SDS-PAGE electrophoresis in a microfluidic chip using Experion automated electrophoresis station (Bio-Rad, USA) under non-reduced conditions. All proteins were observed as single bands in the electrophoregrams.

5.2.2. Methods

5.2.2.1. Aqueous two-phase systems

Stock solutions of PEG8K (50 wt.%), PEG10K (50 wt.%) and Na_2SO_4 (20.3 wt.%) were prepared in water. Sodium phosphate buffer (NaPB; 0.5 M, pH 6.8) was prepared by mixing 3.45 g of $NaH_2PO_4 \cdot H_2O$ and 3.55 g Na_2HPO_4 in 100 mL aqueous solution. Stock solutions of osmolytes: sorbitol (2 M), sucrose (1.8 M), and TMAO (1.8 M), and NaCl (5 M) were prepared in water. A mixture of PEG8K or PEG10K, buffer, and NaCl was prepared by dispensing appropriate amounts of the aqueous stock of polymer, Na_2SO_4 and NaPB solutions into a 1.2 mL microtube using a Hamilton (Reno, NV, USA) ML-4000 four-probe liquid-handling workstation. Appropriate amounts of water and/or stock osmolytes solutions were added to give the required ionic, polymer, and osmolyte composition of the final system with total weight of 0.5 g (after addition of the solute sample, see below). All aqueous PEG8K–Na_2SO_4–NaCl two-phase

systems had a fixed composition of 11.10 wt.% PEG8K, 6.33 wt.% Na_2SO_4, 0.215 M NaCl, and 0.01 M NaPB, pH 6.8, with different 0.5 M osmolyte additive. The aqueous PEG10K–Na_2SO_4–NaCl two-phase system had the same composition of 11.10 wt.% PEG10K, 6.33 wt.% Na_2SO_4, 0.215 M NaCl, and 0.01 M NaPB, pH 6.8.

5.2.2.2. Partitioning experiments

The partitioning experiments were carried out as described in Chapter 4.

Deviation from the average K-value was consistently below 3% and, in most cases, lower than 2%.

5.2.2.3. Solvatochromic studies

The solvatochromic studies were carried out as described in Chapter 4. The procedure was based on the methodology described in [20] and the results analyzed as described in [21].

5.3. Results

Differences between the solvent properties of the coexisting phases (solvent dipolarity/polarizability, $\Delta\pi^*$, hydrogen bond donor acidity, $\Delta\alpha$, and hydrogen bond acceptor basicity, $\Delta\beta$) determined using solvatochromic dyes and calculated as the difference between the measured value of a given solvent feature in the top phase and that of the same feature in the bottom phase are presented in Table 5.1. The difference between the solvent hydrogen bond acidity, $\Delta\alpha$, in the PEG8K–Na_2SO_4 ATPS in the presence of 0.215 M NaCl increases significantly, while the differences between the solvent dipolarity/polarizability, $\Delta\pi^*$, and hydrogen bond basicity, $\Delta\beta$, do not change within the experimental error limits. The effect of same NaCl additive on the differences between the solvent properties in PEG10K–Na_2SO_4 ATPS is much more significant. Similarly, the effects of the NaCl additive in the PEG8K–Na_2SO_4–0.5 M osmolyte ATPSs differ depending on the particular osmolyte used. The NaCl additive does not affect the sequence of the differences between the solvent dipolarity/polarizability of the coexisting phases increasing as:

PEG8K–Na_2SO_4–0.5 M TMAO < PEG10K–Na_2SO_4 < PEG8K–Na_2SO_4 <
< PEG8K–Na_2SO_4–0.5 M sorbitol < PEG8K–Na_2SO_4–0.5 M sucrose

The differences between the hydrogen bond donor acidity of the two phases are affected by the NaCl additive noticeably. The sequence changes from:

PEG10K–Na_2SO_4 < PEG8K–Na_2SO_4 < PEG8K–Na_2SO_4–0.5 M TMAO <
< PEG8K–Na_2SO_4–0.5 M sorbitol < PEG8K–Na_2SO_4–0.5 M sucrose

to

PEG10K–Na_2SO_4–0.215 M NaCl ≤ PEG8K–Na_2SO_4–0.215 M NaCl <
< PEG8K–Na_2SO_4–0.215 M NaCl–0.5 M sucrose ≤
≤ PEG8K–Na_2SO_4–0.215 M NaCl–0.5 M sorbitol < PEG8K–Na_2SO_4–0.215 M NaCl–0.5 M TMAO

The effect of the NaCl additive on the differences between the solvent hydrogen bond basicity of the phases barely exceeds the experimental error limits.

The differences between the hydrophobic and electrostatic properties of the phases were determined in each ATPS by partitioning of a homologous series of sodium salts of dinitrophenylated (DNP-) amino acids with the aliphatic alkyl side-chains of the increasing length (alanine, norvaline, norleucine, and α-amino-*n*-octanoic acid) as described previously [18], [19]. Partition coefficients of these compounds are listed in Table 5.1.

Differences between the solvent properties of the two phases in each ATPS used are presented in Table 5.1 together with partition coefficients for eight organic compounds examined here.

Partitioning of six proteins was examined in all the aforementioned ATPSs and the corresponding partition coefficients are listed in Table 5.2.

Table 5.1. Differences between the solvent properties of the phases and partition coefficients for simple organic compounds and free amino acids in PEG8K–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8, PEG10K–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8, and PEG8K–Na_2SO_4–0.215 M NaCl–0.5 M osmolyte–0.01 M NaPB, pH 6.8 ATPSs.

Solvent Properties	0.01M NaPB[a]	0.01M NaPB[b]	0.5M Sorbitol	0.5M Sucrose	0.5M TMAO
$\Delta G(CH_2)$, cal/mole	-135±10.6	-147±6.5	-180±5.3	-187±2.9	-160±2.4
E	0.102±0.008	0.110±0.005	0.133±0.004	0.138±0.002	0.118±0.002
C	0.490±0.031	0.440±0.019	0.530±0.015	0.525±0.008	0.574±0.006
$\Delta\pi^*$	-0.027±0.003	-0.039±0.002	-0.056±0.001	-0.067±0.001	-0.025±0.004
$\Delta\alpha$	-0.189±0.004	-0.183±0.004	-0.259±0.003	-0.253±0.003	-0.271±0.004
$\Delta\beta$	0.013±0.004	0.021±0.003	0.025±0.002	0.022±0.002	0.015±0.005

a – Data for osmolyte-free PEG8K–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8 ATPS

b – Data for osmolyte-free PEG10K–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8 ATPS

Table 5.1. (cont.) Differences between the solvent properties of the phases and partition coefficients for simple organic compounds and free amino acids in PEG8K–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8, PEG10K–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8, and PEG8K–Na_2SO_4–0.215 M NaCl–0.5 M osmolyte–0.01 M NaPB, pH 6.8 ATPSs.

	Partition coefficients				
COMPOUND	**0.01M NaPB[a]**	**0.01M NaPB[b]**	**0.5M Sorbitol**	**0.5M Sucrose**	**0.5M TMAO**
Benzyl alcohol	4.07±0.008	4.15±0.007	6.08±0.018	6.61±0.038	4.71±0.016
Caffeine	2.11±0.036	2.36±0.006	2.72±0.008	2.55±0.007	2.82±0.008
Coumarin	4.86±0.014	4.55±0.023	8.23±0.024	8.46±0.065	6.27±0.024
Glucoside	2.47±0.033	2.92±0.003	3.96±0.001	4.05±0.010	3.41±0.011
Methyl anthranilate	9.59±0.027	9.58±0.023	14.80±0.290	16.42±0.048	11.77±0.082
Phenol	6.50±0.170	6.54±0.018	10.60±0.030	11.97±0.055	7.38±0.022
2-Phenylethanol	4.98±0.012	5.05±0.013	7.39±0.023	8.52±0.025	5.60±0.025
Vanillin	8.28±0.020	8.31±0.017	13.03±0.04	13.22±0.044	7.58±0.029
DNP-Ala Na	4.43±0.120	3.99±0.015	5.12±0.010	5.09±0.018	5.40±0.020
DNP-NVal Na	5.43±0.099	5.36±0.012	7.68±0.031	7.87±0.021	7.60±0.018
DNP-NLeu Na	7.15±0.032	6.88±0.027	10.23±0.036	10.81±0.048	10.49±0.016
DNP-AO Na	13.93±0.23	14.01±0.035	23.60±0.130	25.04±0.110	20.88±0.080

Table 5.2. Partition coefficients for proteins in PEG8K–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8, PEG10K–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8, and PEG8K–Na_2SO_4–0.215 M NaCl–0.5 M osmolyte–0.01 M NaPB, pH 6.8 ATPSs.

	Partition coefficients				
PROTEIN	**0.01M NaPB[a]**	**0.01M NaPB[b]**	**0.5M Sorbitol**	**0.5M Sucrose**	**0.5M TMAO**
α-chymotrypsinogen A	0.543±0.003	0.472±0.002	0.335±0.001	0.365±0.001	0.601±0.002
α-chymotrypsin	0.110±0.001	0.081±0.0004	0.041±0.0004	0.043±0.0005	0.082±0.0006
Concanavalin A	0.183±0.001	0.173±0.003	0.146±0.001	0.130±0.001	0.167±0.001
Lipase	0.584±0.001	0.585±0.002	0.534±0.002	0.522±0.003	0.538±0.003
Lysozyme	0.983±0.003	0.929±0.003	0.561±0.002	0.335±0.002	0.786±0.003
Papain	1.920±0.007	1.670±0.010	2.060±0.015	1.625±0.005	2.230±0.010

a – Data for osmolyte-free PEG8K–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8 ATPS

b – Data for osmolyte-free PEG10K–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8 ATPS

5.4. Discussion

5.4.1. Effect of NaCl additive on solvent properties of PEG-Na_2SO_4 ATPS

The solvent properties of each ATPS are represented by the set of differences between the solvent features of the coexisting phases: $\Delta\pi^*$, $\Delta\alpha$, $\Delta\beta$, C, and E values, listed in Table 5.1. The set of the $\Delta\pi^*$, $\Delta\alpha$, $\Delta\beta$, C, and E values for a given ATPS may be viewed as a point in a multiple dimensional space of solvent properties. To compare the properties of different ATPSs we calculated the normalized Euclidian distance in the multi-dimensional space represented by the differences between the solvent features of the coexisting phases in different ATPSs:

$$d_{i,0} = \left[\sum_j \left(\frac{\delta_i - \delta_0}{\delta_0}\right)^2\right]^{0.5}$$

(Equation 5.2)

where $d_{i,o}$ is the distance between the solvent properties of i-th ATPS and solvent properties of the o^{th} ATPS chosen as a reference, ∂i and ∂o are the differences between the i-th solvent features in i-th and o-th ATPSs.

To compensate for differences in ∂-values measured for a given solvent property in different ATPSs, we normalized the experimental ∂-values to the reference ∂o-value for each particular solvent property. Therefore, Eq. 5.2 represents the Euclidean distance between the points represented by normalized differences between various solvent features in different ATPSs.

In order to evaluate the effect of the NaCl additive on the properties of the ATPSs used here and previously [18] we selected PEG8K–Na_2SO_4 ATPS as a reference ATPS. The normalized Euclidean distances calculated with Eq. 5.2 are listed in Table 5.3.

Table 5.3. Normalized Euclidean distances between the solvent properties of ATPSs indicated and those of PEG8K–Na_2SO_4 calculated with Eq. 5.2 from the data in Table 5.1 and in [18].

ATPS*	Distance, $d_{i,0}$
PEG8K–Na_2SO_4	0
PEG10K–Na_2SO_4	1.21±0.05
PEG8K–Na_2SO_4–0.215M NaCl	1.24±0.05
PEG10K–Na_2SO_4–0.215M NaCl	1.46±0.06
PEG8K–Na_2SO_4–0.5M TMAO	1.64±0.06
PEG8K–Na_2SO_4–0.5M sorbitol	1.70±0.05
PEG8K–Na_2SO_4–0.215M NaCl–0.5M TMAO	1.86±0.06
PEG8K–Na_2SO_4–0.215M NaCl–0.5M sorbitol	2.35±0.05
PEG8K–Na_2SO_4–0.5M trehalose	2.39±0.06
PEG8K–Na_2SO_4–0.215M NaCl–0.5M sucrose	2.54±0.05
PEG8K–Na_2SO_4–0.5M sucrose	2.61±0.05

***** Each ATPS contains 11.10 %wt. PEG8K, 6.33 %wt. Na_2SO_4 and 0.01M NaPB, pH 6.8

The data in Table 5.3 indicate that the effect of 0.215 M NaCl on the overall solvent properties of the PEG8K–Na_2SO_4 ATPS is rather small in osmolyte-free ATPS, depends on the presence of particular osmolyte, and increases in the sequence:

$$\text{TMAO} < \text{sorbitol} < \text{sucrose}$$

Analysis of partition coefficients determined in this study for various organic compounds and proteins listed in Tables 5.1 and 5.2 shows that the so-called Collander solvent regression equation (see, e.g., in [17]–[19]) holds for all compounds (including proteins) in the PEG8K–Na_2SO_4–0.215 M NaCl and PEG10K–Na_2SO_4–0.215 M NaCl ATPSs and the same NaCl-free ATPSs [18] as shown in Fig. 5.1. The linear relationship plotted in Fig. 5.1 may be described as:

$$\log K_i^{PEG10K-Na_2SO_4(0.215M\ NaCl)} = -0.02_{\pm 0.01} + 1.03_{\pm 0.01} \times \log K_i^{PEG8K-Na_2SO_4(0.215M\ NaCl)}$$

N = 29; r^2 = 0.9955; SD = 0.042; F = 5953

(Equation 5.3)

where $K_i^{PEG8K\text{-}Na2SO4(0.215\ M\ NaCl)}$ and $K_i^{PEG10K\text{-}Na2SO4(0.215\ M\ NaCl)}$ are partition coefficients for the i-th compound in PEG8K–Na_2SO_4 and PEG10K–Na_2SO_4 with and without 0.215 M NaCl ATPSs correspondingly.

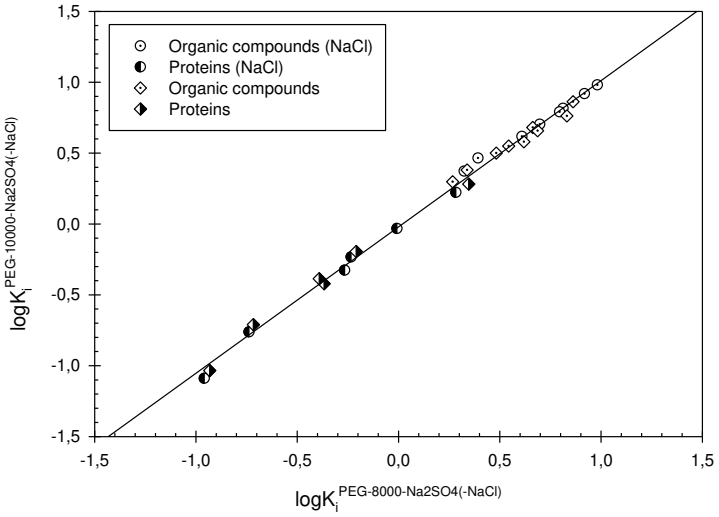


Figure 5.1. Logarithms of partition coefficients for organic compounds and proteins in PEG10K–Na_2SO_4–0.01 M NaPB, pH 6.8 with and without 0.215 M NaCl additive ATPSs versus those for the same compounds and proteins in PEG8K–Na_2SO_4–0.01 M NaPB, pH 6.8 with and without 0.215 M NaCl additive ATPSs.

Comparison of the partition coefficients of organic compounds and proteins in PEG8K–Na_2SO_4–NaCl ATPS with those for the same compounds in the NaCl free PEG8K–Na_2SO_4 ATPS reported previously [15], [17], [18], illustrated graphically in Fig. 5.2 shows that the Collander solvent regression relationship holds for organic compounds but not for proteins. The relationship in Fig. 5.2 may be described as:

$$\log K_i^{PEG8K-Na_2SO_4-0.215M\ NaCl} = -0.08_{\pm 0.013} + 1.33_{\pm 0.026} \times \log K_i^{PEG8K-Na_2SO_4}$$

N = 13; r^2 = 0.9958; SD = 0.032; F = 2583

(Equation 5.4)

where all the parameters are as defined above. The proteins examined do not fit the relationship likely due to the different protein-specific responses to the presence of NaCl additive.

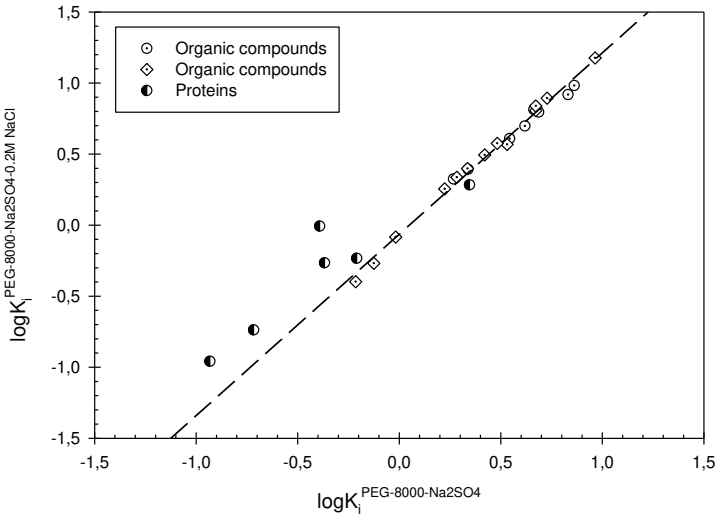


Figure 5.2. Logarithms of partition coefficients for organic compounds and proteins in PEG8K–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8 versus those for the same compounds and proteins in PEG8K–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPSs (line described the linear relationship observed for organic compounds).

Analysis of the data in Tables 5.1 and 5.2 shows that the NaCl additive affects partition behavior of proteins to a much more significant degree than that of organic compounds as may be illustrated by Fig. 5.3 where logarithms of partition coefficients of organic compounds and proteins in the PEG8K–Na_2SO_4–0.5 M sucrose–0.215 M NaCl ATPS are plotted versus those in the same NaCl additive free ATPS.

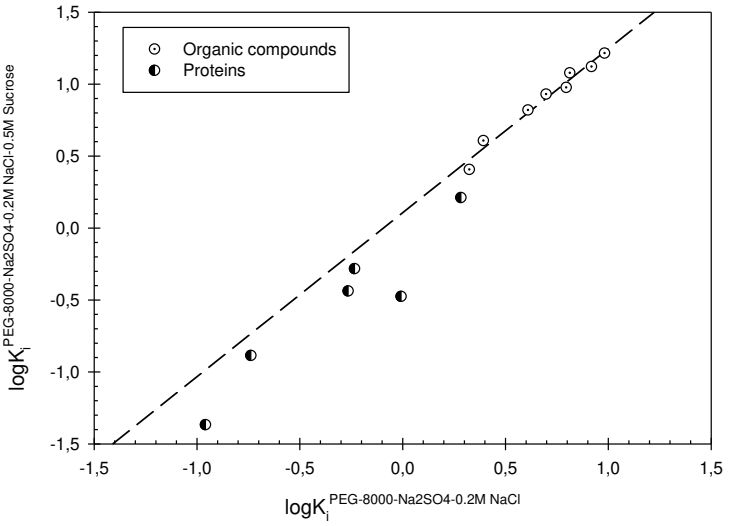


Figure 5.3. Logarithms of partition coefficients for organic compounds and proteins in PEG8K–Na_2SO_4–0.5 M sucrose–0.215 M NaCl–0.01 M NaPB, pH 6.8 versus those for the same compounds and proteins in PEG8K–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8 ATPS (line described the linear relationship observed for organic compounds).

Partition coefficients of lysozyme, α-chymotrypsinogen A (CHTG), and papain clearly do not fit the linear curve representing the Collander solvent regression relationship observed for organic compounds. It should be mentioned that partition coefficients of some of the proteins, such as lipase, concanavalin A, and α-chymotrypsin, are very close to the relationship in question. Similar pattern is observed for the ATPS with 0.5 M TMAO and 0.5 M sorbitol.

It has been shown [18] previously that logarithms of partition coefficients of proteins and organic compounds in PEG–Na_2SO_4–0.5 M osmolyte, all containing 0.01 M K/NaPB, pH 6.8 are linearly interrelated in a three-dimensional space. Analysis of the partition coefficients listed in Tables 5.1 and 5.2 show that similar relationships exists for all compounds examined in PEG–Na_2SO_4–0.5 M osmolyte–0.215 M NaCl ATPS as well. The relationship illustrated graphically in Fig. 5.4 is observed between logarithms of partition coefficients of compounds (including proteins) in PEG–Na_2SO_4–0.215 M NaCl, PEG–Na_2SO_4–0.5 M sucrose–0.215 M NaCl, and PEG–Na_2SO_4–0.5 M sorbitol–0.215 M NaCl and it may be described as:

$$\log K_i^{PEG8K-Na_2SO_4-Sorbitol-NaCl} = -0.01_{\pm 0.02} + 0.50_{\pm 0.14} \times \log K_i^{PEG8K-Na_2SO_4-NaCl} + 0.60_{\pm 0.10} \times \log K_i^{PEG8K-Na_2SO_4-Sucrose-NaCl}$$

N = 14; r^2 = 0.9956; SD = 0.051; F = 1628

(Equation 5.5)

where -NaCl superscript denoted that the logarithms of partition coefficients in the ATPS with 0.215 M NaCl additive were used; all the parameters are as defined above. It should be mentioned that the relationship observed is essentially identical to the one reported [18] previously in the same but NaCl-free ATPSs.

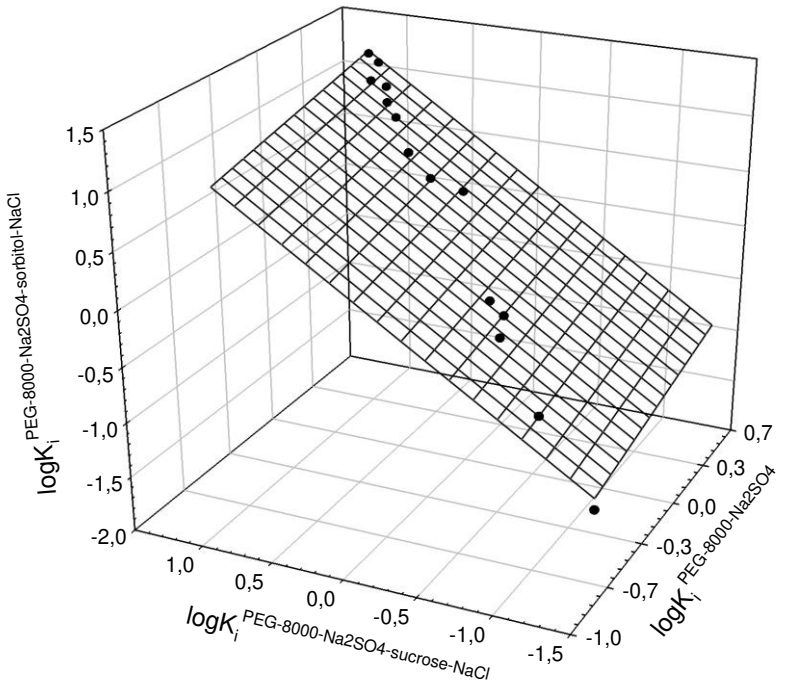


Figure 5.4. Logarithms of partition coefficients for organic compounds and proteins in PEG8K–Na_2SO_4–0.5 M sorbitol–0.215 M NaCl–0.01 M NaPB, pH 6.8 versus those for the same compounds and proteins in PEG8K–Na_2SO_4–0.01 M NaPB pH 6.8 and in PEG8K–Na_2SO_4–0.5 M sucrose–0.215 M NaCl–0.01 M NaPB pH 6.8 ATPSs.

Similar relationship for the PEG8K–Na_2SO_4–TMAO–NaCl, PEG8K–Na_2SO_4–sorbitol–NaCl, and PEG8K–Na_2SO_4–sucrose–NaCl ATPSs is illustrated graphically in Fig. 5.5 and may be described as:

$$\log K_i^{PEG8K-Na_2SO_4-Sorbitol-NaCl} = -0.01_{\pm 0.02} + 0.50_{\pm 0.16} \times \log K_i^{PEG8K-Na_2SO_4-TMAO-NaCl} + 0.60_{\pm 0.13} \times \log K_i^{PEG8K-Na_2SO_4-Sucrose-NaCl}$$

N = 14; r^2 = 0.9961; SD = 0.054; F = 1418

(Equation 5.6)

where all the parameters are as defined above.

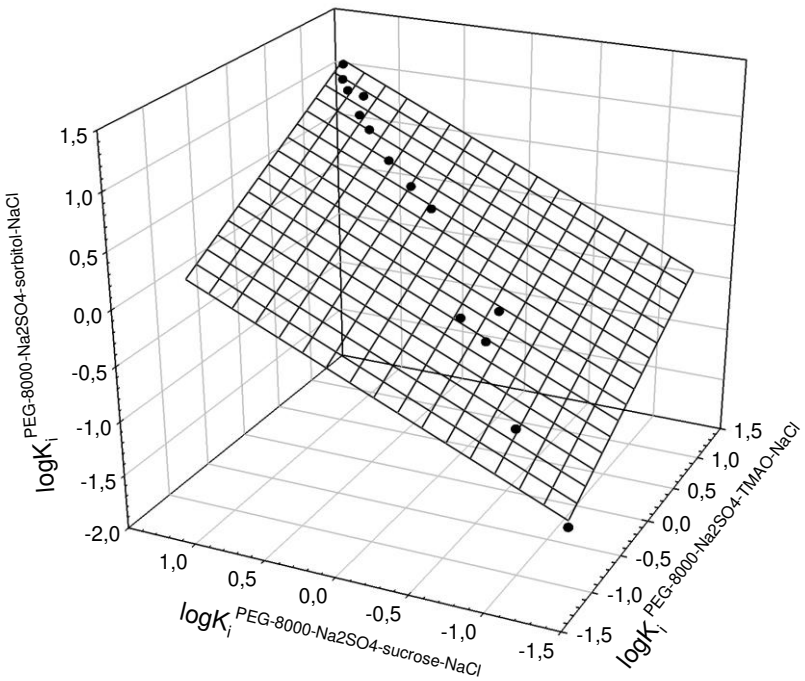


Figure 5.5. Logarithms of partition coefficients for organic compounds and proteins in PEG8K–Na_2SO_4–0.5 M sorbitol–0.215 M NaCl–0.01 M NaPB, pH 6.8 versus those for the same compounds and proteins in PEG8K–Na_2SO_4–0.5 M TMAO–0.215 M NaCl–0.01 M NaPB, pH 6.8 and in PEG8K–Na_2SO_4–0.5 M sucrose–0.215 M NaCl–0.01 M NaPB pH 6.8 ATPSs.

It should be noted that the similar relationships are observed if the logarithms of partition coefficients in NaCl-free ATPS [18] is compared with those in ATPSs with NaCl additive. Typical relationship is illustrated graphically in Fig. 5.6 and it may be described as:

$$\log K_i^{PEG8K-Na_2SO_4-TMAO-NaCl} = 0.02_{\pm 0.02} + 0.24_{\pm 0.17} \times \log K_i^{PEG8K-Na_2SO_4} + 0.85_{\pm 0.17} \times \log K_i^{PEG8K-Na_2SO_4-NaCl}$$

N = 14; r^2 = 0.9922; SD = 0.065; F = 697

(Equation 5.7)

where all the parameters are as defined above.

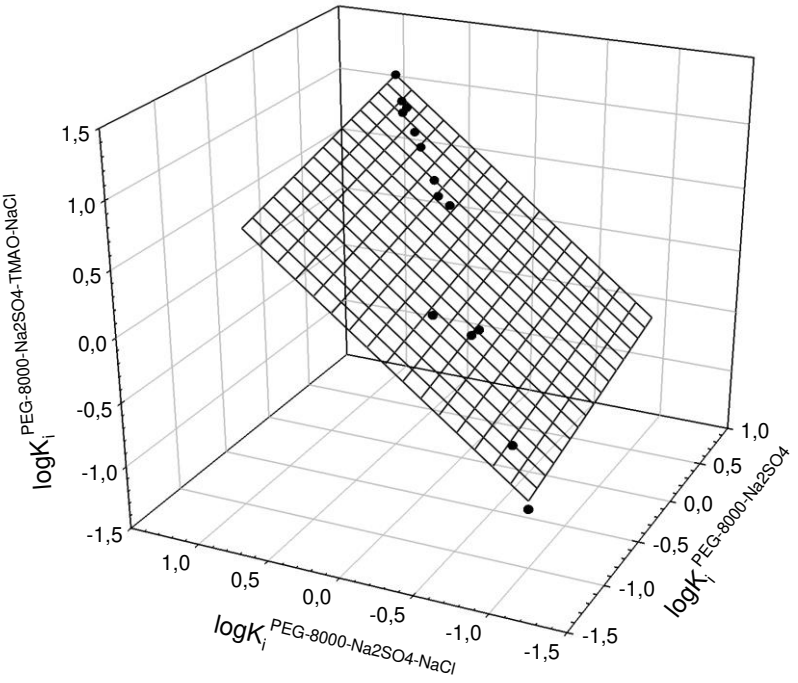


Figure 5.6. Logarithms of partition coefficients for organic compounds and proteins in PEG8K–Na_2SO_4–0.5 M TMAO–0.215 M NaCl–0.01 M NaPB, pH 6.8 versus those for the same compounds and proteins in PEG8K–Na_2SO_4–0.01 M NaPB, pH 6.8 and in PEG8K–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8 ATPSs.

It was suggested previously [22] that the relationships of the type represented by Eqs. 5.3-5.7 imply that the compounds respond to their environment in aqueous solutions in the compound structure- and environment-specific manner, and also that the responses are governed by changes in the compound-water interactions possibly originating from the compound dipole-ion interactions.

5.4.2. Organic compound-water interactions in PEG-Na_2SO_4 ATPS

The partition coefficients for organic compounds listed in Table 5.1 were examined with Eq. 5.1. The solute-specific coefficients were determined by multiple linear regression analysis using the procedure described by Ab Rani *et al.* [23]. According to this procedure [23], the p-value was used for a given compound as a test for significance for each solute-specific coefficient in Eq. 5.1. In view of the small number of five ATPSs employed (and additional condition of partition coefficient K-value = 1 for the compound in the theoretical critical point in an ATPS, when both phases have identical composition; i.e., no difference between each of the solvent properties of the phases [19]), we have used the maximum statistical significance value of $p \leq 0.1$. If all four coefficients (S_s, A_s, B_s, and C_s) proved statistically significant ($p \leq 0.1$), the correlation was accepted. If one or more values reveal a p-value > 0.1, then equations contained different combinations of coefficients were examined. The equation with a set of coefficients providing p-values for all parameters below or equal to 0.1 was accepted.

The solute-specific coefficients determined for each compound and those for the same compounds determined in the absence of NaCl additive [18] are presented in Table 5.4 with the corresponding p-values (except the cases when $p < 0.001$) and the solute-specific coefficients.

Table 5.4. Solute-specific coefficients (see Eq. 5.1) for organic compounds in PEG–Na_2SO_4–0.215 M NaCl–0.01 M NaPB (calculated by multiple linear regression analysis from data in Table 5.1) and in PEG–Na_2SO_4–0.01 M NaPB (data from [18]).

COMPOUND	S_s	A_s	B_s	C_s	N; SD; F
Benzyl alcohol (NaCl) p-value	-4.7±0.15	-	-	1.0±0.01	6; 0.006; 40747
Benzyl alcohol p-value	-2.9±0.55 0.006	-	1.1±0.33 0.03	1.3±0.10 0.0002	7; 0.03; 1304
Caffeine (NaCl) p-value	2.2±0.85 0.080	9.0±2.80 0.050	-1.4±0.15 0.003	-	6; 0.02; 836.1
Caffeine p-value	-	-	0.3±0.10 0.08	0.7±0.05	7; 0.01; 2638
Coumarin (NaCl) p-value	-2.4±0.26 0.003	5.2±0.87 0.009	-	1.3±0.02	6; 0.005; 48324
Coumarin p-value	-	-	-	1.4±0.03	7; 0.05; 1673
Glucoside (NaCl) p-value	-1.3±0.90 0.100	6.1±2.00 0.050	-1.5±0.10 0.0007	-	6; 0.01; 2909
Glucoside p-value	-1.4±0.40 0.020	-	-	0.7±0.03	7; 0.02; 1637
Methyl anthranilate (NaCl) p-value	-4.7±0.33 0.005	3.9±1.11 0.070	1.1±0.16 0.020	2.1±0.07 0.001	6; 0.006; 35509
Methyl anthranilate p-value	-3.0±0.82 0.0200	-	1.7±0.49 0.02	2.1±0.16 0.0002	7; 0.04; 1366

Table 5.4. (cont.) Solute-specific coefficients (see Eq. 5.1) for organic compounds in PEG–Na_2SO_4–0.215 M NaCl–0.01 M NaPB (calculated by multiple linear regression analysis from data in Table 5.1) and in PEG–Na_2SO_4–0.01 M NaPB (data from [18]).

COMPOUND	S_s	A_s	B_s	C_s	N; SD; F
Phenol (NaCl) p-value	-6.6±0.21	-	0.7±0.17 0.030	1.6±0.07 0.0002	6; 0.007; 28494
Phenol p-value	-3.8±0.48 0.001	-	1.0±0.99 0.025	1.5±0.09	7; 0.02; 2839
2-Phenylethanol (NaCl) p-value	-5.7±0.05	-	0.7±0.04 0.0005	1.4±0.02	6; 0.002; 380735
2-Phenylethanol p-value	-2.4 ±0.87 0.040	-	-	1.1±0.07	7; 0.05; 818
Vanillin (NaCl) p-value	-8.1±1.10 0.005	-	2.6±0.90 0.060	2.4±0.38 0.008	6; 0.04; 1233
Vanillin p-value	-3.0±1.40 0.100	-	1.5±0.85 0.100	1.9±0.27 0.002	7; 0.07; 382
DNP-Ala Na (NaCl) p-value	-0.5±0.10 0.040	2.0±0.31 0.020	0.4±0.05 0.010	1.4±0.02 0.0002	6; 0.002; 166699
DNP-Ala Na p-value	-0.5±0.14 0.030	-	0.5±0.08 0.004	1.4±0.03	7; 0.007; 23785
DNP-NVal Na (NaCl) p-value	-2.4±0.39 0.003	-	-	1.4±0.30	6; 0.01; 8231
DNP-NVal Na p-value	1.5±0.31 0.009	-	0.8±0.18 0.010	1.7±0.06	7; 0.01; 7372

Table 5.4. (cont.) Solute-specific coefficients (see Eq. 5.1) for organic compounds in PEG–Na_2SO_4–0.215 M NaCl–0.01 M NaPB (calculated by multiple linear regression analysis from data in Table 5.1) and in PEG–Na_2SO_4–0.01 M NaPB (data from [18]).

COMPOUND	S_s	A_s	B_s	C_s	N; SD; F
DNP-NLeu Na (NaCl) p-value	-2.5±0.38 0.003	-	-	1.7±0.03	6; 0.01; 11183
DNP-NLeu Na p-value	-1.7±0.56 0.040	-	1.0±0.30 0.040	1.9±0.10	7; 0.03; 2876
DNP-AO Na (NaCl) p-value	-3.0±0.63 0.020	6.6±2.10 0.050	-	2.0±0.05	6; 0.01; 16774
DNP-AO Na p-value	-2.8±0.73 0.020	-	2.1±0.44 0.009	2.7±0.14	7; 0.03; 2774

Comparison of the solute-specific coefficients S_s representing the contribution of dipole-dipole solute-solvent interactions into partition coefficients of the compounds in PEG–Na_2SO_4 ATPS in the absence and in the presence of 0.215 M NaCl indicate that there is a linear relationship illustrated graphically in Fig. 5.7 that may be described as:

$$S_S^{i\ PEG-Na_2SO_4-0.215M\ NaCl} = 0.7_{\pm 0.3} + 1.9_{\pm 0.13} \times S_S^{i\ PEG-Na_2SO_4}$$

N = 7; r^2 = 0.9779; SD = 0.35; F = 221

(Equation 5.8)

where $S_S^{iPEG-Na2SO4-0.21\ 5M\ NaCl}$ and $S_S^{iPEG-Na2SO4}$ are the solute-specific coefficients S_s for i-th organic compound determined in PEG–Na_2SO_4–0.215 M NaCl and PEG–Na_2SO_4 ATPS, correspondingly; all the other parameters are as defined above.

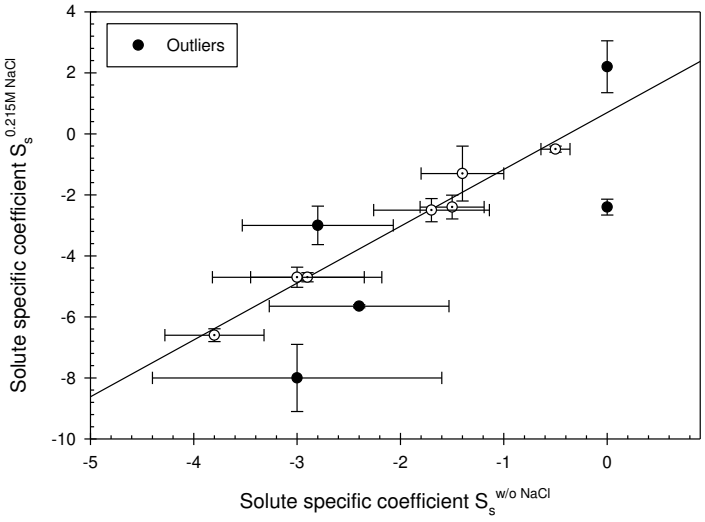


Figure 5.7. Interrelationship between solute-specific coefficients S_s 0.215 M NaCl for organic compounds and solute-specific coefficients S_s without NaCl for the same compounds determined in the presence and absence of 0.215 M NaCl correspondingly.

It should be mentioned that caffeine, coumarin, 2-phenylethanol, vanillin and DNP-AO Na-salt were considered to be outliers and not included in the relationship. Two of these compounds (caffeine and coumarin) show S_s value to be insignificant in the absence of NaCl [18], while the three other compounds might be included taking into account large experimental errors for the S_s values. It should be noted that Eq. 5.8 indicates that the coefficient S_s value for nonionic compounds increases in the presence of NaCl likely due to ion-dipole interactions.

Comparison of the solute-specific coefficient C_s values determined for the same compounds in the PEG–Na_2SO_4 and in PEG–Na_2SO_4–0.215 M NaCl ATPSs shows that the linear relationship similar to the one discussed above is also observed. It is presented graphically in Fig. 5.8 and may be described as:

$$C_S^{i\ PEG-Na_2SO_4-0.215M\ NaCl} = -1.5_{\pm 0.12} + 2.0_{\pm 0.09} \times C_S^{i\ PEG-Na_2SO_4}$$

N = 6; r^2 = 0.9925; SD = 0.092; F = 528

(Equation 5.9a)

where $C_S^{iPEG-Na2SO4-0.215M\ NaCl}$ and $C_S^{iPEG-Na2SO4}$ are the solute-specific coefficients C_s for i-th organic compound determined in PEG–Na_2SO_4–0.215 M NaCl and PEG–Na_2SO_4 ATPS, correspondingly; all the other parameters are as defined above. It should be mentioned that 2-phenylethanol and methyl anthranilate

do not fit the relationship and are considered to be outliers. The relationship seems to hold for nonionic compounds only.

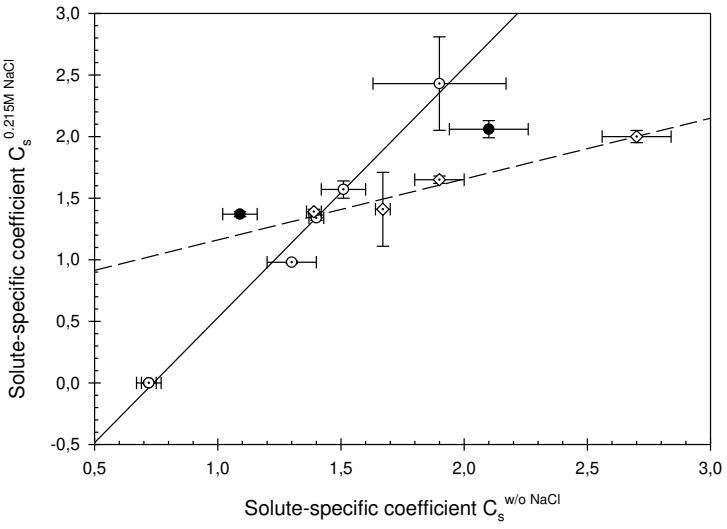


Figure 5.8. Interrelationships between solute-specific coefficients C_s 0.215 M NaCl for nonionic organic compounds (circles) and charged DNP-AA sodium salts (diamonds) and solute-specific coefficients CS without NaCl for the same compounds determined in the presence and absence of 0.215 M NaCl correspondingly.

For sodium salts of DNP-amino acids the NaCl effect is not as strong as shown by the relationship in Fig. 5.8 described as:

$$C_S^{DNP-AA\ PEG-Na_2SO_4-0.215M\ NaCl} = 0.67_{\pm 0.14} + 0.49_{\pm 0.08} \times C_S^{DNP-AA\ PEG-Na_2SO_4}$$

N = 4; r^2 = 0.9586; SD = 0.07; F = 46.4

(Equation 5.9b)

where superscript DNP-AA denotes DNP-amino acids Na-salts; all the other parameters are as defined above. It may be suggested that the small NaCl effect on the solute-specific coefficient C_s for the charged compounds is due to the influence of the NaCl additive on the ionic compositions of the two phases [16], and in this case it should be NaCl concentration dependent. Additional experiments at different NaCl additive concentrations are clearly needed to verify this assumption.

It has been reported [18]–[24] recently that there seems to be a cooperativity between the different types of solute-water interactions due to which the solute-specific coefficients are linearly interrelated. The interrelationship between the solute-specific coefficients presented in Table 5.4 is illustrated graphically in Fig. 5.9, and it may be described as:

$$S_S^i = -5.3_{\pm 0.5} - 1.1_{\pm 0.31} \times A_S^i + 0.6_{\pm 0.12} \times B_S^i$$

N = 8; r^2 = 0.9607; SD = 0.77; F = 61.1

(Equation 5.10)

where B_i^s, A_i^s, and S_i^s are solute-specific coefficients for the i-th compound; all the other parameters are as defined above.

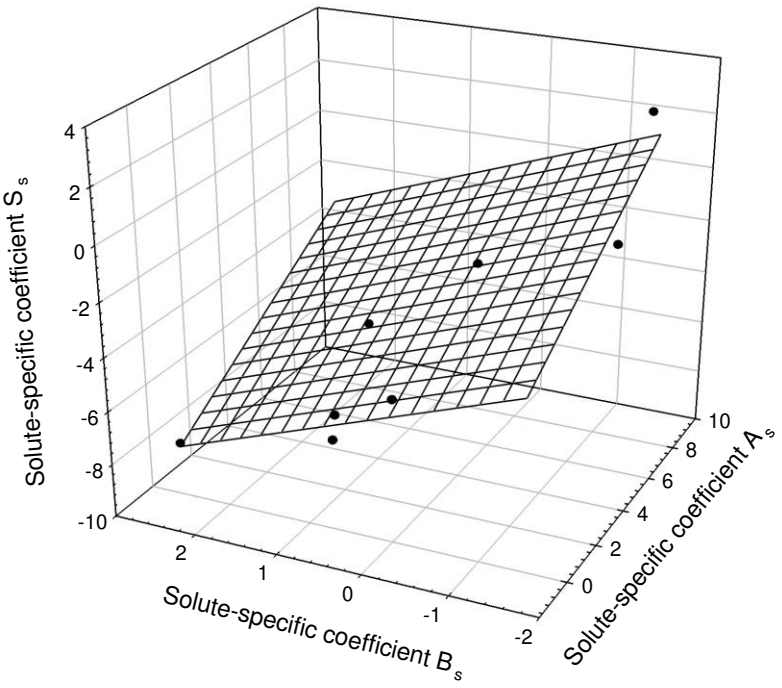


Figure 5.9. Interrelationship between solute specific coefficients S_s, solute-specific coefficients A_s, and solute-specific coefficients B_s for organic compounds determined in the presence of 0.215 M NaCl.

Similar interrelationship is presented in Fig. 5.10 and it may be described as:

$$B_S^i = -1.8_{\pm 0.15} - 0.16_{\pm 0.05} \times S_S^i + 1.1_{\pm 0.14} \times C_S^i$$

N = 7; r^2 = 0.9861; SD = 0.17; F = 141.9

(Equation 5.11)

where B_i^s, C_i^s, and S_i^s are solute-specific coefficients for the i-th compound; all the other parameters are as defined above. It should be noted that the data for caffeine do not fit the relationship.

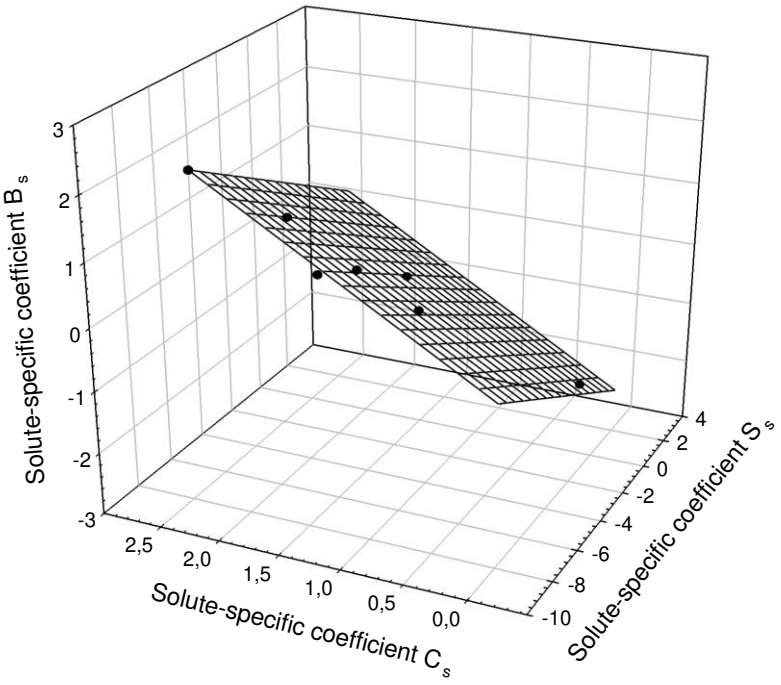


Figure 5.10. Interrelationship between solute-specific coefficients B_s for organic compounds and solute-specific coefficients S_s and C_s for the same compounds determined in the presence of 0.215 M NaCl.

The above data indicate that the NaCl additive in PEG–Na_2SO_4 ATPS does affect the solute-solvent interactions for organic compounds. The effects of the additive on the partition behavior of proteins seem to indicate it would affect the protein-solvent interactions even more.

Table 5.5. Solute-specific coefficients (see Eq. 5.1) for proteins in PEG–Na_2SO_4–0.215 M NaCl–0.01 M NaPB (calculated by multiple linear regression analysis from data in Table 5.1) and in PEG–Na_2SO_4–0.01 M NaPB (data from [18]).

COMPOUND	S_s	A_s	B_s	C_s	N; SD; F
α-chymotrypsinogen A (NaCl)	3.3±1.40	-10.5±3.20	-	-	6; 0.03; 373.2
p-value	0.070	0.030			
α-chymotrypsinogen A	9.6±0.96	24.0±7.10	-	-1.2±0.21	7; 0.04; 518
p-value	0.040	0.030		0.006	
α-chymotrypsin (NaCl)	5.3±0.57	-17.3±1.90	-	-1.2±0.04	6; 0.01; 17401
p-value	0.003	0.003			
α-chymotrypsin	9.9±1.10	33.2±7.70	-2.3±0.50	-3.2 ±0.30	7; 0.04; 1221
p-value	0.003	0.020	0.020	0.001	
Concanavalin A (NaCl)	4.5±0.78	-	-1.8±0.64	-2.0±0.27	6; 0.03; 1557
p-value	0.010		0.060	0.060	
Concanavalin A	2.4±1.10	-	-2.2±0.60	-1.9±0.20	7; 0.05; 469.8
p-value	0.100		0.030	0.0007	
Lipase (NaCl)	0.8±0.14	-	-	-0.4±0.01	6; 0.005; 6087
p-value	0.004				
Lipase	0.8±0.28	-	-0.4±0.17	-0.5±0.05	7; 0.01; 644.5
p-value	0.040		0.070	0.0008	
Lysozyme (NaCl)	12.7±0.90	24.2±2.90	3.2±0.43	1.2±0.19	6; 0.02; 241.1
p-value	0.005	0.010	0.020	0.020	
Lysozyme	12.0±2.40	-	1.7±0.58	-	7; 0.11; 174.8
p-value	0.005		0.040		
Papain (NaCl)	1.8±0.79	-	-	0.7±0.07	6; 0.03; 222.8
p-value	0.050			0.0006	
Papain	4.0±2.00	23.0 ±4.60	-	-	7; 0.09; 36.2
p-value	0.100				

5.4.3. Protein-water interactions in PEG-Na_2SO_4 ATPS

The solute-specific coefficients for proteins presented in Table 5.5 indicate that the effect of NaCl additive is protein specific. As an example, in the presence of 0.215 M NaCl the solute specific coefficient B_s for α-chymotrypsin is displayed as change from -2.3 to 0, and for lysozyme from 1.7 to 3.2. There seems to be no general trend in the NaCl effect on any of the solute-specific coefficients.

Nevertheless, proteins seem to demonstrate cooperativity of different types of solute-water interactions similar to that displayed by simple organic compounds. Analysis of the data in Table 5.5 shows that the linear relationship similar to the one established for organic compounds is observed for proteins as well. This relationship illustrated graphically in Fig. 5.11 may be described as:

$$B_S^i = -0.5_{\pm 0.45} + 0.20_{\pm 0.07} \times S_S^i + 0.9_{\pm 0.25} \times C_S^i$$

N = 6; r^2 = 0.9092; SD = 0.63; F = 15

(Equation 5.12)

where all the parameters are as defined above.

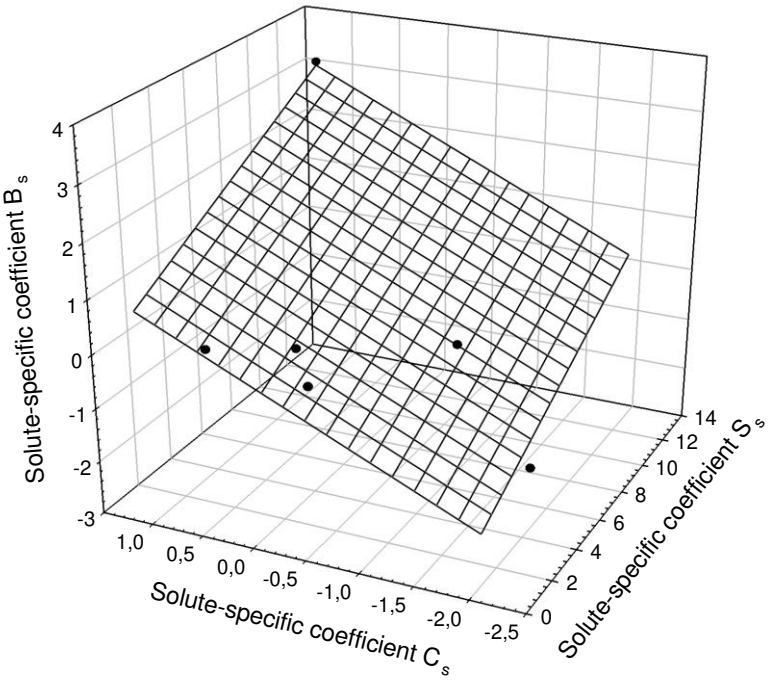


Figure 5.11. Interrelationship between solute-specific coefficients B_s for proteins and solute-specific coefficients S_s and C_s for the same proteins determined in the presence of 0.215 M NaCl.

It should be noted that the regression coefficients in Eq. 5.12 have the same absolute values as those in Eq. 5.11 for organic compounds, but most of the proteins examined have some of the solute-specific coefficients with zero values. Hence the above equation should be viewed as describing the trend rather than the reliable relationship. More proteins must be examined in order to ensure that the relationship in question does exist.

It is of interest to note that the severity of the effect of NaCl addition on the partition behavior of globular proteins in PEG–sulfate ATPSs is correlated with the protein charge. This observation is illustrated by Fig. 5.12 that represents dependence of the NaCl-induced change in the protein partition coefficient (calculated as $logK_{NaCl}-logK$), where K and K_{NaCl} correspond to the partition coefficients of a given protein in the absence and presence of 0.215 M NaCl) on the protein charge at pH 6.8 evaluated using the InCharge software from the Aptium Biologics Ltd. (Southampton, UK). Fig. 5.12 clearly shows that the NaCl efficiency to modulate partition of a protein is dependent on the protein's net charge.

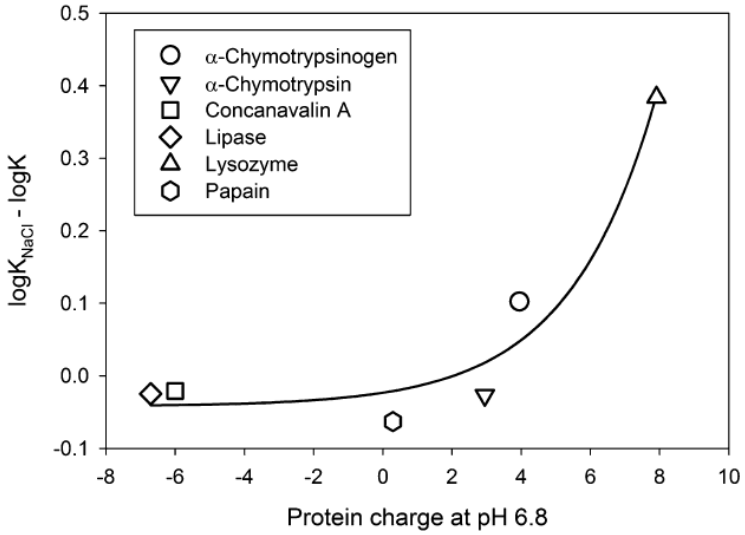


Figure 5.12. Difference between logarithms of partition coefficients for proteins in the presence of 0.215 M NaCl and in the absence of NaCl additive in PEG–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPSs as a function of the protein net charge at pH 6.8. Line plotted for eye-guidance only.

Here, the addition of NaCl does not affect the behavior of the negatively charged proteins in the PEG–sulfate ATPS, whereas the partition of the positively-charged proteins in the same ATPS is strongly affected by NaCl, suggesting the existence of some preferential binding of Cl^- ions to the positively charged proteins. Curiously, this preferential binding happens in the PEG–sulfate ATPS, which already contains a very high Na_2SO_4 concentration (0.45 M overall, and 0.68 M Na_2SO_4 and 0.2 M NaCl in the bottom phase). In other words, NaCl seems to act as if no sulfate is present in the solution. This phenomenon can be explained using the results of earlier studies of Cremer's group, who, based on the analysis of interaction between Hofmeister ions with an uncharged 600-residue elastin-like polypeptide, $(VPGVG)_{120}$, revealed that Cl^- binds to the amide nitrogen/α-carbon binding site, whereas SO_4^{2-} was repelled from both the backbone and hydrophobic side chains of the polypeptide [25]. Additional experimental data indicating specificity of the effects of different ions on proteins and colloids are presented in the recent review by Salis and Ninham [26]. Although proteins analyzed in our study are expected to be differently charged in aqueous solutions, their charges are efficiently screened by high concentrations of sulfate, and, therefore, they might behave similar to the aforementioned uncharged polypeptide, repelling SO_4^{2-} and attracting Cl^-. This preferential global attraction of the Cl^- ions might create potential platform for the observed correlation between the effects of NaCl on partition behavior of globular proteins in PEG–sulfate ATPS and the protein charge in aqueous media.

It should be noted that the partition behavior of both small organic compounds and proteins in PEG–Na_2SO_4–0.215 M NaCl ATPS can be described in terms of solute-solvent interactions.

The results of our study indicate that the solute-specific coefficients representing contributions of different types of solute-solvent interactions under the conditions explored differ from those determined in the absence of NaCl additive [18]. This fact confirms the previously suggested hypothesis [22]–[24] that the solute-solvent interactions depend upon the solvent environment as well as upon the solute structure. Studies of the effects of different salt additives are necessary in order to better understand molecular mechanisms of these interactions, and these studies are currently in progress in our laboratories.

References

[1] R. R. G. Soares, A. M. Azevedo, J. M. Van Alstine, and M. R. Aires-Barros, "Partitioning in aqueous two-phase systems: Analysis of strengths, weaknesses, opportunities and threats," *Biotechnol. J.*, vol. 10, no. 8, pp. 1158–1169, Aug. 2015.

[2] S. A. Oelmeier, F. Dismer, and J. Hubbuch, "Application of an aqueous two-phase systems high-throughput screening method to evaluate mAb HCP separation," *Biotechnol. Bioeng.*, vol. 108, no. 1, pp. 69–81, 2011.

[3] P. Diederich, M. Hoffmann, and J. Hubbuch, "High-throughput process development of purification alternatives for the protein avidin," *Biotechnol. Prog.*, vol. 31, no. 4, pp. 957–973, Jul. 2015.

[4] B. A. Andrews, A. S. Schmidt, and J. A. Asenjo, "Correlation for the partition behavior of proteins in aqueous two-phase systems: Effect of surface hydrophobicity and charge," *Biotechnol. Bioeng.*, vol. 90, no. 3, pp. 380–390, May 2005.

[5] P. A. J. Rosa, A. M. Azevedo, S. Sommerfeld, M. Mutter, M. R. Aires-Barros, and W. Bäcker, "Application of aqueous two-phase systems to antibody purification: A multi-stage approach," *J. Biotechnol.*, vol. 139, no. 4, pp. 306–313, Feb. 2009.

[6] G. Tubio, G. A. Picó, and B. B. Nerli, "Extraction of trypsin from bovine pancreas by applying polyethyleneglycol/sodium citrate aqueous two-phase systems," *J. Chromatogr. B*, vol. 877, no. 3, pp. 115–120, 2009.

[7] A. M. Azevedo, P. A. J. Rosa, I. F. Ferreira, and M. R. Aires-Barros, "Optimisation of aqueous two-phase extraction of human antibodies," *J. Biotechnol.*, vol. 132, no. 2, pp. 209–217, 2007.

[8] G. Bassani, P. Fuciños, G. Picó, and B. Farruggia, "Candida rugosa lipase Lip1–polyethyleneglycol interaction and the relation with its partition in aqueous two-phase systems," *Colloids Surfaces B Biointerfaces*, vol. 75, no. 2, pp. 532–537, 2010.

[9] H. Cao, M. Yuan, L. Wang, J. Yu, and F. Xu, "Coupling purification and in situ immobilization process of monoclonal antibodies to clenbuterol for immunosensor application," *Anal. Biochem.*, vol. 476, pp. 59–66, 2015.

[10] C. A. S. da Silva, J. S. R. Coimbra, E. E. G. Rojas, L. A. Minim, and L. H. M. da Silva, "Partitioning of caseinomacropeptide in aqueous two-phase systems," *J. Chromatogr. B*, vol. 858, no. 1, pp. 205–210, 2007.

[11] R. L. de Souza, J. M. P. Barbosa, G. M. Zanin, M. W. N. Lobão, C. M. F. Soares, and Á. S. Lima, "Partitioning of Porcine Pancreatic Lipase in a Two-Phase Systems of Polyethylene Glycol/Potassium Phosphate Aqueous," *Appl. Biochem. Biotechnol.*, vol. 161, no. 1, pp. 288–300, 2010.

[12] T. Oshima, A. Suetsugu, and Y. Baba, "Extraction and separation of a lysine-rich protein by formation of supramolecule between crown ether and protein in aqueous two-phase system," *Anal. Chim. Acta*, vol. 674, no. 2, pp. 211–219, 2010.

[13] R. L. Pérez, D. B. Loureiro, B. B. Nerli, and G. Tubio, "Optimization of pancreatic trypsin extraction in PEG/citrate aqueous two-phase systems," *Protein Expr. Purif.*, vol. 106, pp. 66–71, 2015.

[14] D. M. Peričin, S. Z. Ma\djarev-Popović, and L. M. Radulović-Popović, "Optimization of conditions for acid protease partitioning and purification in aqueous two-phase systems using response surface methodology," *Biotechnol. Lett.*, vol. 31, no. 1, p. 43, 2008.

[15] L. A. Ferreira, J. A. Teixeira, L. M. Mikheeva, A. Chait, and B. Y. Zaslavsky, "Effect of salt additives on partition of nonionic solutes in aqueous PEG–sodium sulfate two-phase system," *J. Chromatogr. A*, vol. 1218, no. 31, pp. 5031–5039, Aug. 2011.

[16] L. A. Ferreira, P. Parpot, J. A. Teixeira, L. M. Mikheeva, and B. Y. Zaslavsky, "Effect of NaCl additive on properties of aqueous PEG–sodium sulfate two-phase system," *J. Chromatogr. A*, vol. 1220, pp. 14–20, 2012.

[17] N. R. da Silva, L. A. Ferreira, L. M. Mikheeva, J. A. Teixeira, and B. Y. Zaslavsky, "Origin of salt additive effect on solute partitioning in aqueous polyethylene glycol-8000–sodium sulfate two-phase system," *J. Chromatogr. A*, vol. 1337, no. 0, pp. 3–8, Apr. 2014.

[18] N. R. da Silva, L. A. Ferreira, P. P. Madeira, J. A. Teixeira, V. N. Uversky, and B. Y. Zaslavsky, "Analysis of partitioning of organic compounds and proteins in aqueous polyethylene glycol-sodium sulfate aqueous two-phase systems in terms of solute–solvent interactions," *J. Chromatogr. A*, vol. 1415, pp. 1–10, 2015.

[19] B. Y. Zaslavsky, *Aqueous Two-Phase Partitioning: Physical Chemistry and Bioanalytical Applications*. Taylor & Francis, 1994.

[20] J. G. Huddleston, H. D. Willauer, and R. D. Rogers, "The solvatochromic properties, α, β, and π*, of PEG-salt aqueous biphasic systems," *Phys. Chem. Chem. Phys.*, vol. 4, no. 16, pp. 4065–4070, 2002.

[21] Y. Marcus, "The properties of organic liquids that are relevant to their use as solvating solvents," *Chem. Soc. Rev.*, vol. 22, no. 6, pp. 409–416, 1993.

[22] L. A. Ferreira, P. P. Madeira, A. V Uversky, V. N. Uversky, and B. Y. Zaslavsky, "Responses of proteins to different ionic environment are linearly interrelated.," *J. Chromatogr. A*, vol. 1387, pp. 32–41, Mar. 2015.

[23] M. A. Ab Rani *et al.*, "Understanding the polarity of ionic liquids," *Phys. Chem. Chem. Phys.*, vol. 13, no. 37, pp. 16831–16840, 2011.

[24] P. P. Madeira, A. Bessa, J. A. Loureiro, L. Álvares-Ribeiro, A. E. Rodrigues, and B. Y. Zaslavsky, "Cooperativity between various types of polar solute-solvent interactions in aqueous media," *J. Chromatogr. A*, vol. 1408, p. 108—117, 2015.

[25] K. B. Rembert, J. Paterová, J. Heyda, C. Hilty, P. Jungwirth, and P. S. Cremer, "Molecular Mechanisms of Ion-Specific Effects on Proteins," *J. Am. Chem. Soc.*, vol. 134, no. 24, pp. 10039–10046, Jun. 2012.

[26] A. Salis and B. W. Ninham, "Models and mechanisms of Hofmeister effects in electrolyte solutions, and colloid and protein systems revisited," *Chem. Soc. Rev.*, vol. 43, no. 21, pp. 7358–7377, 2014.

CHAPTER 6 | Interrelationship between types of ATPSs

This chapter is based on the following paper

Luisa A. Ferreira[a], **Nuno R. da Silva**[b], Samarina R. Wlodarczyk[c], Joana A. Loureiro[d], Pedro P. Madeira[e,f], José A. Teixeira[b], Vladimir N. Uversky[g] and Boris Y. Zaslavsky[a] (2016). Interrelationship between partition behavior of organic compounds and proteins in aqueous dextran-polyethylene glycol and polyethylene glycol-sodium sulfate two-phase systems. *Journal of Chromatography A*, 1443, 21-25.

[a] Analiza Inc./Cleveland Diagnostics, Superior Ave, Cleveland, OH (USA)

[b] Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga (Portugal)

[c] Laboratório de Biologia Molecular Aplicada à Biotecnologia Farmacêutica, Faculdade de Ciências Farmacêuticas da Universidade de São Paulo, São Paulo (Brazil)

[d] LEPABE, Department of Chemical Engineering, Faculty of Engineering of the University of Porto, Porto (Portugal)

[e] Laboratory of Separation and Reaction Engineering, Department of Chemical Engineering, Faculty of Engineering of the University of Porto, Porto (Portugal)

[f] Centro de Investigação em Materiais Cerâmicos e Compósitos, Department of Chemistry, University of Aveiro, Aveiro (Portugal)

[g] Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, Tampa, FL (USA)

Highlights

- Partition behavior of mononucleotides shows that phosphate group is not a good probe for estimation of electrostatic properties of phases in ATPS;
- Linear interrelationship between partition coefficients of solutes in two polymer and polymer–salt ATPS is established;
- Generality of linear relationships between solute properties in aqueous media is discussed;
- It is suggested that the linear relationships may exist between biological properties of chemical compounds.

6.1. Introduction

There are two main types of ATPS [1]–[5]. The first type includes those formed in aqueous mixtures of two different polymers, such as dextran and Ficoll or PEG. These ATPS are used for separation of cells and other biological particles [1]–[4]. The second type of ATPS includes systems formed in aqueous mixtures of a single polymer, such as PEG, and a salt, such as sodium sulfate, phosphate, citrate, and these ATPS are generally used for protein separation [6]. Both these types of ATPS may be used for analytical applications [7]. The third type of ATPS being recently developed and characterized [8] is the ATPS formed in water by mixing ionic liquids and inorganic salts or polymers. This family of ATPS is used successfully for separation purposes but presents a particular challenge in regard to the mechanism of solute partitioning. It has been established [7] that solute partition behavior in polymer–polymer ATPS and to a lesser degree in polymer–salt ATPS is governed by the solute-water interactions without direct interactions of the solute with the phase-forming components of ATPS. The question of direct solute-ionic liquid interactions in the ionic liquid–salt ATPS, however, remains open.

It has been reported by us recently that various properties of polar organic compounds and proteins in aqueous solutions (solubility [9], lipophilicity (expressed as logD values in octanol–water system) [10], partition coefficients in ATPS [11]) in the presence of different salt additives, and optical rotation of enantiomeric amino acids and glucose in aqueous solutions in the presence of different salts [12], [13] are interrelated as:

$$\log SP_{salt-1} = k_1 + k_2 \times \log SP_{salt-2} + k_3 \times \log SP_{salt-3}$$

(Equation 6.1)

where SP_{salt} is the property of a solute in the presence of a given salt additive, k_1, k_2, and k_3 are constants depending on the solute property under consideration and the salt compositions employed.

From a practical point of view, the most important aspect of the relationship described by Eq. 6.1 is that once the relationship is established it may be used to predict protein partition coefficient in a system with a certain ionic composition without need to perform experiments saving the necessary protein quantity to be used, time and labor. It may be important when the optimal separation conditions for a target protein are designed. From the theoretical viewpoint the relationship described by Eq. 6.1 if applicable to both polymer–polymer and polymer–salt ATPSs may be used to gain better understanding

in what respect these two different subfamilies of ATPSs are similar or different and derive better insight into mechanisms of phase separation and fundamental properties of ATPSs.

Analysis of the partition coefficients of small organic compounds and proteins in PEG–DEX and PEG–Na_2SO_4 ATPSs was one of the aims of this study. In addition, the second aim of the study was to explore if the homologous series of mononucleotides may be used for characterization of ATPSs. Partitioning of adenine and adenosine mono-, di-, and tri-phosphates and guanosine mono-, di-, and tri-phosphates in PEG–DEX and PEG–Na_2SO_4 with and without 0.215 M NaCl additive ATPSs were examined to explore if contribution of a phosphate group into logarithm of partition coefficient of mononucleotide may be used for characterization of electrostatic properties of coexisting phases in ATPSs.

6.2. Experimental

6.2.1. Material and methods

6.2.1.1. Materials

Polyethylene glycol 8000 (Lot 091M01372 V), Mw = 8000, adenine, adenosine, adenosine 5'-monophosphate disodium salt (AMP), adenosine 5'-diphosphate sodium salt (ADP), adenosine triphosphate sodium salt (ATP), guanosine, guanosine 5'-monophosphate disodium salt (GMP), guanosine 5'-diphosphate sodium salt (GDP), guanosine 5'-triphosphate sodium salt (GTP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dextran 75 (Lot 119945) with an average molecular weight (Mw) 75,000 by light scattering were purchased from USB Corporation (Cleveland, OH, USA).

6.2.1.2. Aqueous two-phase systems

PEG–DEX systems of polymer composition of 6.0 wt.% PEG8K and 12.0 wt.% DEX75 and ionic composition of 0.01 M K/NaPB, pH 7.4 and PEG8K–Na_2SO_4 systems with composition of 11.10 wt.% PEG8K, 6.33 wt.% Na_2SO_4 (~0.45 M) and 0.01 M NaPB, pH 6.8, with or without 0.215 M NaCl additive were prepared as described previously [11], [14], [15] by dispensing appropriate amounts of the aqueous

stock polymer and salt solutions into a 1.2 mL microtube using a Hamilton Company (Reno, NV, USA) ML-4000 four-probe liquid-handling workstation. With total weight of 0.5 g (total volume 470 µL).

6.2.1.3. Partitioning

The partitioning experiments were carried out as described in Chapter 3.

Deviation from the average K-value was consistently below 3% and, in most cases, lower than 1%.

6.3. Results and discussion

The partition coefficients of AMP, ADP, ATP, GMP, GDP, and GTP in three ATPSs with previously characterized differences between the hydrophobic and electrostatic properties of the phases [14]–[16] are listed in Table 6.1. Some of the partition coefficients for adenine, adenosine, guanosine, AMP, ADP, and ATP in the same ATPSs were reported previously [17], [18].

Table 6.1. Differences between the solvent properties of the phases and partition coefficients for indicated organic compounds in PEG–DEX–0.01 M K/NaPB, pH 7.4, PEG–Na_2SO_4–0.01 M NaPB, pH 6.8, and PEG–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8 ATPSs.

Solvent Properties	PEG–DEX–0.01M K/NaPB[a]	PEG–Na_2SO_4–0.01M NaPB[b]	PEG–Na_2SO_4–0.01M NaPB–0.215M NaCl[b]
$\Delta G(CH_2)$, cal/mole	-45±1.2	-122±1.8	-135±0.6
E	0.033±0.001	0.090±0.002	0.102±0.008
C	0.058±0.003	0.445±0.005	0.490±0.031

a – Reported in [11]

b – Reported in [7]

Table 6.1. (cont.) Differences between the solvent properties of the phases and partition coefficients for indicated organic compounds in PEG–DEX–0.01 M K/NaPB, pH 7.4, PEG–Na_2SO_4–0.01 M NaPB, pH 6.8, and PEG–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8 ATPSs.

	Partition coefficients		
COMPOUND	**PEG–DEX–0.01M K/NaPB[a]**	**PEG–Na_2SO_4–0.01M NaPB[b]**	**PEG–Na_2SO_4–0.01M NaPB–0.215M NaCl[b]**
Adenine	1.220±0.006[a]	3.400±0.120[b]	3.700±0.160[b]
Adenosine	1.128±0.004[a]	2.630±0.020[c]	3.120±0.040[c]
AMP	0.714±0.001	1.020±0.050[b]	0.830±0.040[b]
ADP	0.769±0.002	0.770±0.020[b]	0.540±0.030[b]
ATP	0.826±0.002	0.610±0.010[b]	0.400±0.020[b]
Guanosine	1.068±0.007	1.673±0.008[c]	1.800±0.030[c]
GMP	0.709±0.005	0.783±0.007	0.606±0.003
GDP	0.753±0.004	0.577±0.002	0.444±0.003
GTP	0.789±0.005	0.514±0.003	0.410±0.004

c – Reported in [6]

Commonly, the differences between the electrostatic and hydrophobic properties of the coexisting phases of ATPS are estimated by analysis of partitioning of a homologous series of dinitrophenylated (DNP-) amino acids Na salts with aliphatic alkyl side-chains of the increasing length [5], [10], [11], [14]–[18]. The typical data in a given ATPS may be described as [3], [8]–[13]:

$$\log K^i_{DNP-AA} = C^i + E^i \times N_C$$

(Equation 6.2)

where $K_{DNP\text{-}AA}$ is the partition coefficient of a DNP-amino acids Na-salt in ith ATPS; N_C is the equivalent number of CH_2 groups in the side-chain [13]. Parameter E represents an average contribution of a CH_2

group into *logK* and characterizes the difference between the hydrophobic properties of the phases [5], [10], [11], [14]–[18]. Parameter *C* represents the contribution of the polar DNP-NH-CH-COONa moiety. The advantages and drawbacks of using this moiety as a probe for electrostatic properties of the phases were considered in detail in [5]. The obvious drawbacks of using the DNP-NH-CH-COONa moiety as a probe for electrostatic interactions are the volume of the moiety and the presence of substituted aromatic group. It would be preferable to replace this moiety with a moiety capable to participate solely in electrostatic interactions.

We used here the similar approach to analysis of partitioning of mononucleotides with the difference that the number of charged phosphate groups is varied. The data obtained in PEG–DEX ATPSs are illustrated in Fig. 6.1 where logarithms of partition coefficients for the two series of mononucleotides are plotted against the number of phosphate groups.

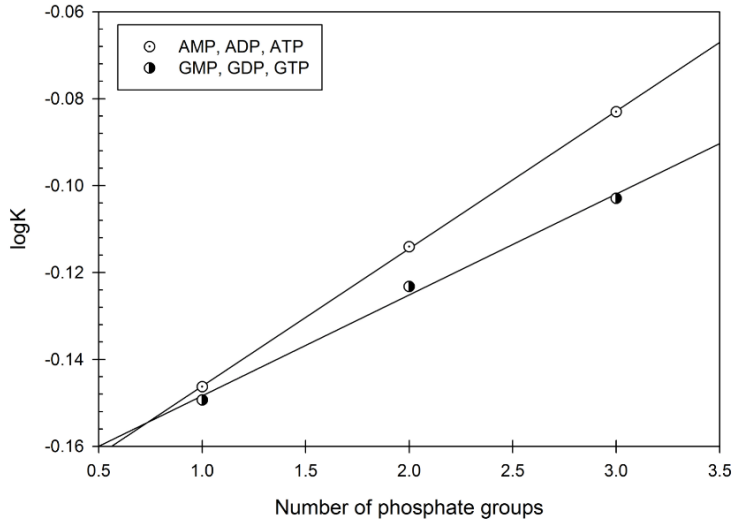


Figure 6.1. Logarithm of the partition coefficient value, *logK*, for adenosine and guanine mononucleotides with different number of phosphate groups in PEG–DEX ATPS as a function of number of phosphate groups.

An increase in the number of phosphate groups results in the increase of the nucleotide partition coefficient for both series in PEG–DEX ATPS. The data plotted in Fig. 6.1 may be described as:

$$\log K_A = A_A + B_A \times N_P \quad \text{and} \quad \log K_G = A_G + B_G \times N_P$$

(Equation 6.3)

where K_A and K_G are the partition coefficients of a given adenosine phosphate and guanine phosphate respectively; N_P is the number of phosphate groups; A_A, A_G, B_A, and B_G are coefficients with values dependent on the particular mononucleotides examined.

It can be seen in Fig. 6.1, however, that the partition behavior of adenosine mononucleotides differs from that of guanine mononucleotides. The parameters B_A and B_G representing the average contribution of a phosphate group into *logK* of the corresponding nucleotides are 0.0316±0.0004 and 0.023±0.002 respectively. The observed dependence of the average contributions of a phosphate group on the nature of nucleobase makes it questionable whether contribution of a phosphate group may be used for evaluation of the difference between the electrostatic properties of the phases in ATPS.

The data obtained for the same two series of mononucleotides in PEG-Na_2SO_4 ATPS are illustrated graphically in Fig. 6.2.

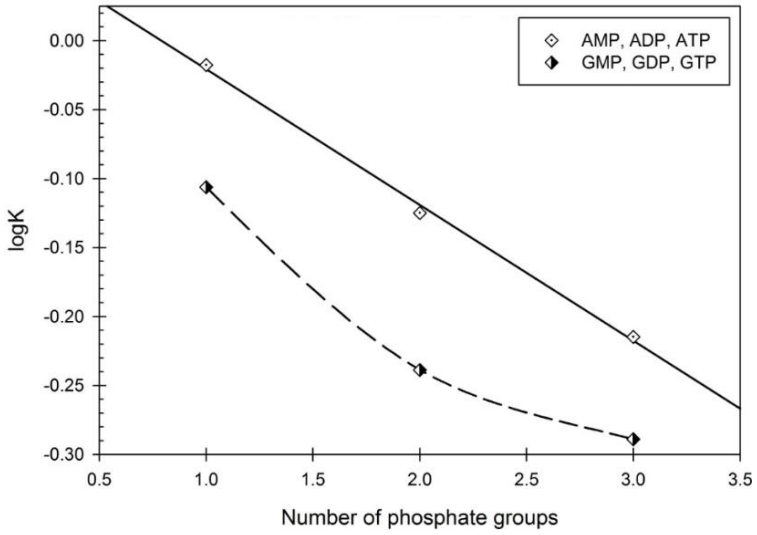


Figure 6.2. Logarithm of the partition coefficient value, *logK*, for adenosine and guanine mononucleotides with different number of phosphate groups in PEG–Na_2SO_4 ATPS as a function of number of phosphate groups.

The partition behavior of adenosine mononucleotides as a function of the number of phosphate groups may be described by Eq. 6.3, while the same dependence for guanine mononucleotides is clearly nonlinear and may be described as:

$$\log K_G = a_G + b_G \times e^{-N_P}$$

(Equation 6.4)

where a_G and b_G are constants; all the other parameters are as defined above.

Similar dependences in PEG–Na_2SO_4–0.215 M NaCl ATPS illustrated in Fig. 6.3 appear to be nonlinear for both adenosine and guanine mononucleotides series.

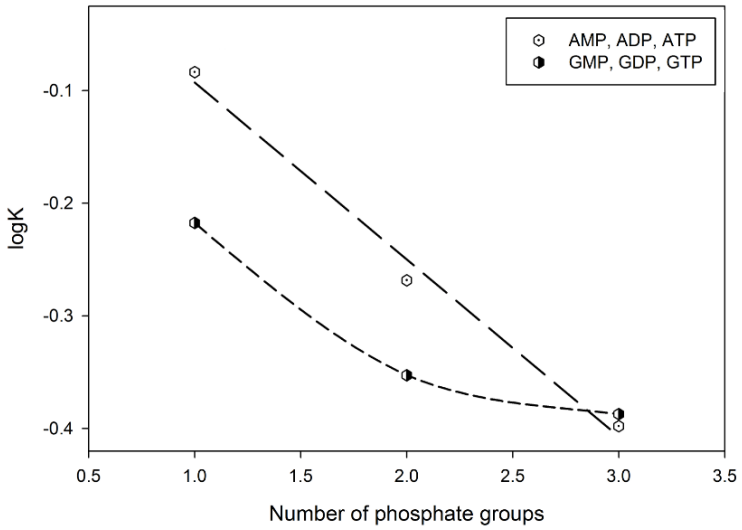


Figure 6.3. Logarithm of the partition coefficient value, *logK*, for adenosine and guanine mononucleotides with different number of phosphate groups in PEG–Na_2SO_4–0.215 M NaCl ATPS as a function of number of phosphate groups.

An increase in the number of phosphate groups results in the decrease of the nucleotide partition coefficient for both series in PEG–Na_2SO_4 ATPS with and without NaCl additive.

If we assume that the partition behavior of adenosine mononucleotides as a function of the number of phosphate groups may be described by Eq. 6.3 under all conditions used here and plot the slope of the linear function (coefficient B_A) in all three ATPS versus the parameter C (Eq. 6.2), Fig. 6.4, used as the estimate of the difference between the electrostatic properties of the phases [5] we observe nonlinear relationship.

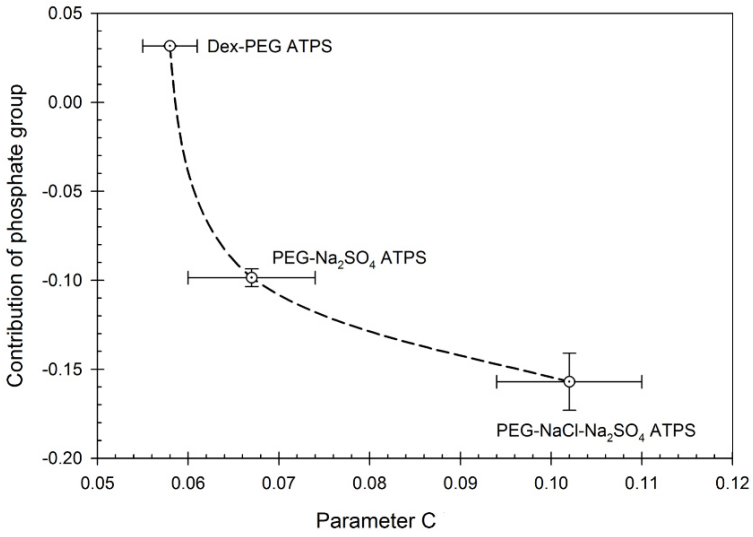


Figure 6.4. An average contribution of phosphate group into logarithm of partition coefficient of adenosine mononucleotides versus parameter C in Eq. 6.2 characterizing the difference between electrostatic properties of two phases in ATPS indicated.

This nonlinearity is not as important as the change of the sign of the difference between the electrostatic properties of the two phases between PEG–DEX and PEG–Na_2SO_4 ATPS observed with adenosine mononucleotides and not detected with DNP-amino acids Na-salts. The previously reported [14]–[20] studies of partition behavior of small organic compounds and proteins in PEG–Na_2SO_4 ATPS with and without NaCl additive showed that using the difference between the electrostatic properties of the coexisting phases characterized with parameter C (Eq. 6.2) allowed to estimate the solute-solvent interactions. Hence it seems possible to conclude that the contribution of a phosphate group into partition coefficient of mononucleotide does not provide the reliable estimate of the difference between the electrostatic properties of the phases in PEG–salt ATPS.

All the partition coefficients for small organic compounds and proteins in the three ATPS under consideration reported previously [3], [10]–[12] together with those obtained in this study are presented in Table 6.2.

Table 6.2. Partition coefficients for organic compounds and proteins in PEG–DEX–0.01 M K/NaPB, pH 7.4 [16]; PEG–Na_2SO_4–0.01 M NaPB, pH 6.8 [14], [17], [18]; and PEG–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8 ATPSs [15], [17].

	Partition coefficients		
COMPOUND	**PEG–DEX–0.01M K/NaPB**	**PEG–Na_2SO_4–0.01M NaPB**	**PEG–Na_2SO_4–0.01M NaPB–0.215M NaCl**
Adenine	1.220±0.006	3.400±0.120	3.700±0.160
Adenosine	1.128±0.004	2.630±0.020	3.120±0.040
AMP	0.714±0.001	1.020±0.050	0.830±0.040
ADP	0.769±0.002	0.770±0.020	0.540±0.030
ATP	0.826±0.002	0.610±0.010	0.400±0.020
Benzyl alcohol	1.409±0.009	3.500±0.007	4.067±0.008
Caffeine	1.154±0.009	1.850±0.039	2.110±0.036
Coumarin	1.490±0.009	4.860±0.014	4.860±0.014
Glucoside	1.232±0.003	2.180±0.023	2.470±0.033
Guanosine	1.068±0.007	1.673±0.008	1.800±0.030
GMP	0.709±0.005	0.783±0.007	0.606±0.003
GDP	0.753±0.004	0.577±0.002	0.444±0.003
GTP	0.789±0.005	0.514±0.003	0.410±0.004
Methyl anthranilate	1.770±0.010	7.280±0.035	9.590±0.027
Phenol	1.700±0.020	4.600±0.084	6.500±0.170
2-Phenylethanol	1.469±0.005	4.160±0.020	4.980±0.012
Vanillin	1.709±0.009	6.780±0.027	8.280±0.020

Table 6.2. (cont.) Partition coefficients for organic compounds and proteins in PEG–DEX–0.01 M K/NaPB, pH 7.4 [16]; PEG–Na_2SO_4–0.01 M NaPB, pH 6.8 [14], [17], [18]; and PEG–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8 ATPSs [15], [17].

	Partition coefficients		
PROTEIN	**PEG–DEX–0.01M K/NaPB**	**PEG–Na_2SO_4–0.01M NaPB**	**PEG–Na_2SO_4–0.01M NaPB–0.215M NaCl**
α-chymotrypsin	0.420±0.010	0.117±0.001	0.110±0.001
α-chymotrypsinogen A	1.000±0.010	0.429±0.003	0.543±0.003
Concanavalin A	0.236±0.003	0.192±0.001	0.183±0.001
Lysozyme	0.230±0.003	0.406±0.003	0.983±0.003
Papain	1.050±0.010	2.220±0.011	1.920±0.007

Analysis of these data shows that there is a linear relationship illustrated in Fig. 6.5 corresponding to Eq. 6.1 and described as:

$$\log K_i^{PEG-Na_2SO_4} = 0.05_{\pm 0.02} + 0.50_{\pm 0.12} \times \log K_i^{PEG-DEX} + 0.71_{\pm 0.05} \times \log K_i^{PEG-Na_2SO_4-0.215M\ NaCl}$$

N = 22; r^2 = 0.9775; SD = 0.079; F = 412.3

(Equation 6.5)

where $K_i^{PEG-Na2SO4}$ is the partition coefficient of the i-th compound in PEG–Na_2SO_4–0.01 M NaPB ATPS; $K_i^{PEG-DEX}$ is the partition coefficient of the i-th compound in PEG–DEX–0.01 M K/NaPB ATPS; $K_i^{PEG-Na2SO4-0.215\ M\ NaCl}$ is the partition coefficient of the i-th compound in PEG–Na_2SO_4–0.215 M NaCl–0.01 M NaPB ATPS.

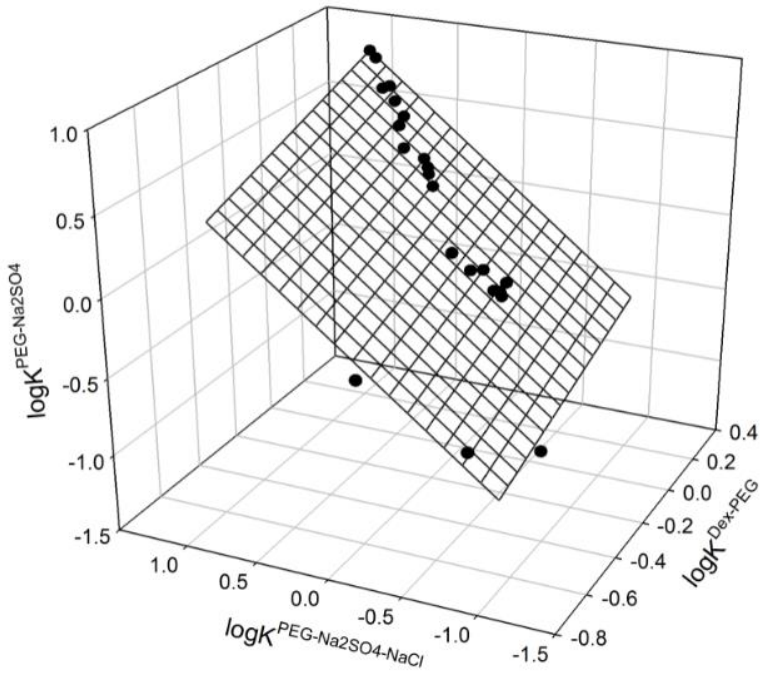


Figure 6.5. Logarithms of partition coefficients for organic compounds and proteins in PEG–DEX8K–0.01 M K/NaPB, pH 7.4 versus those for the same compounds and proteins in PEG8K–Na_2SO_4–0.215M NaCl–0.01 M NaPB, pH 6.8 and in PEG8K–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPSs.

It should be noted that the above relationship holds for partition coefficients of proteins in PEG–Na_2SO_4–0.01 M NaPB and PEG–Na_2SO_4–0.215 M NaCl–0.01 M NaPB ATPS at pH 6.8 and in PEG–DEX–0.01 M K/NaPB at pH 7.4. This fact seems to imply that the pH differences as well as the differences in the buffer composition do not affect the nature and spatial arrangement of the solvent exposed groups in the proteins examined.

Previously the similar relationship was reported for partition coefficients of organic compounds and proteins in PEG–DEX ATPS with different ionic composition [11], and in PEG–Na_2SO_4 ATPS formed by PEG8K and PEG600 with different salt additives [20]. Analysis of the data reported previously [16], [20] shows also that the similar relationship for proteins may be found between polymer–polymer and polymer–salt ATPS. For example, the relationship illustrated graphically in Fig. 6.6 may be described as:

$$\log K_i^{PEG-DEX-0.8\,M\,CsCl-0.01M\,NaPB} = 0.12_{\pm 0.10} + 0.30_{\pm 0.07} \times \log K_i^{PEG600-Na_2SO_4-0.4M\,NaSCN-0.17M\,K/NaPB} + 0.46_{\pm 0.08} \times \log K_i^{PEG8K-Na_2SO_4-0.33M\,NaCl-0.01M\,UB}$$

N = 10; r^2 = 0.9416; SD = 0.18; F = 56.4

(Equation 6.6)

where $K_i^{PEG-DEX-0.8\,M\,CsCl-0.01\,M\,NaPB}$ is the partition coefficient of the i-th compound in PEG–DEX–0.8 M CsCl–0.01 M NaPB ATPS; $K_i^{PEG600-Na2SO4-0.4\,M\,NaSCN-0.17\,M\,K/NaPB}$ is the partition coefficient of the i-th compound in PEG600–Na_2SO_4–0.4 M NaSCN–0.17 M K/NaPB ATPS; and $K_i^{PEG8K-Na2SO4-0.33\,M\,NaCl-0.01\,M\,UB}$ is the partition coefficient of the i-th compound in PEG8K–Na_2SO_4–0.33 M NaCl–0.01 M UB ATPS; all the other parameters are as defined above.

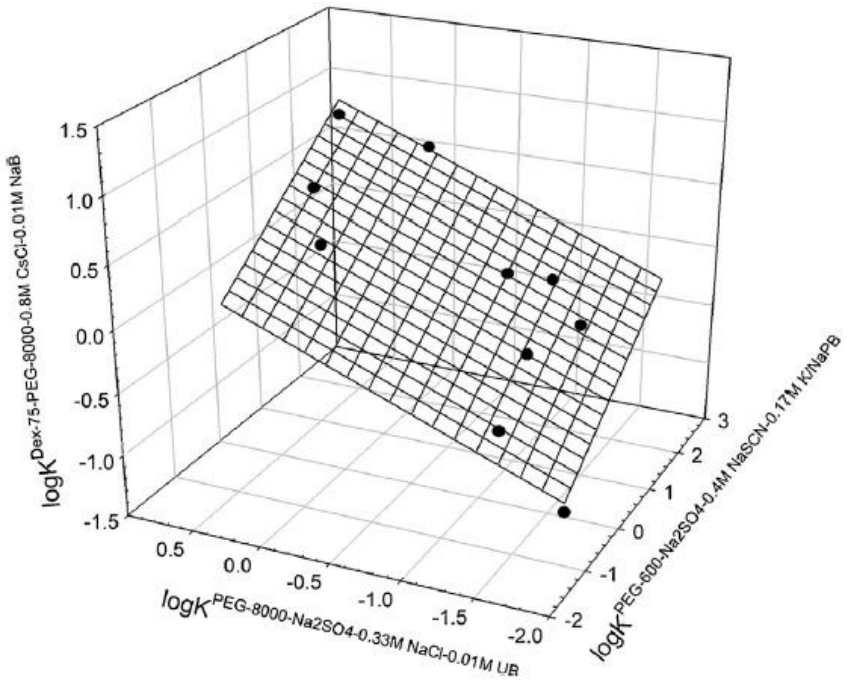


Figure 6.6. Logarithms of partition coefficients for organic compounds and proteins in PEG–DEX8K–0.8 M CsCl–0.01 M NaPB, pH 7.4 versus those for the same compounds and proteins in PEG600–Na_2SO_4–0.4 M NaSCN–0.17 M NaPB, pH 7.4 and in PEG8K–Na_2SO_4–0.33 M NaCl–0.01 M UB, pH 7.4 ATPSs.

The above relationships seem to indicate that Eq. 6.1 is rather universal. These correlations suggest that the properties and mechanism of phase separation in both types of polymer–polymer and polymer–salt ATPSs are of the same nature. We do not know yet the boundaries of its applicability to physicochemical and biological properties. The work in this regard is currently in progress in our laboratories.

References

[1] T. J. Peters, "Partition of cell particles and macromolecules: Separation and purification of biomolecules, cell organelles, membranes and cells in aqueous polymer two phase systems and their use in biochemical analysis and biotechnology. P-A. Albertsson. Third Edition," *Cell Biochem. Funct.*, vol. 5, no. 3, pp. 233–234, 1987.

[2] H. Walter, *Partitioning In Aqueous Two – Phase System: Theory, Methods, Uses, and Applications To Biotechnology*. Elsevier Science, 2012.

[3] H. HUSTEDT, K. H. KRONER, and M.-R. KULA, "15 - Applications of Phase Partitioning in Biotechnology," H. WALTER, D. E. BROOKS, and D. B. T.-P. in A. T.-P. S. FISHER, Eds. Academic Press, 1985, pp. 529–587.

[4] R. Hatti-Kaul, *Aqueous Two-phase Systems: Methods and Protocols*. Humana Press, 2000.

[5] B. Y. Zaslavsky, *Aqueous Two-Phase Partitioning: Physical Chemistry and Bioanalytical Applications*. Taylor & Francis, 1994.

[6] R. R. G. Soares, A. M. Azevedo, J. M. Van Alstine, and M. R. Aires-Barros, "Partitioning in aqueous two-phase systems: Analysis of strengths, weaknesses, opportunities and threats," *Biotechnol. J.*, vol. 10, no. 8, pp. 1158–1169, Aug. 2015.

[7] B. Y. Zaslavsky, V. N. Uversky, and A. Chait, "Analytical applications of partitioning in aqueous two-phase systems: Exploring protein structural changes and protein-partner interactions in vitro and in vivo by solvent interaction analysis method.," *Biochim. Biophys. Acta*, vol. 1864, no. 5, pp. 622–644, May 2016.

[8] M. G. Freire *et al.*, "Aqueous biphasic systems: a boost brought about by using ionic liquids," *Chem. Soc. Rev.*, vol. 41, no. 14, pp. 4966–4995, 2012.

[9] L. A. Ferreira, A. Chervenak, S. Placko, A. Kestranek, P. P. Madeira, and B. Y. Zaslavsky, "Responses of polar organic compounds to different ionic environments in aqueous media are interrelated," *Phys. Chem. Chem. Phys.*, vol. 16, no. 42, pp. 23347–23354, 2014.

[10] L. A. Ferreira, A. Chervenak, S. Placko, A. Kestranek, P. P. Madeira, and B. Y. Zaslavsky, "Effect of ionic composition on the partitioning of organic compounds in octanol–buffer systems," *RSC Adv.*, vol. 5, no. 26, pp. 20574–20582, 2015.

[11] L. A. Ferreira, P. P. Madeira, A. V Uversky, V. N. Uversky, and B. Y. Zaslavsky, "Responses of proteins to different ionic environment are linearly interrelated.," *J. Chromatogr. A*, vol. 1387, pp. 32–41, Mar. 2015.

[12] P. Lo Nostro, B. W. Ninham, S. Milani, L. Fratoni, and P. Baglioni, "Specific anion effects on the optical rotation of glucoseand serine," *Biopolymers*, vol. 81, no. 2, pp. 136–148, Feb. 2006.

[13] S. Rossi, P. Lo Nostro, M. Lagi, B. W. Ninham, and P. Baglioni, "Specific Anion Effects on the Optical Rotation of α-Amino Acids," *J. Phys. Chem. B*, vol. 111, no. 35, pp. 10510–10519, Sep. 2007.

[14] N. R. da Silva, L. A. Ferreira, P. P. Madeira, J. A. Teixeira, V. N. Uversky, and B. Y. Zaslavsky, "Analysis of partitioning of organic compounds and proteins in aqueous polyethylene glycol-sodium sulfate aqueous two-phase systems in terms of solute–solvent interactions," *J. Chromatogr. A*, vol. 1415, pp. 1–10, 2015.

[15] N. R. da Silva, L. A. Ferreira, P. P. Madeira, J. A. Teixeira, V. N. Uversky, and B. Y. Zaslavsky, "Effect of sodium chloride on solute–solvent interactions in aqueous polyethylene glycol–sodium sulfate two-phase systems," *J. Chromatogr. A*, vol. 1425, pp. 51–61, 2015.

[16] L. A. Ferreira, X. Fan, P. P. Madeira, L. Kurgan, V. N. Uversky, and B. Y. Zaslavsky, "Analyzing the effects of protecting osmolytes on solute–water interactions by solvatochromic comparison method: II. Globular proteins," *RSC Adv.*, vol. 5, no. 73, pp. 59780–59791, 2015.

[17] L. A. Ferreira, J. A. Teixeira, L. M. Mikheeva, A. Chait, and B. Y. Zaslavsky, "Effect of salt additives on partition of nonionic solutes in aqueous PEG–sodium sulfate two-phase system," *J. Chromatogr. A*, vol. 1218, no. 31, pp. 5031–5039, Aug. 2011.

[18] N. R. da Silva, L. A. Ferreira, L. M. Mikheeva, J. A. Teixeira, and B. Y. Zaslavsky, "Origin of salt additive effect on solute partitioning in aqueous polyethylene glycol-8000–sodium sulfate two-phase system," *J. Chromatogr. A*, vol. 1337, no. 0, pp. 3–8, Apr. 2014.

[19] L. A. Ferreira, P. Parpot, J. A. Teixeira, L. M. Mikheeva, and B. Y. Zaslavsky, "Effect of NaCl additive on properties of aqueous PEG–sodium sulfate two-phase system," *J. Chromatogr. A*, vol. 1220, pp. 14–20, 2012.

[20] L. Ferreira, P. P. Madeira, L. Mikheeva, V. N. Uversky, and B. Zaslavsky, "Effect of salt additives on protein partition in polyethylene glycol–sodium sulfate aqueous two-phase systems," *Biochim. Biophys. Acta - Proteins Proteomics*, vol. 1834, no. 12, pp. 2859–2866, Dec. 2013.

CHAPTER 7 | Salt additives and osmolytes combined effects

This chapter is based on the following paper

Nuno R. da Silva[a], Luisa A. Ferreira[b], José A. Teixeira[a], Vladimir N. Uversky[c] and Boris Y. Zaslavsky[b] (2019). Effects of sodium chloride and sodium perchlorate on properties and partition behavior of solutes in aqueous dextran-polyethylene glycol and polyethylene glycol-sodium sulfate two-phase systems. *Journal of Chromatography A*, 1583, 28-38.

[a] Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga (Portugal)

[b] Analiza Inc./Cleveland Diagnostics, Superior Ave, Cleveland, OH (USA)

[c] Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, Tampa, FL (USA)

Highlights

- Solvent properties of PEG–DEX and PEG–Na_2SO_4 ATPSs examined;
- Effects of NaCl and $NaClO_4$ on the properties of PEG–DEX and PEG–Na_2SO_4 ATPSs compared;
- With each salt additive different osmolytes additives were used;
- Effects of additives on partitioning of organic compounds and proteins studied;
- Partition coefficients ranges in the systems compared.

7.1. Introduction

ATPSs are typically formed in mixtures of two compounds in water. The phase-forming compounds commonly include two polymers, a single polymer and a salt [1]–[5] or surfactant [6], [7], two different surfactants [8], and ionic liquids [9]–[11]. The most thoroughly studied ATPSs include those formed by two polymers, such as DEX and PEG, or a single polymer and inorganic or organic salt such as PEG and Na_2SO_4 [1]–[5]. These ATPSs are generally applicable for separation of various biological materials ranging from small biomolecules, proteins, and nucleic acids to cells and viruses [1]–[5], as well as for the analysis of proteins [5], [12]–[14], discovery and monitoring of biomarkers in biological fluids [14], [15], and for clinical diagnostics [16].

It is currently impossible to predict partition behavior of a protein in a given ATPS with a fixed composition. It is possible, however, and important in many cases to manipulate protein partitioning in order to increase/decrease its distribution into one of the phases (e.g., for improved separation or for analytical purposes). The partition behavior of proteins and other biomacromolecules may be manipulated by changing concentrations of phase-forming compounds or by introducing various additives in a given ATPS. Inorganic salts additives are well-known to affect the partition behavior of proteins by changing the properties of the coexisting phases on the one hand, and also modulating properties of proteins on the other hand. The other type of additives capable of manipulation of the partition behavior of solutes includes some nonionic compounds, such as osmolytes. These additives affect the solvent properties of phases, but not the properties of the solutes [17], [18].

The purpose of this work was to study and compare the effects of two salt additives, NaCl and $NaClO_4$, at the fixed concentrations of ca. 0.2 M on the properties of two ATPSs formed by DEX and PEG and by PEG and Na_2SO_4, and on partition of 12 low molecular organic compounds and five proteins in these systems. In addition, in each system with a given salt additives, the osmolyte additives, such as 0.5 M sorbitol, 0.5 M sucrose, 0.5 M trehalose, and 0.5 and/or1.5 M trimethylamine N-oxide (TMAO), were used. The obtained results were considered in terms of the solute-solvent interactions.

7.2. Experimental

7.2.1. Materials

7.2.1.1. Polymers

Polyethylene glycol (PEG8K, Lot 091M01372V) with an average molecular weight (Mn) of 8000 and polyethylene glycol (PEG10K, Lot 043K2522) with an average molecular weight (Mn) of 10,000 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Dextran 75 (DEX75, Lot 119945) with an average molecular weight (Mw) 75,000 by light scattering was purchased from USB Corporation (Cleveland, OH, USA).

7.2.1.2. Amino acids

Dinitrophenylated (DNP) amino acids — DNP-alanine, DNP-norvaline, DNP-norleucine, and DNP-α-amino-*n*-octanoic acid, were purchased from Sigma-Aldrich. The sodium salts of the DNP-amino acids were prepared by titration.

7.2.1.3. Organic compounds

Benzyl alcohol, caffeine, coumarin, methyl anthranilate, *p*-nitrophenyl-α-D-glucopyranoside, sorbitol, sucrose, phenol, 2-phenylethanol, trimethylamine N-oxide (TMAO), and vanillin were purchased from Sigma-Aldrich and used without further purification. *o*-Phthaldialdehyde (OPA) reagent solution (complete) was purchased from Sigma.

7.2.1.4. Solvatochromic dyes

The solvatochromic probe 4-nitrophenol (spectrophotometric grade) was purchased from Sigma-Aldrich and 4-nitroanisole (GC, > 99%) was supplied by Acros Organic (New Jersey, USA). Reichardt's

carboxylated betaine dye, sodium 2,6-diphenyl-4-[4-(4-carboxylato-phenyl)-2,6-diphenylpyridinium-1-yl]phenolate, was synthesized according to the procedure reported previously [19].

7.2.1.5. Proteins

α-chymotrypsin, α-chymotrypsinogen A from bovine pancreas, and concanavalin A from *Canavalia ensiformis* (jack beans) were purchased from Sigma-Aldrich. Lysozyme (salt free) from chicken egg white was obtained from Worthington Biochemical Corp. (Lakewood, NJ, USA). Porcine pancreatic lipase was purchased from USB Corp. (Solon, OH, USA). Purity of all proteins was verified by electrophoresis.

7.2.1.6. Other chemicals

All salts and other chemicals used were of analytical-reagent grade and used without further purification.

7.2.2. Methods

7.2.2.1. Preparation of aqueous two-phase systems

ATPSs were prepared as described previously [20], [21]. Stock solutions of PEG8K (50 wt.%), PEG10K (50 wt.%) and Na_2SO_4 (20.3 wt.%) were prepared in water. Sodium phosphate buffer (NaPB; 0.5 M, pH 6.8) was prepared by mixing appropriate amounts of NaH_2PO_4 and Na_2HPO_4. Stock solution of 2.0 M $NaClO_4$ was prepared in water. Stock solutions of osmolytes: sorbitol (2 M), sucrose (1.8 M), and TMAO (1.8 M and 5.0 M), were prepared in water. A mixture of PEG8K or PEG10K, buffer, and $NaClO_4$ was prepared by dispensing appropriate amounts of the aqueous stock PEG8K, Na_2SO_4, NaPB, and $NaClO_4$ solutions into a 1.2 ml microtube using a Hamilton (Reno, NV, USA) ML-4000 four-probe liquid-handling workstation. Appropriate amounts of water and/or stock solutions of osmolytes were added to give the required ionic, polymer, and osmolyte composition of the final system with total weight of 0.5 g (after

addition of the solute sample, see below). All aqueous PEG8K–Na_2SO_4–$NaClO_4$ two-phase systems had a fixed composition of 11.10 wt.% PEG8K, 6.33 wt.% Na_2SO_4, 0.215 M $NaClO_4$, and 0.01 M NaPB, pH 6.8, with different 0.5 M osmolyte or 1.5 M TMAO additive. The aqueous PEG10K–Na_2SO_4–$NaClO_4$ two-phase system had the same composition of 11.10 wt.% PEG10K, 6.33 wt.% Na_2SO_4, 0.215 M $NaClO_4$, and 0.01 M NaPB, pH 6.8.

Similar protocol was used to prepare PEG–DEX ATPS. Stock solutions of PEG8K (50 wt.%), DEX75 (42 wt.%), 1.5 M NaCl, 2.0 M $NaClO_4$ and osmolytes (as indicated above) were prepared in deionized (DI) water. Stock sodium/potassium phosphate buffer (K/NaPB; 0.5 M, pH 7.4) was prepared by mixing appropriate amounts of KH_2PO_4 and Na_2HPO_4. Using the Hamilton Company ML-4000 four-probe liquid-handling workstation, proper amounts of stock solutions of polymers, salt (NaCl or $NaClO_4$), osmolyte, stock buffer solutions, and water were added to give the ionic, polymer, and osmolyte composition required for the final system (after the sample addition – see below) with total weight of 0.5 g. All the two polymer-based ATPSs used in this study had the same polymer composition of 6.0 wt.% PEG8K and 12.0 wt.% DEX75 and same ionic composition of 0.01 M K/NaPB, pH7.4, 0.215 M NaCl (or 0.215 M $NaClO_4$) with 0.5 M osmolyte or 1.5 M TMAO additive.

7.2.2.2. Partitioning

The partitioning experiments were carried out as described in Chapter 4.

Deviation from the average K-value was consistently below 3% and, in most cases, lower than 1%.

7.2.2.3. Analysis of hydrophobic and electrostatic properties of the phases

Analysis of hydrophobic and electrostatic properties of the coexisting phases of all ATPSs used in this study was performed as described previously [5] using results of partitioning of DNP-AA sodium salts. The detailed description is provided in Supplementary Information – section A.

7.2.2.4. Solvatochromic measurements

All solvatochromic measurements in the phases of ATPSs used were performed as described previously [20], [21].

7.3. Results and discussion

7.3.1. Solvent properties of ATPSs

Partitioning of the homologous series of Na-salts of dinitrophenylated (DNP-) amino acids was examined in order to estimate the difference between the relative hydrophobic and electrostatic properties of the phases as described previously [5], [20], [21]. Partition coefficients of Na-salts of DNP-AA with the aliphatic alkyl side-chains of the increasing length (alanine, norvaline, nor-leucine, and α-amino-*n*-octanoic acid) in the ATPS under study are listed below in Tables 7.1-7.3 and Tables S7.1 (Supplementary Information – section A), 4.1 (Chapter 4) and 5.1 (Chapter 5) and shown graphically as functions of the equivalent number of methylene groups representing the length of the alkyl side-chain in Fig. S7.1-S7.3. The data obtained may be described as:

$$\log K_{DNP-AA}^{(i)} = C^{(i)} + E^{(i)} \times N_C$$

(Equation 7.1)

where $K_{DNP\text{-}AA}$ is the partition coefficient of a sodium salt of DNP-amino acid with aliphatic side-chain; superscript *(i)* denotes the particular i-th ATPSs used for the partition experiments; N_c is equivalent number of CH_2 groups in the aliphatic alkyl side-chain of a given DNP-AA; E is an average logK increment per CH_2 group; C represents the total contribution of the non-alkyl part of the structure of a DNP-amino acid into $logK_{DNP\text{-}AA}$ and used to characterize the difference between the electrostatic properties of the coexisting phases as described previously [5], [20], [21].

Table 7.1. Partition coefficients for organic compounds and proteins in PEG–DEX–0.215 M NaCl–0.01 M K/NaPB, pH 7.4 and PEG–DEX–0.215 M NaCl–osmolyte–0.01 M K/NaPB, pH 7.4 ATPSs.

	Partition coefficients				
COMPOUND	**0.01 M K/NaPB**	**0.5 M Sorbitol**	**0.5 M Sucrose**	**0.5 M TMAO**	**1.5 M TMAO**
DNP-Ala Na	0.986±0.003	1.100±0.001	1.144±0.002	1.000±0.002	1.110±0.001
DNP-NVal Na	1.077±0.002	1.227±0.001	1.251±0.002	1.107±0.001	1.271±0.003
DNP-NLeu Na	1.131±0.003	1.300±0.003	1.346±0.003	1.170±0.002	1.406±0.002
DNP-AO Na	1.308±0.002	1.557±0.002	1.624±0.003	1.372±0.004	1.722±0.004
Benzyl alcohol	1.547±0.002	1.630±0.002	1.722±0.003	1.563±0.005	1.554±0.004
Caffeine	1.154±0.002	1.176±0.003	1.184±0.003	1.208±0.004	1.311±0.005
Coumarin	1.611±0.003	1.742±0.003	1.777±0.004	1.660±0.002	1.923±0.006
Glucoside	1.222±0.002	1.310±0.002	1.350±0.002	1.260±0.002	1.360±0.004
Methyl anthranilate	1.929±0.004	2.135±0.004	2.218±0.004	1.932±0.005	2.467±0.012
2-Phenylethanol	1.592±0.003	1.779±0.005	1.846±0.005	1.667±0.003	1.701±0.003
Phenol	1.932±0.003	2.116±0.007	2.234±0.006	1.906±0.007	1.998±0.008
Vanillin	1.702±0.004	1.939±0.005	1.987±0.007	1.641±0.004	1.818±0.006

Table 7.1. (cont.) Partition coefficients for organic compounds and proteins in PEG–DEX–0.215 M NaCl–0.01 M K/NaPB, pH 7.4 and PEG–DEX–0.215 M NaCl–osmolyte–0.01 M K/NaPB, pH 7.4 ATPSs.

	Partition coefficients				
PROTEIN	**0.01 M K/NaPB**	**0.5 M Sorbitol**	**0.5 M Sucrose**	**0.5 M TMAO**	**1.5 M TMAO**
α-chymotrypsin	0.927±0.002	4.148±0.024	5.184±0.041	0.967±0.004	1.560±0.008
α-chymotrypsinogen A	2.750±0.007	1.177±0.003	1.072±0.002	3.100±0.011	4.45±0.021
Concanavalin A	1.481±0.003	1.170±0.002	1.156±0.004	1.539±0.004	0.928±0.007
Lipase	0.801±0.004	0.801±0.003	0.817±0.001	0.830±0.002	0.757±0.003
Lysozyme	2.380±0.025	3.584±0.017	3.766±0.012	2.455±0.005	3.848±0.018

Table 7.2. Partition coefficients for organic compounds and proteins in PEG–DEX–0.215 M $NaClO_4$–0.01M K/NaPB, pH 7.4 and PEG–DEX–0.215 M $NaClO_4$–osmolyte–0.01M K/NaPB, pH 7.4 ATPSs.

	Partition coefficients				
COMPOUND	**0.01 M K/NaPB**	**0.5 M Sorbitol**	**0.5 M Sucrose**	**0.5 M TMAO**	**1.5 M TMAO**
DNP-Ala Na	0.959±0.004	0.966±0.003	0.971±0.002	0.965±0.003	0.994±0.002
DNP-NVal Na	1.029±0.002	1.064±0.002	1.074±0.003	1.059±0.004	1.175±0.002
DNP-NLeu Na	1.092±0.002	1.154±0.003	1.170±0.003	1.142±0.004	1.346±0.001
DNP-AO Na	1.249±0.004	1.393±0.005	1.419±0.002	1.361±0.003	1.854±0.003
Benzyl alcohol	1.620±0.003	1.767±0.006	1.857±0.004	1.586±0.004	1.855±0.006
Caffeine	1.197±0.003	1.266±0.002	1.246±0.003	1.252±0.004	1.432±0.007
Coumarin	1.669±0.005	1.974±0.004	1.977±0.003	1.781±0.005	2.445±0.008
Glucoside	1.249±0.007	1.396±0.005	1.409±0.004	1.303±0.004	1.515±0.007
Methyl anthranilate	1.985±0.006	2.453±0.007	2.581±0.011	2.130±0.008	3.099±0.015
2-Phenylethanol	1.663±0.005	1.895±0.006	2.035±0.009	1.726±0.004	2.034±0.008
Phenol	1.909±0.011	1.233±0.009	2.396±0.006	1.939±0.005	2.265±0.007
Vanillin	1.653±0.003	1.973±0.007	2.047±0.005	1.591±0.003	1.778±0.007

Table 7.2. (cont.) Partition coefficients for organic compounds and proteins in PEG–DEX–0.215 M $NaClO_4$–0.01M K/NaPB, pH 7.4 and PEG–DEX–0.215 M $NaClO_4$–osmolyte–0.01M K/NaPB, pH 7.4 ATPSs.

	Partition coefficients				
PROTEIN	**0.01 M K/NaPB**	**0.5 M Sorbitol**	**0.5 M Sucrose**	**0.5 M TMAO**	**1.5 M TMAO**
α-chymotrypsin	1.220±0.004	1.634±0.006	1.845±0.007	1.400±0.003	1.603±0.005
α-chymotrypsinogen A	3.646±0.014	6.519±0.023	6.245±0.032	4.550±0.018	7.451±0.041
Concanavalin A	0.213±0.001	0.246±0.003	0.267±0.003	0.227±0.002	0.235±0.003
Lipase	0.727±0.002	0.791±0.003	0.849±0.002	0.739±0.003	0.670±0.001
Lysozyme	20.3±0.15	46.4±0.22	49.10±0.23	22.3±0.13	38.9±0.34

Table 7.3. Partition coefficients for organic compounds and proteins in PEG–Na_2SO_4–0.215 M $NaClO_4$–0.01M NaPB, pH 6.8 formed by PEG8K and PEG10K and PEG–Na_2SO_4–0.215 M $NaClO_4$–0.5M osmolyte–0.01M NaPB, pH 6.8 ATPSs.

	Partition coefficients				
COMPOUND	**0.01 M NaPB**	**0.5 M Sorbitol**	**0.5 M Sucrose**	**0.5 M TMAO**	**PEG10K**
DNP-Ala Na	3.511±0.009	4.023±0.011	3.485±0.008	3.908±0.033	3.673±0.029
DNP-NVal Na	5.188±0.032	6.095±0.044	5.398±0.021	6.067±0.064	5.358±0.052
DNP-NLeu Na	7.145±0.047	8.610±0.087	7.727±0.053	8.166±0.104	7.244±0.081
DNP-AO Na	13.932±0.104	19.099±0.145	17.730±0.092	18.030±0.213	14.997±0.103
Benzyl alcohol	6.081±0.058	7.989±0.116	6.397±0.014	6.237±0.044	5.802±0.026
Caffeine	2.582±0.006	3.500±0.032	2.938±0.006	3.467±0.028	2.985±0.017
Coumarin	10.740±0.087	15.171±0.124	12.078±0.023	12.794±0.135	10.304±0.108
Glucoside	2.897±0.007	4.150±0.045	3.855±0.014	3.639±0.011	3.303±0.027
Methyl anthranilate	18.576±0.116	29.522±0.178	21.627±0.111	12.794±0.128	15.922±0.215
2-Phenylethanol	8.185±0.035	9.452±0.086	9.670±0.071	7.690±0.064	7.325±0.068
Phenol	9.920±0.048	11.780±0.109	12.430±0.086	8.854±0.085	8.627±0.097
Vanillin	12.471±0.083	19.138±0.127	14.859±0.115	8.640±0.073	12.372±0.107

Table 7.3. (cont.) Partition coefficients for organic compounds and proteins in PEG–Na_2SO_4–0.215 M $NaClO_4$–0.01M NaPB, pH 6.8 formed by PEG8K and PEG10K and PEG–Na_2SO_4–0.215 M $NaClO_4$–0.5M osmolyte–0.01M NaPB, pH 6.8 ATPSs.

	Partition coefficients				
PROTEIN	**0.01 M NaPB**	**0.5 M Sorbitol**	**0.5 M Sucrose**	**0.5 M TMAO**	**PEG10K**
α-chymotrypsin	0.070±0.004	0.058±0.003	0.094±0.003	0.054±0.002	0.064±0.003
α-chymotrypsinogen A	0.191±0.003	0.171±0.002	0.241±0.002	0.159±0.001	0.180±0.041
Concanavalin A	0.136±0.001	0.132±0.002	0.139±0.003	0.141±0.002	0.154±0.003
Lipase	0.512±0.002	0.487±0.003	0.468±0.002	0.501±0.003	0.550±0.002
Lysozyme	28.710±0.176	43.251±0.370	27.290±0.372	20.941±0.251	22.92±0.228

The differences between the hydrophobic and electrostatic properties of the phases characterized by the *C* and *E* values are listed for all the ATPSs under comparison in Table 7.4. The free energies of transfer of a CH_2 group from the lower to the upper phases determined from Eq. 7.1 as described in Supplementary Information – section A are also present in Table 7.4. It has been shown previously [5], [20], [21] that partition behavior of solutes in any given ATPS is affected by the solvent features of the coexisting phases, such as the solvent dipolarity/polarizability (π^*) characterizing the ability of water to participate in dipole-dipole and dipole-induced dipole interactions with a solute, solvent hydrogen bond donor acidity (α), and hydrogen bond acceptor basicity (β). The differences between these solvent features of the phases determined as described in before are also listed in Table 7.4.

Table 7.4. Differences between the solvent properties of the coexisting phases PEG–DEX–0.01 M K/NaPB, pH 7.4 and PEG–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPSs with indicated salts and osmolytes additives.

	PEG–DEX (data from [20])				
Solvent Properties	**0.01M K/NaPB**	**0.5M Sorbitol**	**0.5M Sucrose**	**0.5M TMAO**	**0.5M Trehalose**
$\Delta G(CH_2)$, cal/mole	-45±1.3	-43±1.1	-39.4±0.44	-40.9±0.6	-47.7±0.6
E	0.033±0.001	0.032±0.002	0.029±0.001	0.028±0.001	0.035±0.001
C	0.058±0.003	0.090±0.003	0.110±0.002	0.083±0.002	0.113±0.002
$\Delta\pi^*$	-0.042±0.002	-0.042±0.004	-0.073±0.004	-0.031±0.002	-0.042±0.003
$\Delta\alpha$	-0.051±0.003	-0.066±0.003	-0.046±0.005	-0.074±0.003	-0.081±0.003
$\Delta\beta$	0.006±0.004	0.006±0.003	0.023±0.006	0.009±0.008	0.006±0.005

Table 7.4. (cont.) Differences between the solvent properties of the coexisting phases PEG–DEX–0.01 M K/NaPB, pH 7.4 and PEG–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPSs with indicated salts and osmolytes additives.

	PEG–DEX–0.215 M NaCl				
Solvent Properties	**0.01M K/NaPB**	**0.5M Sorbitol**	**0.5M Sucrose**	**0.5M TMAO**	**1.5M TMAO**
$\Delta G(CH_2)$, cal/mole	-33±1.2	-41±1.8	-41±2.9	-37±1.2	-51±2.4
E	0.024±0.001	0.030±0.001	0.031±0.001	0.027±0.001	0.038±0.002
C	-0.036±0.004	0.005±0.005	0.017±0.002	-0.032±0.004	0.0006±0.007
$\Delta\pi^*$	-0.037±0.003	-0.041±0.002	-0.040±0.003	-0.033±0.002	-0.026±0.001
$\Delta\alpha$	-0.054±0.002	-0.063±0.002	-0.080±0.002	-0.065±0.002	-0.023±0.002
$\Delta\beta$	0.002±0.002	0.007±0.002	0.004±0.003	-0.019±0.002	-0.105±0.002

Table 7.4. (cont.) Differences between the solvent properties of the coexisting phases PEG–DEX–0.01 M K/NaPB, pH 7.4 and PEG–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPSs with indicated salts and osmolytes additives.

	PEG–DEX–0.215 M $NaClO_4$				
Solvent Properties	**0.01M K/NaPB**	**0.5M Sorbitol**	**0.5M Sucrose**	**0.5M TMAO**	**1.5M TMAO**
$\Delta G(CH_2)$, cal/mole	-31.2±0.1	-43±0.2	-44.7±0.2	-40.6±0.1	-73.5±0.2
E	0.023±0.003	0.032±0.002	0.033±0.001	0.067±0.001	0.054±0.001
C	-0.049±0.001	-0.056±0.009	-0.056±0.003	0.0546±0.0007	-0.074±0.005
$\Delta\pi^*$	-0.037±0.002	-0.040±0.002	-0.044±0.002	-0.031±0.003	-0.020±0.003
$\Delta\alpha$	-0.031±0.003	-0.067±0.003	-0.072±0.003	-0.075±0.003	-0.104±0.003
$\Delta\beta$	0.004±0.002	0.006±0.002	0.008±0.002	0.003±0.004	-0.002±0.003

Table 7.4. (cont.) Differences between the solvent properties of the coexisting phases PEG–DEX–0.01 M K/NaPB, pH 7.4 and PEG–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPSs with indicated salts and osmolytes additives.

	PEG–Na_2SO_4 (Data from [23])				
Solvent Properties	**0.01M NaPB**	**0.5M Sorbitol**	**0.5M Sucrose**	**0.5M TMAO**	**PEG10K**
$\Delta G(CH_2)$, cal/mole	-122±1.8	-144±3.4	-178±3.5	-146±2.4	-137±5.8
E	0.048±0.002	0.102±0.003	0.123±0.006	0.108±0.002	0.100±0.004
C	0.445±0.005	0.670±0.011	0.670±0.020	0.625±0.007	0.440±0.017
$\Delta\pi^*$	-0.029±0.003	-0.046±0.004	-0.077±0.005	-0.010±0.003	-0.020±0.003
$\Delta\alpha$	-0.128±0.004	-0.248±0.005	-0.228±0.007	-0.208±0.004	-0.075±0.004
$\Delta\beta$	0.015±0.004	0.021±0.008	0.028±0.008	0.021±0.009	0.013±0.004

Table 7.4. (cont.) Differences between the solvent properties of the coexisting phases PEG–DEX–0.01 M K/NaPB, pH 7.4 and PEG–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPSs with indicated salts and osmolytes additives.

	PEG–Na_2SO_4–0.215 M NaCl (Data from [21])				
Solvent Properties	**0.01M NaPB**	**0.5M Sorbitol**	**0.5M Sucrose**	**0.5M TMAO**	**PEG10K**
$\Delta G(CH_2)$, cal/mole	-152.4±0.6	-180±5.3	-187±2.9	-161±1.8	-149±4.1
E	0.112±0.001	0.133±0.004	0.138±0.002	0.119±0.001	0.110±0.003
C	0.435±0.002	0.530±0.015	0.525±0.008	0.571±0.005	0.438±0.011
$\Delta\pi^*$	-0.027±0.003	-0.056±0.002	-0.067±0.003	-0.025±0.002	-0.039±0.001
$\Delta\alpha$	-0.189±0.002	-0.259±0.002	-0.253±0.002	-0.271±0.002	-0.183±0.002
$\Delta\beta$	0.013±0.002	0.025±0.002	0.022±0.003	0.015±0.002	0.021±0.002

Table 7.4. (cont.) Differences between the solvent properties of the coexisting phases PEG–DEX–0.01 M K/NaPB, pH 7.4 and PEG–Na_2SO_4–0.01 M NaPB, pH 6.8 ATPSs with indicated salts and osmolytes additives.

	PEG–Na_2SO_4–0.215 M $NaClO_4$				
Solvent Properties	**0.01M NaPB**	**0.5M Sorbitol**	**0.5M Sucrose**	**0.5M TMAO**	**PEG10K**
$\Delta G(CH_2)$, cal/mole	-162±3.5	-184±1.2	-191.9±0.6	-179±3.5	-166±0.6
E	0.112±0.003	0.136±0.001	0.142±0.001	0.132±0.002	0.122±0.001
C	0.395±0.010	0.426±0.001	0.357±0.001	0.423±0.010	0.404±0.002
$\Delta\pi^*$	-0.042±0.003	-0.053±0.003	-0.068±0.002	-0.018±0.002	-0.030±0.003
$\Delta\alpha$	-0.173±0.004	-0.107±0.012	-0.194±0.002	-0.242±0.003	-0.161±0.019
$\Delta\beta$	0.006±0.003	0.026±0.002	0.023±0.002	0.009±0.002	0.018±0.002

The differences between the solvent hydrogen bond donor acidity, $\Delta\alpha$, in the PEG–Na_2SO_4 ATPSs exceed those observed in PEG–DEX ATPSs in the presence of all osmolytes additives used. The salts additives effects on $\Delta\alpha$ appears to be more pronounced in PEG–Na_2SO_4 ATPSs than in PEG–DEX systems. In PEG–Na_2SO_4 ATPSs the salt additive effect depends on the presence of osmolyte additives. Addition of ~0.2 M NaCl increases the difference, while addition of $NaClO_4$ may reduce or increase it, depending on the particular osmolyte present.

The differences between the solvent hydrogen bond acceptor basicity, $\Delta\beta$, in the PEG–Na_2SO_4 ATPSs generally exceed those observed in PEG–DEX ATPSs in the presence of all osmolytes except in the presence of 0.5 M or 1.5 M TMAO in PEG–DEX–NaCl ATPSs.

The differences between the solvent dipolarity/polarizability, $\Delta\pi^*$, vary in both types of ATPSs within the same range from -0.020 to -0.077 and the effects of salt and osmolyte additives do not display any noticeable trend.

The differences between electrostatic properties of the phases (parameter *C*) in PEG–Na_2SO_4 ATPSs exceed those observed in PEG–DEX ATPSs as expected. Surprisingly, osmolyte additives affect the differences between electrostatic properties in both types of ATPSs rather strongly. In PEG–DEX ATPSs, both NaCl and $NaClO_4$ additives reduce the difference. Both salts additives appear also to reduce the difference between the electrostatic properties of the phases in PEG–Na_2SO_4 ATPSs, and their effects seem to depend on the particular osmolyte additive present.

The differences between the relative hydrophobicity of the phases (parameter *E*) in PEG–Na_2SO_4 ATPSs exceed those observed in PEG–DEX ATPSs in the presence of all additives. The effects of salt additives in these two types of ATPSs are different, however. In osmolyte free PEG–DEX ATPSs both NaCl and $NaClO_4$ additives reduce it. In PEG–Na_2SO_4 ATPSs, both salt additives increase the difference between the relative hydrophobicity of the phases.

In order to simplify the comparison, we estimated the normalized Euclidean distances between all the ATPSs under consideration as described previously [21].

The solvent properties of each ATPS are represented by the set of differences between the solvent features of the coexisting phases: $\Delta\pi^*$, $\Delta\alpha$, $\Delta\beta$, *C*, and *E* values, listed in Table 7.4. The set of the $\Delta\pi^*$, $\Delta\alpha$, $\Delta\beta$, *C*, and *E* values for a given ATPS may be viewed as a point in a multi-dimensional space of solvent properties. To compare the properties of different ATPSs, we calculated the normalized Euclidian distance in the multi-dimensional space represented by the differences between the solvent features of the coexisting phases in different ATPSs:

$$d_{i,0} = \left[\sum_j \left(\frac{\delta_i - \delta_0}{\delta_0}\right)^2\right]^{0.5}$$

(Equation 7.2)

where $d_{i,o}$ is the distance between the solvent properties of i-th ATPS and solvent properties of the o-th ATPS chosen as a reference, ∂_i and ∂_o are the differences between the j-th solvent features in i-th and o-th ATPSs.

To compensate for differences in ∂-values measured for a given solvent property in different ATPSs, we normalized the experimental ∂-values to the reference ∂_o-value for each particular solvent property. Therefore, Eq. 7.2 represents the Euclidean distance between the points characterized by normalized differences between various solvent features in different ATPSs.

In order to compare the properties of the multiple ATPSs used here and previously [20]–[23], we selected the osmolyte and salt additive-free PEG–DEX ATPS as the reference ATPS. The normalized Euclidean distances calculated with Eq. 7.2 are listed in Table 7.5.

Table 7.5. Normalized Euclidean distances between the solvent properties of ATPSs calculated with Eq. 7.2 from the data in Table 7.4. (The ATPSs compositions presented in Table 7.4.).

ATPS	Distance, d_{io} [a]	Distance, d_{io}* [b]
PEG–DEX	0	0
PEG–DEX–0.5 M sorbitol	0.63	0.63
PEG–DEX–0.5 M TMAO	0.85	0.64
PEG–DEX–0.5 M trehalose	1.12	0.91
PEG–DEX–0.215 M NaCl–0.5 M sucrose	1.54	1.12
PEG–DEX–0.215 M NaCl–0.5 M sorbitol	1.61	1.51
PEG–DEX–0.215 M $NaClO_4$	1.91	1.60
PEG–DEX–0.215 M NaCl	1.93	1.80
PEG–DEX–0.215 M $NaClO_4$–0.5 M sorbitol	1.99	1.87
PEG–DEX–0.215 M $NaClO_4$–0.5 M TMAO	2.04	1.88
PEG–DEX–0.215 M $NaClO_4$–0.5 M sucrose	2.08	1.99
PEG–DEX–0.215 M $NaClO_4$–1.5 M TMAO	2.95	2.00
PEG–DEX–0.5 M sucrose	3.07	2.01
PEG–DEX–0.215 M NaCl–0.5 M TMAO	4.57	2.41
PEG–Na_2SO_4–0.215 M $NaClO_4$	6.75	2.58
PEG–Na_2SO_4	7.03	6.75
PEG10K–Na_2SO_4	7.03	6.75
PEG10K–Na_2SO_4–0.215 M $NaClO_4$	7.18	6.86
PEG–Na_2SO_4–0.215 M $NaClO_4$–0.5 M TMAO	7.35	6.89
PEG–Na_2SO_4–0.215 M $NaClO_4$–0.5 M sorbitol	7.89	6.91
PEG–Na_2SO_4–0.215 M $NaClO_4$–0.5 M sucrose	7.95	7.14

Table 7.5. (cont.) Normalized Euclidean distances between the solvent properties of ATPSs calculated with Eq. 7.2 from the data in Table 7.4. (The ATPSs compositions presented in Table 7.4.).

ATPS	Distance, d_{io} [a]	Distance, d_{io}* [b]
PEG–Na_2SO_4–0.215 M NaCl	8.29	7.91
PEG–Na_2SO_4–0.215 M NaCl–0.5 M sorbitol	8.69	8.09
PEG–Na_2SO_4–0.215 M NaCl–0.5 M TMAO	9.80	8.20
PEG–Na_2SO_4–0.215 M NaCl–0.5 M sucrose	9.93	9.54
PEG10K–Na_2SO_4–0.215 M NaCl	9.94	9.62
PEG–Na_2SO_4–0.5 M TMAO	10.82	9.67
PEG–Na_2SO_4–0.5 M sorbitol	11.70	10.50
PEG–Na_2SO_4–0.5 M sucrose	12.04	11.43
PEG–Na_2SO_4–0.5 M trehalose	12.51	11.44
PEG–DEX–1.5 M TMAO	18.69	12.20

a – Distances d_{io} calculated with Eq. 7.2 using PEG–DEX ATPS as a reference and all solvent properties of ATPSs presented in Table 7.4

b – Distances d_{io}* calculated with Eq. 7.2 using PEG–DEX ATPS as a reference and only three solvent properties of ATPSs (parameters C, E, and $\Delta\alpha$)

In order to explore what solvent features dominate in the ATPS under comparison, we explored how the calculated distances vary with reducing the number of different solvent features included in calculation.

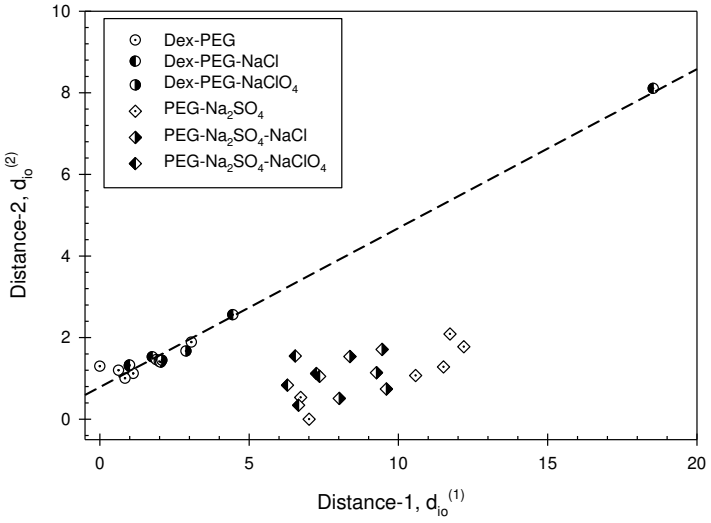


Figure 7.1. Euclidian distance for ATPSs, $d_{io}^{(2)}$, calculated using three solvent features (parameters E, C, and $\Delta\alpha$) plotted against Euclidian distance, $d_{io}^{(1)}$, calculated using all the solvent features (C, E, $\Delta\alpha$, $\Delta\pi^*$, and $\Delta\beta$).

The result illustrated graphically in Fig. 7.1 shows that there are three solvent features, the differences between electrostatic and hydrophobic properties (parameters C and E) and the difference between the solvent hydrogen bond donor acidity ($\Delta\alpha$) sufficient for estimating the distance between the ATPS under comparison. Only three ATPSs (PEG–DEX–0.215 M NaCl–1.5 M TMAO, PEG–DEX–0.215 M NaCl–0.5 M TMAO, and PEG–DEX–0.5 M sucrose) are exceptions due to extraordinary $\Delta\beta$ of -0.105, negative value of $\Delta\beta$ -0.019, and high value of $\Delta\pi^*$-0.073, correspondingly.

7.3.2. Partition behavior of organic compounds and proteins

Partition coefficients of 12 organic compounds and five proteins in PEG–DEX–0.215 M NaCl–0.01 M K/NaPB, pH 7.4, PEG–DEX–0.215 M $NaClO_4$–0.01 M K/NaPB, pH 7.4, and PEG–Na_2SO_4–0.215 M $NaClO_4$–0.01 M NaPB, pH 6.8 are presented in Tables 7.1-7.3. The partition coefficients for the same compounds and proteins in PEG–DEX–0.01 M K/NaPB, pH 7.4 reported previously [20], [22], in PEG–Na_2SO_4–0.01 M NaPB, pH 6.8 [23] and in PEG–Na_2SO_4–0.215 M NaCl–0.01 M NaPB, pH 6.8 [21] are listed in Tables S7.1 (Supplementary Information – section A), 4.1 (Chapter 4) and 5.1 (Chapter 5). In

order to compare partition behavior of the compounds utilized in the study, we used the so-called Collander linear relationship or solvent regression equation [5], [24]. This equation describes the linear relationship between partition coefficients of various solutes in two different ATPSs as:

$$\log K_j^{(i)} = a_{jo} \log K_o^i + b_{jo}$$

(Equation 7.3)

where K_j and K_o are partition coefficients for a solute i in the ATPSs denoted by subscripts j and o; a_{jo} and b_{jo} are constants, the values of which depend on the particular composition of the two-phase systems under comparison. It should be emphasized that both ATPSs under comparison have the same ionic composition.

Typical Collander relationships for two ATPSs different in regard to the presence of nonionic osmolyte additive TMAO are shown in Fig. 7.2.

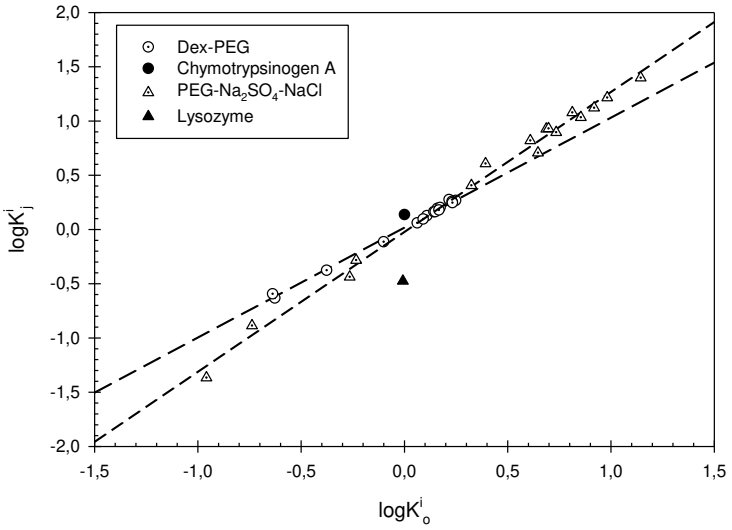


Figure 7.2. Logarithms of partition coefficients for compounds (including proteins) in PEG–DEX and PEG–Na_2SO_4 ATPSs with 0.5 M TMAO versus logarithms of partition coefficients for the same compounds in the corresponding systems without TMAO additive.

Similar relationships for various ATPS are observed. These relationships are characterized by coefficients a_{jo} and b_{jo} listed in Table 7.6.

Table 7.6. Coefficients of Collander relationships (Eq. 7.3) for compounds in ATPS with osmolytes.

ATPS	Salt	Osmolyte*	b_{jo}	a_{jo}	N	r^2	SD	F	Outliers**
PEG–DEX	-	TMAO	0.017±0.005	1.01±0.02	16	0.9963	0.019	3737	P2
		Sorbitol	0.032±0.005	1.07±0.02	14	0.9964	0.017	3307	P2, P5, 1
		Sucrose	0.041±0.005	1.08±0.02	14	0.9957	0.019	2764	P2, P5, 1
PEG–DEX	NaCl	TMAO	0.015±0.004	0.96±0.02	16	0.9945	0.010	2543	P2
		1.5M TMAO	0.05±0.02	1.32±0.07	10	0.9758	0.034	323	2, 3, 4, 5, P1, P3. P4
		Sorbitol	0.03±0.01	1.04±0.05	13	0.9754	0.022	396	P1, P2, P3, P5
		Sucrose	0.06±0.01	1.01±0.06	12	0.9682	0.020	305	P1, P2, P3, P4, P5
PEG–DEX	$NaClO_4$	TMAO	0.018±0.007	1.29±0.02	14	0.9982	0.019	6794	P1, P3, P4
		1.5M TMAO	0.07±0.01	1.14±0.04	16	0.9854	0.056	946	P2
		Sorbitol	0.02±0.01	1.26±0.04	15	0.9894	0.046	1211	4, P5
		Sucrose	0.017±0.005	1.01±0.01	16	0.9976	0.020	5881	P2

Table 7.6. (cont.) Coefficients of Collander relationships (Eq. 7.3) for compounds in ATPS with osmolytes.

ATPS	Salt	Osmolyte*	b_{jo}	a_{jo}	N	r^2	SD	F	Outliers**
PEG–Na_2SO_4	-	TMAO	0.06±0.02	1.11±0.03	16	0.9872	0.077	1077	P5
		Sorbitol	0.04±0.02	1.29±0.02	14	0.9964	0.048	3343	1, P2, P5
		Sucrose	0.04±0.02	1.36±0.03	14	0.9938	0.066	1934	1, P2, P5
PEG–Na_2SO_4	NaCl	TMAO	0	1.29±0.03	16	0.9925	0.073	1863	P5
		Sorbitol	-0.03±0.01	1.04±0.02	16	0.9966	0.040	4083	1
		Sucrose	0	1.26±0.03	14	0.9934	0.069	1796	1, P2, P5
PEG–Na_2SO_4	$NaClO_4$	TMAO	0.06±0.02	0.99±0.02	17	0.9959	0.052	3626	-
		Sorbitol	0	0.96±0.02	11	0.9929	0.067	1575	1, P5, 1a-4a
		Sucrose	0	1.00± 0.03	17	0.9858	0.098	1044	-

* Concentrations of each osmolyte – 0.5 M except in the indicated case of 1.5 M TMAO

** Abbreviations: 1 – 2-phenylethanol, 2 – Benzyl alcohol, 3 – *p*-nitrophenyl-D-glucopyranoside, 4 – Phenol, 5 – Vanillin, 1a – DNP-Ala Na, 2a – DNP-NVal Na, 3a – DNP-NLeu Na, 4a – DNP-AO Na, P1 – α-chymotrypsin, P2 – α-chymotrypsinogen A, P3 – Concanavalin A, P4 – Lipase, P5 – Lysozyme

In order to explain the relationships observed and the fact that some of the compounds do not fit these relationships it is necessary to mention that as established previously [25], [26] the partition coefficient of a solute in a given ATPS is governed by different types of interactions of the solute with the aqueous media in the coexisting phases and may be described as:

$$\log K_j^i = {S_S}^i \, \Delta\pi^*_j + {B_S}^i \, \Delta\alpha_j + {A_S}^i \, \Delta\beta_j + {C_S}^i \, c_j$$

(Equation 7.4)

where K_j^i is the solute *i* partition coefficient in j-th ATPS; $\Delta\pi^*_j$, $\Delta\alpha_j$, $\Delta\beta_j$ and c_j are the differences between the solvent properties of the top and bottom phases in the j-th ATPS (solvent dipolarity/polarizability, hydrogen-bond donor acidity, hydrogen-bond acceptor basicity, and electrostatic interactions, respectively; S_s^i, B_s^i, A_s^i, and C_s^i are constants (solute-specific coefficients) that describe the contributions of the complementary interactions of the solute *i* with the solvent media in the coexisting phases; the subscript *s* designates the solute.

All the data in the set of ATPSs with the same ionic composition were used to determine solute-specific coefficients S_s, A_s, B_s, and C_s in Eq. 7.4 for each compound by the multiple linear regression analysis. The p-value was used to estimate the significance for each solute-specific coefficient in Eq. 7.4 for a given compound taking into account the small number of ATPSs with the same ionic composition utilized. If all four coefficients (S_s, A_s, B_s, and C_s) proved statistically significant ($p < 0.1$), then the correlation was accepted. If one or more values reveal a p-value > 0.1, then the equations contained different combinations of coefficients were examined. The equation with a set of coefficients providing p-values for all parameters below 0.1 was accepted. The solute-specific coefficients determined for each compound from the data obtained in the chosen ATPS set are presented in Table S7.2 (Supplementary Information – section B).

Taking Eq. 7.4 into account, it seems reasonable that the changes in the differences between the solvent properties of the phases brought by the presence of a given osmolyte may affect various compounds in a different manner. That is the likely reason why some compounds do not fit the Collander relationship under discussion (see in Table 7.6). As an example, the reason for α-chymotrypsinogen A (P2) not to fit the linear relationship in the presence of 0.5 M TMAO in the salt additive free PEG–DEX ATPS seems to be the negative contribution of A_s solute-specific coefficient differing this protein from the other proteins examined (see in Table S7.2 in Supplementary Information – section B). The same protein

does not fit the Collander relationship in PEG–DEX–0.215 M NaCl ATPS containing 0.5 M TMAO likely due to the relatively high negative A_s value together with the high negative value of the C_s solute-specific coefficient.

If a given salt additive does not interact directly with certain compounds, the partition coefficients of these compounds in the systems with different ionic composition may fit the linear Collander relationship [21], [27]. Typical relationships for partition coefficients in PEG–DEX and PEG–DEX–0.215 M NaCl ATPSs and in PEG–Na_2SO_4 and PEG–Na_2SO_4–0.215 M NaCl ATPSs are illustrated graphically in Fig. 7.3a and 7.3b.

The data presented in Fig. 7.3a and 7.3b show that proteins and Na-salts of DNP-AA do not fit the linear relationship shown for organic compounds. Vanillin is the only ionized compound among the other organic compounds examined, and therefore the effect of NaCl additive on its partition behavior might be expected. In order to explain why methyl anthranilate and *p*-nitrophenyl-α-D-glucopyranoside do not fit the relationship in question, it is necessary to explore the effect of NaCl additive on the solute-specific coefficients in Eq. 7.4 for these compounds relative to the other organic compounds examined.

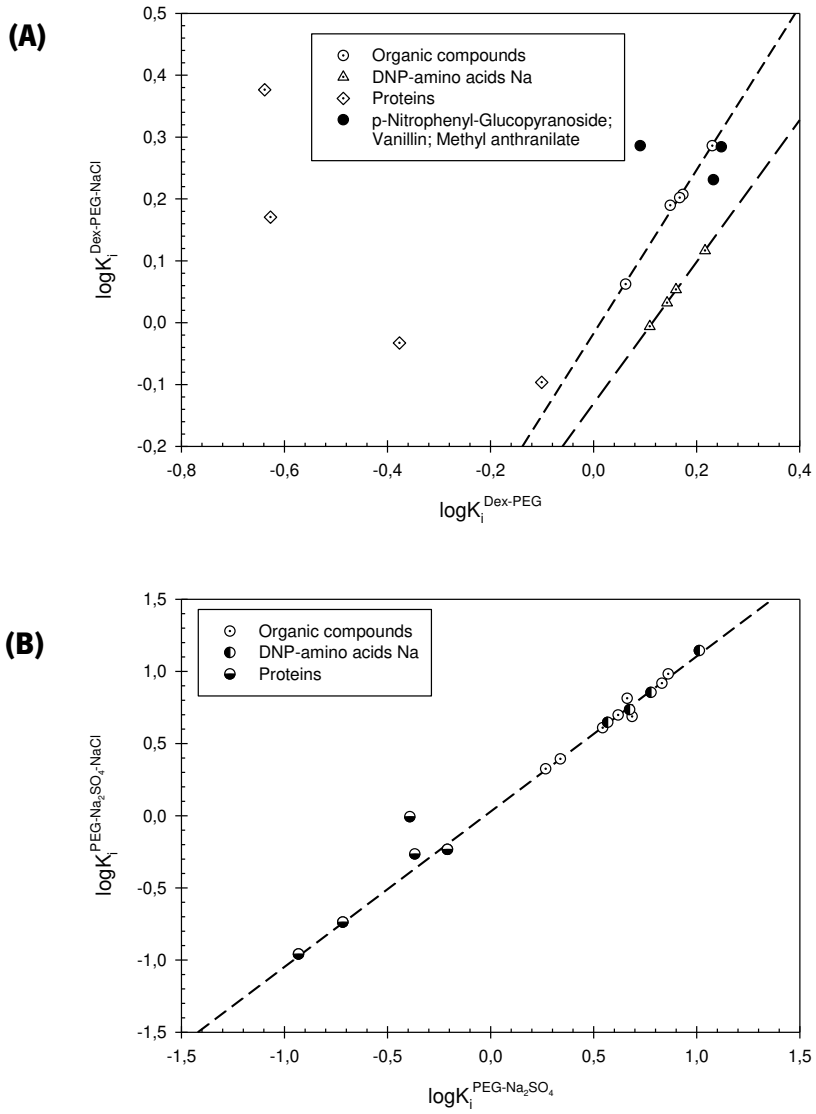


Figure 7.3. (A) Logarithms of partition coefficients for compounds (including proteins) in PEG–DEX–0.215 M NaCl ATPS versus logarithms of partition coefficients for the same compounds in the PEG–DEX ATPS without NaCl additive. **(B)** Logarithms of partition coefficients for compounds (including proteins) in PEG–Na_2SO_4–0.215 M NaCl ATPS versus logarithms of partition coefficients for the same compounds in the PEG–Na_2SO_4 ATPS without NaCl additive.

The data for all four solute-specific coefficients in the presence of 0.215 M NaCl for all organic compounds examine (except Na-salts of DNP-amino acids) are plotted against the data for same compounds in the absence of the NaCl additive in Fig. S7.4.A-D (Supplementary Information – section B). It can be seen that in the presence of 0.215 M NaCl additive, the solute-water interactions of *p*-nitrophenyl-α-D-glucopyranoside deviates from the trends observed for other compounds in regard to dipole-dipole interactions (coefficient S_s), hydrogen bonding (coefficient A_s), and electrostatic interactions (coefficient C_s), whereas methyl anthranilate deviates from the trends observed in regard to hydrogen bonding (coefficients A_s and B_s). Other compounds, such as caffeine and coumarin, show changes in only one of the solute-specific coefficients only, B_s and A_s, correspondingly.

The effect of NaCl additive on partition behavior of compounds in PEG–Na_2SO_4 ATPSs causes the deviation from linear relationship only for two proteins, lysozyme (HEL) and α-chymotrypsinogen A (CHTG), likely due to the direct interactions of these proteins with NaCl. Based on the obtained data, we explored the effects of salt additives on linear relationships for only organic compounds in PEG–DEX ATPSs and on those for all compounds in PEG–Na_2SO_4 ATPSs. The coefficients of Eq. 7.3 for the observed relationships are listed in Table 7.7.

Table 7.7. Coefficients for Eq. 7.3 ($\log K_2 = b + a \log K_1$) for ATPSs with different ionic composition (concentrations of NaCl and $NaClO_4$ additives – 0.215 M)[a].

ATPS-1	ATPS-2	Osmolyte	b	a	N	r^2	SD	F	Outlier*
PEG–DEX	PEG–DEX–NaCl	-	0	1.32±0.05	5	0.9949	0.007	590	3, 5, 6
PEG–DEX	PEG–DEX–NaCl	TMAO	0.03±0.01	0.98±0.06	6	0.9848	0.010	259	3; 5
PEG–DEX	PEG–DEX–NaCl	Sorbitol	0	1.08±0.05	6	0.9925	0.009	528	1; 3
PEG–DEX	PEG–DEX–NaCl	Sucrose	0	1.03±0.06	7	0.9832	0.013	293	3
PEG–DEX	PEG–DEX–$NaClO_4$		0.07±0.01	0.92±0.05	5	0.9926	0.004	405	3; 4, 7
PEG–DEX	PEG–DEX–$NaClO_4$	TMAO	0	1.47±0.07	6	0.9914	0.013	461	1; 5
PEG–DEX	PEG–DEX–$NaClO_4$	Sorbitol	0	1.25±0.06	6	0.9922	0.010	508	5; 4
PEG–DEX	PEG–DEX–$NaClO_4$	Sucrose	0.03±0.02	0.88±0.08	7	0.9606	0.019	122	5
PEG–DEX	PEG–Na_2SO_4	-	0.06±0.03	3.3±0.2	7	0.9880	0.027	413	4
PEG–DEX	PEG–Na_2SO_4	TMAO	0.20±0.04	2.7±0.2	7	0.9676	0.041	150	4
PEG–DEX	PEG–Na_2SO_4	Sorbitol	0.16±0.03	3.2±0.1	7	0.9914	0.027	579	4
PEG–DEX	PEG–Na_2SO_4	Sucrose	0.22±0.03	2.9±0.1	7	0.9925	0.025	658	4

Table 7.7. (cont.) Coefficients for Eq. 7.3 ($logK_2 = b + a\ logK_1$) for ATPSs with different ionic composition (concentrations of NaCl and $NaClO_4$ additives – 0.215 M)[a].

ATPS-1	ATPS-2	Osmolyte	b	a	N	r^2	SD	F	Outlier*
PEG–Na_2SO_4	PEG–Na_2SO_4–NaCl	-	0.03±0.01	1.08±0.02	15	0.9972	0.034	4609	p2; p5
PEG–Na_2SO_4	PEG–Na_2SO_4–NaCl	TMAO	0	1.34±0.03	8	0.9962	0.061	2101	p5;7;8;aa
PEG–Na_2SO_4	PEG–Na_2SO_4–NaCl	Sorbitol	-0.03±0.01	0.87±0.01	14	0.9968	0.039	3796	p2;p5;8
PEG–Na_2SO_4	PEG–Na_2SO_4–NaCl	Sucrose	0	0.97±0.03	13	0.9926	0.055	1484	p1;p2;p5;7
PEG–Na_2SO_4	PEG–Na_2SO_4–$NaClO_4$	-	0.09±0.01	1.34±0.01	8	0.9993	0.026	9106	p2;p4;p5;3;5;aa
PEG–Na_2SO_4	PEG–Na_2SO_4–$NaClO_4$	TMAO	0.12±0.01	1.25±0.02	8	0.9987	0.038	4467	p2;p4;p5; 3;7;aa
PEG–Na_2SO_4	PEG–Na_2SO_4–$NaClO_4$	Sorbitol	0.08±0.02	1.14±0.03	11	0.9951	0.071	1835	p4; p5;aa
PEG–Na_2SO_4	PEG–Na_2SO_4–$NaClO_4$	Sucrose	0	0.96±0.01	9	0.9985	0.038	4766	p5;1;7;8; aa
PEG–DEX–NaCl	PEG–Na_2SO_4–NaCl	-	0.20±0.04	2.2±0.2	5	0.9723	0.035	105	4; 5; 6
PEG–DEX–NaCl	PEG–Na_2SO_4–NaCl	TMAO	0.07±0.03	3.9±0.1	5	0.9971	0.018	1036	3; 4; 5
PEG–DEX–NaCl	PEG–Na_2SO_4–NaCl	Sorbitol	0.25±0.02	1.75±0.07	5	0.9958	0.012	713	3; 5; 6
PEG–DEX–NaCl	PEG–Na_2SO_4–NaCl	Sucrose	0.25±0.08	2.4±0.3	6	0.9442	0.066	68	3; 5

Table 7.7. (cont.) Coefficients for Eq. 7.3 ($\log K_2 = b + a \log K_1$) for ATPSs with different ionic composition (concentrations of NaCl and $NaClO_4$ additives – 0.215 M)[a].

ATPS-1	ATPS-2	Osmolyte	b	a	N	r^2	SD	F	Outlier*
PEG–DEX–$NaClO_4$	PEG–Na_2SO_4–$NaClO_4$	-	0	3.6±0.3	5	0.9881	0.047	250	2; 4; 5
PEG–DEX–$NaClO_4$	PEG–Na_2SO_4–$NaClO_4$	TMAO	0.19±0.07	2.8±0.3	5	0.9750	0.066	117	2; 4; 5
PEG–DEX–$NaClO_4$	PEG–Na_2SO_4–$NaClO_4$	Sorbitol	0.16±0.07	3.3±0.3	5	0.9825	0.059	169	1; 4; 5
PEG–DEX–$NaClO_4$	PEG–Na_2SO_4–$NaClO_4$	Sucrose	0.30±0.03	2.4±0.1	5	0.9892	0.029	275	1; 5; 8

a – For the PEG–DEX ATPSs only small organic compounds (excluding DNP-AA Na salts) were considered, for PEG–Na_2SO_4 ATPSs all compounds (including proteins) were considered

* – Abbreviations: 1 – 2-phenylethanol, 2 – Benzyl alcohol, 3 – *p*-nitrophenyl-D-glucopyranoside, 4 – Phenol, 5 – Vanillin, 6 – Methyl anthranilate; 7 – Caffeine; 8 – Coumarin; aa – Na salts of DNP-AA (DNP-Ala Na, DNP-NVal Na, DNP-NLeu Na, DNP-AO Na), p1 – α-chymotrypsin, p2 – α-chymotrypsinogen A, p3 – Concanavalin A, p4 – Lipase, p5 – Lysozyme

As aforementioned, the presence of salt additives affects partition behavior of solutes in the solute-specific manner for more compounds in the PEG–DEX ATPSs than in the PEG–Na_2SO_4 ATPSs likely because of the absence of excessive Na_2SO_4 concentration in the former ATPSs. It is important to note that the effects of salt additives appear to depend on the particular osmolyte present in a given ATPS. As an example, in PEG–DEX ATPSs, the effect of NaCl additive is observed on three compounds (*p*-nitrophenyl-α-D-glucopyranoside, vanillin, and methyl anthranilate) in the absence of any osmolyte, on *p*-nitrophenyl-α-D-glucopyranoside and vanillin in the presence of 0.5 M TMAO, and on 2-phenylethanoland *p*-nitrophenyl-α-D-glucopyranoside in the presence of 0.5 M sorbitol. Many other examples of the same effects of osmolytes maybe found in Table 7.7. These osmolyte-specific effects are likely due to the different degrees of influence of various osmolytes on different solvent features of the phases in ATPSs.

It has been shown previously [27], [28] that the logarithms of partition coefficients of proteins and organic compounds in three ATPSs with different ionic compositions may be linearly interrelated. Analysis of the partition coefficients presented in Tables 7.1-7.3 and Tables S7.1 (Supplementary Information – section A), 4.2 (Chapter 4) and 5.2 (Chapter 5) shows that the relationship between the logarithms of partition coefficients of compounds in PEG–DEX ATPSs illustrated in Fig. 7.4a may be described as:

$$\log K_i^{PEG-DEX-NaCl} = 0.22_{\pm 0.02} \times \log K_i^{PEG-DEX-NaClO_4} + 0.77_{\pm 0.02} \times \log K_i^{PEG-DEX}$$

N = 14; r^2 = 0.9952; SD = 0.005; F = 1240

(Equation 7.5a)

where K_i is the partition coefficient of the i-th compound, superscripts denote the ATPS composition. Three compounds (concanavalin A, lysozyme, and *p*-nitrophenyl-α-D-glucopyranoside) do not fit the relationship.

Similar relationship is observed for the logarithms of partition coefficients of compounds in PEG–Na_2SO_4 ATPSs. This relationship shown in Fig. 7.4b may be described as:

$$\log K_i^{PEG-Na_2SO_4} = -0.03_{\pm 0.01} + 0.13_{\pm 0.06} \times \log K_i^{PEG-Na_2SO_4-NaClO_4} + 0.78_{\pm 0.07} \times \log K_i^{PEG-Na_2SO_4-NaCl}$$

N = 16; r^2 = 0.9971; SD = 0.034; F = 2238

(Equation 7.5b)

where all parameters are as defined above. One compound (lysozyme) does not fit the relationship.

(A)

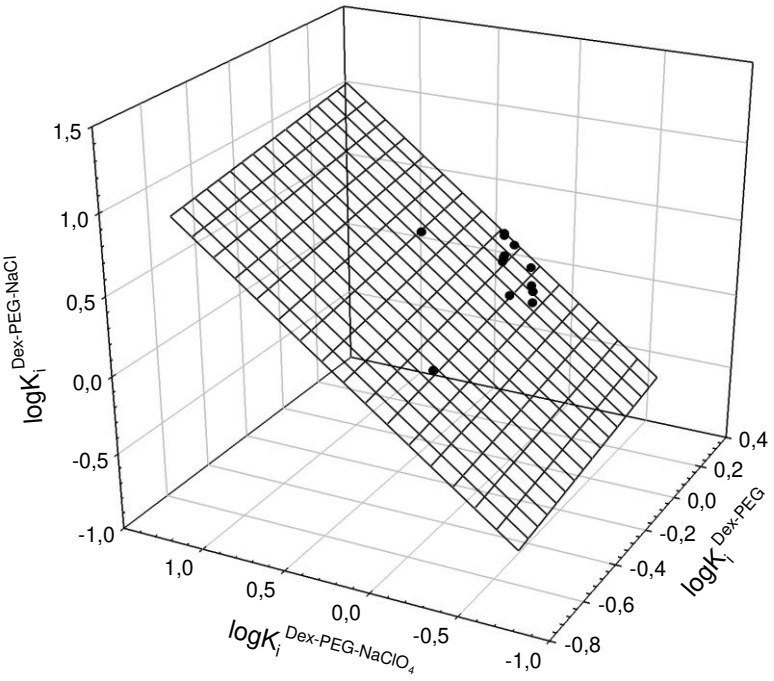


Figure 7.4. (A) Interrelationship between logarithms of partition coefficients for organic compounds in PEG–DEX–0.215 M NaCl ATPS, logarithms of partition coefficients for the same compounds in PEG–DEX–0.215 M $NaClO_4$ ATPS, and logarithms of partition coefficients for the same compounds in PEG–DEX ATPS without salt additive.

(B)

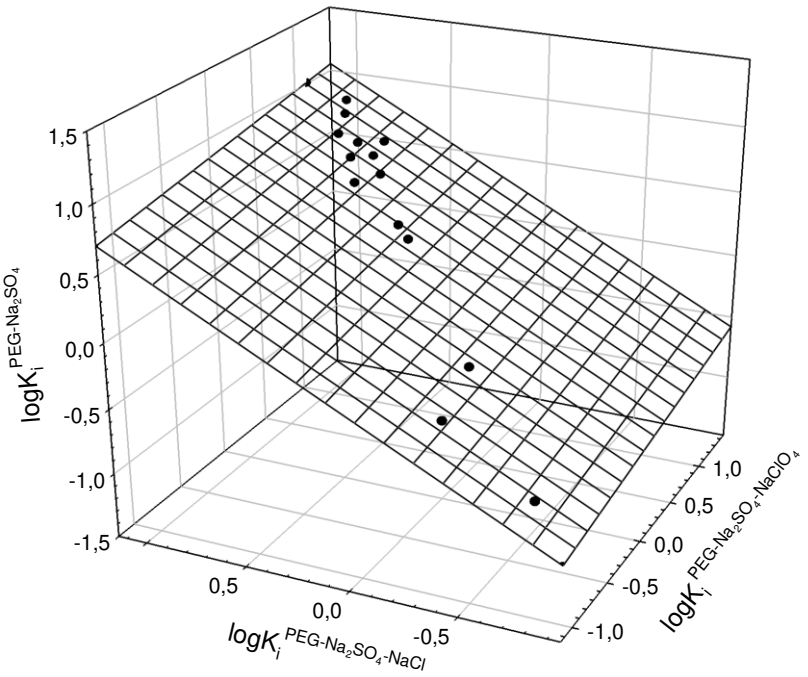


Figure 7.4. (cont.) (B) Interrelationship between logarithms of partition coefficients for organic compounds and proteins in PEG–Na_2SO_4 ATPS, logarithms of partition coefficients for the same compounds in PEG–Na_2SO_4–0.215 M NaCl ATPS and in PEG–Na_2SO_4–0.215 M $NaClO_4$ ATPS.

Analysis of the solute-specific coefficients for all organic compounds (including Na-salts of DNP-amino acids) examined in the Na_2SO_4-free aqueous media (i.e., in PEG–DEX ATPSs) shows the linear interrelationship illustrated graphically in Fig. 7.5 and described as:

$$C_s^i = -2.30_{\pm 0.30} - 0.45_{\pm 0.07} S_s^i - 1.26_{\pm 0.06} B_s^i$$

N = 48; r^2 = 0.9444; SD = 1.65; F = 382

(Equation 7.6a)

where all parameters are as defined above. It should be emphasized that the solute-specific coefficients for proteins do not fit the relationship.

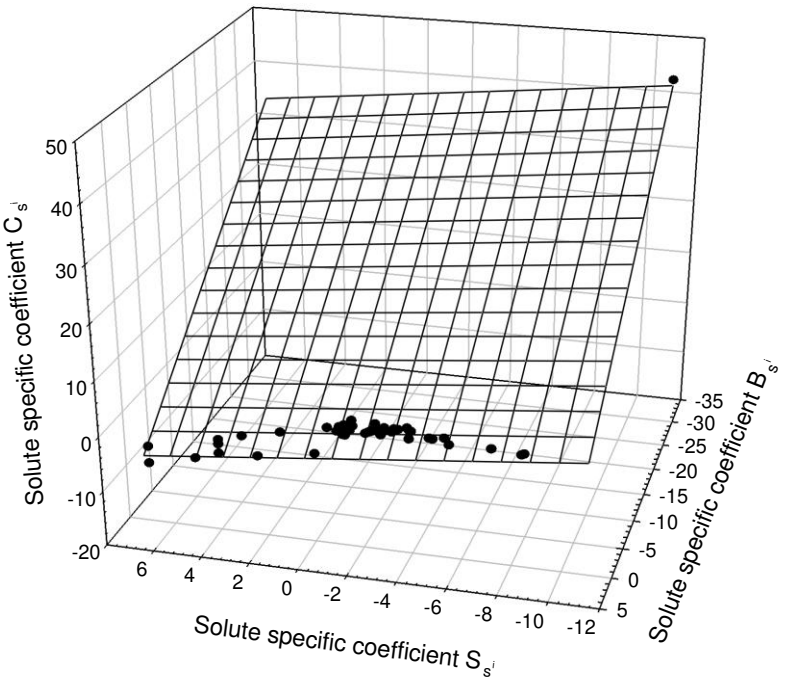


Figure 7.5. Interrelationship between solute-specific coefficients C_s^i, B_s^i, and S_s^i for organic compounds calculated using Eq. 7.4 from the partition coefficients of the compounds in PEG–DEX ATPSs with different salt additives (see data in Table S7.4).

For solute-specific coefficients for the same compounds and certain proteins in aqueous media containing ~0.45 M Na_2SO_4 (i.e. in PEG–Na_2SO_4 ATPSs) is observed the similar linear interrelationship shown graphically in Fig. 7.6 and described as:

$$C_s^i = 0.76_{\pm 0.08} - 0.20_{\pm 0.02} S_s^i + 0.73_{\pm 0.03} B_s^i$$

N = 41; r^2 = 0.9413; SD = 0.49; F = 305

(Equation 7.6b)

where all parameters are as defined above.

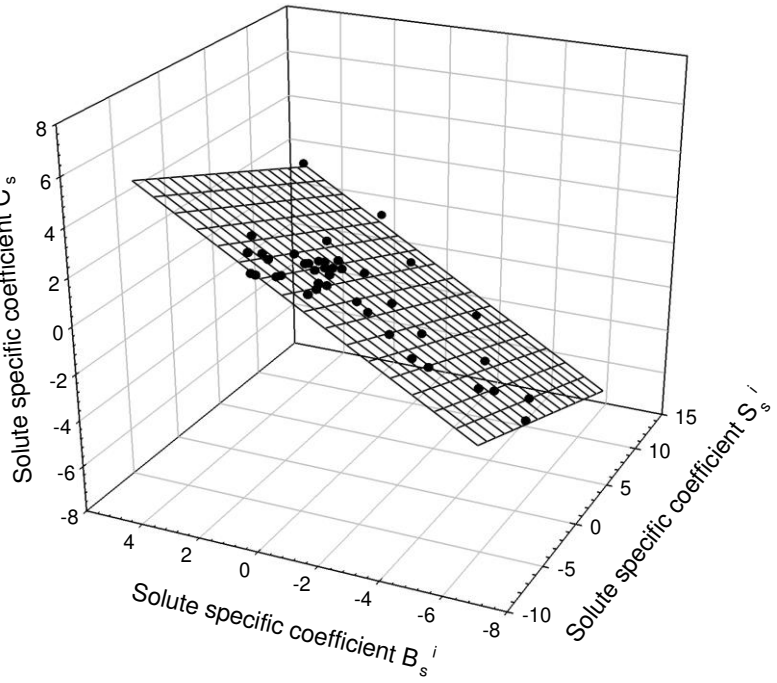


Figure 7.6. Interrelationship between solute-specific coefficients C_s^i, B_s^i, and S_s^i for organic compounds and proteins calculated using Eq. 7.4 from the partition coefficients of the compounds in PEG–Na_2SO_4 ATPSs with different salt additives (see data in Table S7.4).

It should be noted that there are 10 outliers (Na-salts of DNP-Nval, DNP-NIeu, and DNP-AO, *p*-nitrophenyl-α-D-glucopyranoside, α-chymotrypsin, α-chymotrypsinogen A, concanavalin A, and lipase (all in the presence of $NaClO_4$), and chymotrypsin and vanillin in the presence of NaCl. It should be noted that the direct interactions of salt additives with these compounds may be the reason for their solute-specific coefficients not fitting the relationship.

The number of compounds with determined solute-specific coefficients so far prevents one from drawing any general conclusion, and this issue is beyond the scope of the present study.

Finally, we examined the range of the partition coefficients values determined in the two types of ATPSs (PEG–DEX and PEG–Na_2SO_4). The highest (K_{max}) and lowest (K_{min}) K-values for each compound determined in the PEG–DEX ATPSs and in the PEG–Na_2SO_4 ATPSs with all different salt and osmolyte additives are listed in Table 7.8. The differences between these values (ΔK) represent the range of K-vales for a given compound in a given type of ATPSs.

Table 7. 8. The overall range of partition coefficients for examined compounds in PEG–DEX–0.01 M K/NaPB and PEG–Na_2SO_4–0.01 M NaPB ATPSs with salt and osmolyte additives*.

	PEG–DEX ATPSs				PEG–Na_2SO_4 ATPSs			
COMPOUND	**K_{min}**	**Additives**	**K_{max}**	**Additives**	**K_{min}**	**Additives**	**K_{max}**	**Additives**
DNP-Ala Na	0.96	$NaClO_4$	1.44	w/o salt, trehalose	3.49	$NaClO_4$, trehalose	6.88	w/o salt, trehalose
DNP-NVal Na	1.03	$NaClO_4$	1.62	w/o salt, trehalose	4.73	w/o salt	10.7	w/o salt, trehalose
DNP-NLeu Na	1.09	$NaClO_4$	1.77	w/o salt, trehalose	6.00	w/o salt	15.0	w/o salt, trehalose
DNP-AO Na	1.25	$NaClO_4$	2.15	w/o salt, trehalose	10.3	w/o salt	32.1	w/o salt, trehalose
Benzyl alcohol	1.41	w/o salt	1.86	$NaClO_4$, TMAO[c]	3.50	w/o salt	7.99	$NaClO_4$, sorbitol
Caffeine	1.15	w/o salt[a]	1.43	$NaClO_4$, TMAO[c]	1.85	w/o salt	3.50	$NaClO_4$, sorbitol
Coumarin	1.49	w/o salt	2.45	$NaClO_4$, TMAO[c]	4.55	w/o salt, sorbitol	15.2	$NaClO_4$, sorbitol
Glucoside	1.23	w/o salt	2.23	NaCl, sucrose	2.18	w/o salt	4.15	$NaClO_4$, sorbitol
Methyl anthranilate	1.77	w/o salt	3.10	$NaClO_4$, TMAO[c]	7.28	w/o salt	29.5	$NaClO_4$, sorbitol
2-Phenylethanol	1.47	w/o salt	2.03	$NaClO_4$, TMAO[c]	3.80	w/o salt, sorbitol	9.67	$NaClO_4$, TMAO
Phenol	1.23	$NaClO_4$, sorbitol	2.40	$NaClO_4$, TMAO	4.60	w/o salt	12.4	$NaClO_4$, TMAO
Vanillin	1.59	$NaClO_4$, sucrose	2.11	w/o salt, trehalose	5.79	w/o salt[b]	19.1	$NaClO_4$, sorbitol

Table 7.8. (cont.) The overall range of partition coefficients for examined compounds in PEG–DEX–0.01 M K/NaPB and PEG–Na_2SO_4–0.01 M NaPB ATPSs with salt and osmolyte additives*.

	PEG–DEX ATPSs				PEG–Na_2SO_4 ATPSs			
PROTEIN	**K_{min}**	**Additives**	**K_{max}**	**Additives**	**K_{min}**	**Additives**	**K_{max}**	**Additives**
α-chymotrypsin	0.41	w/o salt, trehalose	5.18	NaCl, sucrose	0.027	w/o salt, trehalose	0.117	w/o salt
α-chymotrypsinogen A	1.00	w/o salt	7.45	$NaClO_4$, TMAO[c]	0.120	w/o salt, trehalose	0.60	NaCl[b]
Concanavalin A	0.21	w/o salt, trehalose	1.54	NaCl, TMAO	0.116	w/o salt, trehalose	0.195	w/o salt[b]
Lipase	0.67	$NaClO_4$, TMAO	0.85	$NaClO_4$, TMAO	0.47	$NaClO_4$, TMAO	0.64	w/o salt[b]
Lysozyme	0.23	w/o salt	49.1	$NaClO_4$, TMAO	0.045	w/o salt, trehalose	43.3	$NaClO_4$, sorbitol

* Salt additives concentrations – 0.215 M; osmolyte additives concentrations – 0.5 M except for TMAO where indicated

a – Same K-value in PEG–DEX with NaCl additive

b – PEG10K–Na_2SO_4 ATPS

c – TMAO at concentration 1.5 M

These differences observed in PEG–Na_2SO_4 ATPSs are plotted against those observed in PEG–DEX ATPSs for all studied compounds (except lysozyme) in Fig. 7.7.

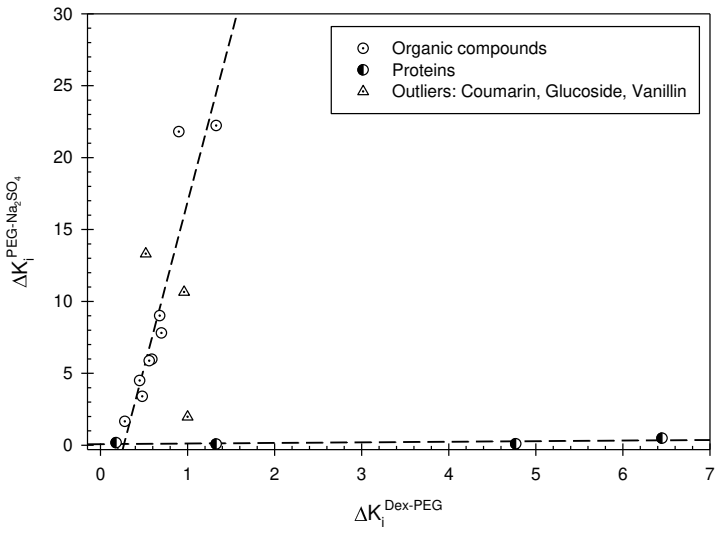


Figure 7.7. The ranges of K-values variability for organic compounds and proteins in PEG–Na_2SO_4 ATPSs with different salt additives, $\Delta K_i^{PEG\text{-}Na2SO4}$, versus ranges of K-values variability for the same compounds and proteins in PEG–DEX ATPSs with different salt additives, $\Delta K_i^{DEX\text{-}PEG}$.

The data plotted in Fig. 7.7 show that for small organic compounds, the ranges of K-values observed in PEG–Na_2SO_4 ATPSs exceed those determined in PEG–DEX ATPSs quite significantly (the slope of the linear curve in the plot is ca. 23), while for proteins the range of K-values in PEG–DEX ATPSs exceed those in PEG–Na_2SO_4 ATPSs for three proteins (α-chymotrypsin, α-chymotrypsinogen A, and concanavalin A) or are very similar (for lipase and lysozyme). This observation confirms the suggestion that the ATPSs formed by two polymers are more useful for protein analysis in comparison with the ATPSs formed by a single polymer and a salt. The ATPSs of the latter type have an advantage for protein isolation/separation.

References

[1] T. J. Peters, "Partition of cell particles and macromolecules: Separation and purification of biomolecules, cell organelles, membranes and cells in aqueous polymer two phase systems and their use in biochemical analysis and biotechnology. P-A. Albertsson. Third Edition," *Cell Biochem. Funct.*, vol. 5, no. 3, pp. 233–234, 1987.

[2] H. Walter, *Partitioning In Aqueous Two – Phase System: Theory, Methods, Uses, and Applications To Biotechnology*. Elsevier Science, 2012.

[3] H. HUSTEDT, K. H. KRONER, and M.-R. KULA, "15 - Applications of Phase Partitioning in Biotechnology," H. WALTER, D. E. BROOKS, and D. B. T.-P. in A. T.-P. S. FISHER, Eds. Academic Press, 1985, pp. 529–587.

[4] H.-K. Rajni, *Aqueous Two-Phase Systems: Methods and Protocols*, 1st ed. Totowa, NJ: Humana Press, 2000.

[5] B. Y. Zaslavsky, *Aqueous Two-Phase Partitioning: Physical Chemistry and Bioanalytical Applications*. Taylor & Francis, 1994.

[6] H. Everberg, T. Leiding, A. Schiöth, F. Tjerneld, and N. Gustavsson, "Efficient and non-denaturing membrane solubilization combined with enrichment of membrane protein complexes by detergent/polymer aqueous two-phase partitioning for proteome analysis," *J. Chromatogr. A*, vol. 1122, no. 1, pp. 35–46, 2006.

[7] U. Sivars and F. Tjerneld, "Mechanisms of phase behaviour and protein partitioning in detergent/polymer aqueous two-phase systems for purification of integral membrane proteins11This work was carried out in the Swedish Center for Bioseparation.," *Biochim. Biophys. Acta - Gen. Subj.*, vol. 1474, no. 2, pp. 133–146, 2000.

[8] J.-X. Xiao, U. Sivars, and F. Tjerneld, "Phase behavior and protein partitioning in aqueous two-phase systems of cationic–anionic surfactant mixtures," *J. Chromatogr. B Biomed. Sci. Appl.*, vol. 743, no. 1, pp. 327–338, 2000.

[9] S. Oppermann, F. Stein, and U. Kragl, "Ionic liquids for two-phase systems and their application for purification, extraction and biocatalysis," *Appl. Microbiol. Biotechnol.*, vol. 89, no. 3, pp. 493–499, 2011.

[10] C. M. S. S. Neves, A. M. S. Silva, A. M. Fernandes, J. A. P. Coutinho, and M. G. Freire, "Toward an Understanding of the Mechanisms behind the Formation of Liquid–liquid Systems formed by Two Ionic Liquids," *J. Phys. Chem. Lett.*, vol. 8, no. 13, pp. 3015–3019, Jul. 2017.

[11] F. A. Vicente *et al.*, "Impact of Surface Active Ionic Liquids on the Cloud Points of Nonionic

Surfactants and the Formation of Aqueous Micellar Two-Phase Systems," *J. Phys. Chem. B*, vol. 121, no. 37, pp. 8742–8755, Sep. 2017.

[12] A. Zaslavsky, N. Gulyaeva, A. Chait, and B. Zaslavsky, "A new method for analysis of components in a mixture without preseparation: evaluation of the concentration ratio and protein-protein interaction.," *Anal. Biochem.*, vol. 296, no. 2, pp. 262–269, Sep. 2001.

[13] A. Zaslavsky, P. Madeira, L. Breydo, V. N. Uversky, A. Chait, and B. Zaslavsky, "High throughput characterization of structural differences between closely related proteins in solution," *Biochim. Biophys. Acta - Proteins Proteomics*, vol. 1834, no. 2, pp. 583–592, Feb. 2013.

[14] B. Y. Zaslavsky, V. N. Uversky, and A. Chait, "Analytical applications of partitioning in aqueous two-phase systems: Exploring protein structural changes and protein-partner interactions in vitro and in vivo by solvent interaction analysis method.," *Biochim. Biophys. Acta*, vol. 1864, no. 5, pp. 622–644, May 2016.

[15] B. Y. Zaslavsky, V. N. Uversky, and A. Chait, "Solvent interaction analysis as a proteomic approach to structure-based biomarker discovery and clinical diagnostics," *Expert Rev. Proteomics*, vol. 13, no. 1, pp. 9–17, Jan. 2016.

[16] E. A. Klein *et al.*, "The Single-parameter, Structure-based IsoPSA Assay Demonstrates Improved Diagnostic Accuracy for Detection of Any Prostate Cancer and High-grade Prostate Cancer Compared to a Concentration-based Assay of Total Prostate-specific Antigen: A Preliminary Repo," *Eur. Urol.*, vol. 72, no. 6, pp. 942–949, Dec. 2017.

[17] L. A. Ferreira, P. P. Madeira, L. Breydo, C. Reichardt, V. N. Uversky, and B. Y. Zaslavsky, "Role of solvent properties of aqueous media in macromolecular crowding effects," *J. Biomol. Struct. Dyn.*, vol. 34, no. 1, pp. 92–103, Jan. 2016.

[18] L. A. Ferreira, V. N. Uversky, and B. Y. Zaslavsky, "Role of solvent properties of water in crowding effects induced by macromolecular agents and osmolytes," *Mol. BioSyst.*, vol. 13, no. 12, pp. 2551–2563, 2017.

[19] C. Reichardt, E. Harbusch-Görnert, and G. SchWäfer, "Über Pyridinium-N-phenolat-Betaine und ihre Verwendung zur Charakterisierung der Polarität von Lösungsmitteln, XI. Herstellung und UV/VIS-spektroskopische Eigenschaften eines wasserlöslichen Carboxylat-substituierten Pyridinium-N-phenolat-Betainfarbstoffs," *Liebigs Ann. der Chemie*, vol. 1988, no. 9, pp. 839–844, Sep. 1988.

[20] L. A. Ferreira, P. P. Madeira, V. N. Uversky, and B. Y. Zaslavsky, "Analyzing the effects of protecting osmolytes on solute–water interactions by solvatochromic comparison method: I. Small organic compounds," *RSC Adv.*, vol. 5, no. 74, pp. 59812–59822, 2015.

[21] N. R. da Silva, L. A. Ferreira, P. P. Madeira, J. A. Teixeira, V. N. Uversky, and B. Y. Zaslavsky,

"Effect of sodium chloride on solute–solvent interactions in aqueous polyethylene glycol–sodium sulfate two-phase systems," *J. Chromatogr. A*, vol. 1425, pp. 51–61, 2015.

[22] L. A. Ferreira, O. Fedotoff, V. N. Uversky, and B. Y. Zaslavsky, "Effects of osmolytes on protein–solvent interactions in crowded environments: study of sucrose and trehalose effects on different proteins by solvent interaction analysis," *RSC Adv.*, vol. 5, no. 34, pp. 27154–27162, 2015.

[23] N. R. da Silva, L. A. Ferreira, P. P. Madeira, J. A. Teixeira, V. N. Uversky, and B. Y. Zaslavsky, "Analysis of partitioning of organic compounds and proteins in aqueous polyethylene glycol-sodium sulfate aqueous two-phase systems in terms of solute–solvent interactions," *J. Chromatogr. A*, vol. 1415, pp. 1–10, 2015.

[24] P. P. Madeira, J. A. Teixeira, E. A. Macedo, L. M. Mikheeva, and B. Y. Zaslavsky, "'On the Collander equation': Protein partitioning in polymer/polymer aqueous two-phase systems," *J. Chromatogr. A*, vol. 1190, no. 1–2, pp. 39–43, May 2008.

[25] P. P. Madeira *et al.*, "Solvatochromic relationship: prediction of distribution of ionic solutes in aqueous two-phase systems," *J. Chromatogr. A*, vol. 1271, no. 1, pp. 10–16, 2013.

[26] P. P. Madeira *et al.*, "Study of organic compounds–water interactions by partition in aqueous two-phase systems," *J. Chromatogr. A*, vol. 1322, pp. 97–104, 2013.

[27] L. A. Ferreira, P. P. Madeira, A. V Uversky, V. N. Uversky, and B. Y. Zaslavsky, "Responses of proteins to different ionic environment are linearly interrelated.," *J. Chromatogr. A*, vol. 1387, pp. 32–41, Mar. 2015.

[28] P. P. Madeira, A. Bessa, J. A. Loureiro, L. Álvares-Ribeiro, A. E. Rodrigues, and B. Y. Zaslavsky, "Cooperativity between various types of polar solute-solvent interactions in aqueous media," *J. Chromatogr. A*, vol. 1408, p. 108—117, 2015.

[29] L. A. Ferreira, X. Fan, P. P. Madeira, L. Kurgan, V. N. Uversky, and B. Y. Zaslavsky, "Analyzing the effects of protecting osmolytes on solute–water interactions by solvatochromic comparison method: II. Globular proteins," *RSC Adv.*, vol. 5, no. 73, pp. 59780–59791, 2015.

[30] J. G. Huddleston, H. D. Willauer, and R. D. Rogers, "The solvatochromic properties, α, β, and π^*, of PEG-salt aqueous biphasic systems," *Phys. Chem. Chem. Phys.*, vol. 4, no. 16, pp. 4065–4070, 2002.

[31] Y. Marcus, "The properties of organic liquids that are relevant to their use as solvating solvents," *Chem. Soc. Rev.*, vol. 22, no. 6, pp. 409–416, 1993.

Supplementary Information

A. Analysis of hydrophobic and electrostatic properties of the phases

The difference between the hydrophobic and electrostatic properties of the coexisting phases was determined in each ATPS by partitioning of a homologous series of sodium salts of dinitrophenylated (DNP-) amino acids with the aliphatic alkyl side-chains of the increasing length (alanine, norvaline, norleucine, and α-amino-*n*-octanoic acid). Partition coefficients of these compounds are presented graphically in Fig. S7.1-S7.3, where the logarithms of their partition coefficients are plotted against the length of the side-chain expressed in equivalent number of methylene groups, N_c. The N_c values for the DNP-amino acids used are: DNP-Alanine Na – 1.31; DNP-norvaline Na – 2.65; DNP-norleucine Na – 3.75; and DNP-α-amino-*n*-octanoic acid Na – 6.30.

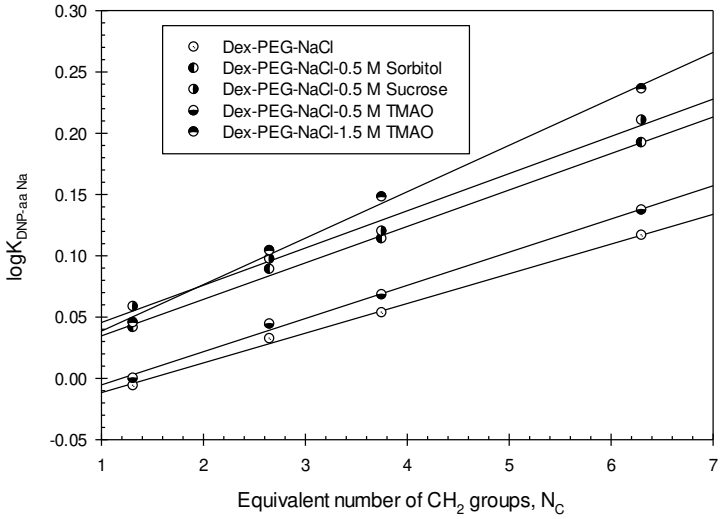


Figure S7.1. Logarithm of the partition coefficient value, $logK_{DNP\text{-}AA}$, for sodium salts of DNP-AA with aliphatic side-chains in 12 wt.% DEX – 6 wt.% PEG8K – 0.215 M NaCl – 0.01 M K/NaPB, pH 7.4 and 12 wt.% DEX – 6 wt.% PEG8K – 0.215 M NaCl – osmolyte – 0.01 M K/NaPB, pH 7.4 ATPSs as a function of equivalent length of the side-chain, N_c. Data from Table 7.1.

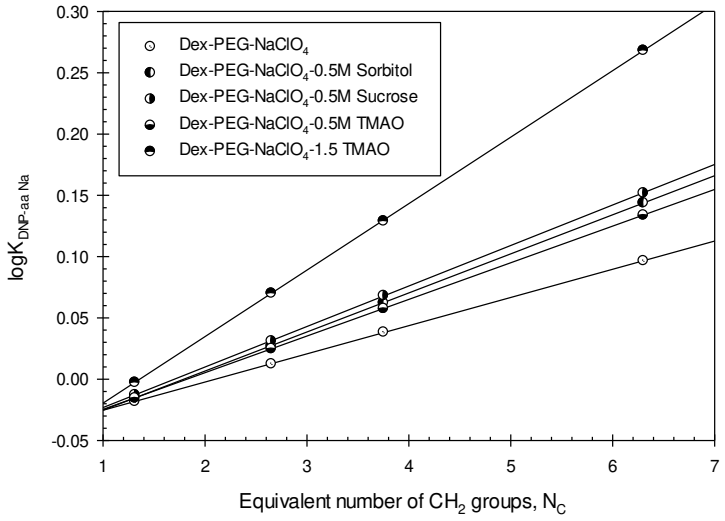


Figure S7.2. Logarithm of the partition coefficient value, $logK_{DNP\text{-}AA}$, for sodium salts of DNP-AA with aliphatic side-chains in 12 wt.% DEX – 6 wt.% PEG8K – 0.215 M $NaClO_4$ – 0.01 M K/NaPB, pH 7.4 and 12 wt.% DEX – 6 wt.% PEG8K – 0.215 M $NaClO_4$ – osmolyte – 0.01 M K/NaPB, pH 7.4 ATPSs as a function of equivalent length of the side-chain, N_C. Data from Table 7.2.

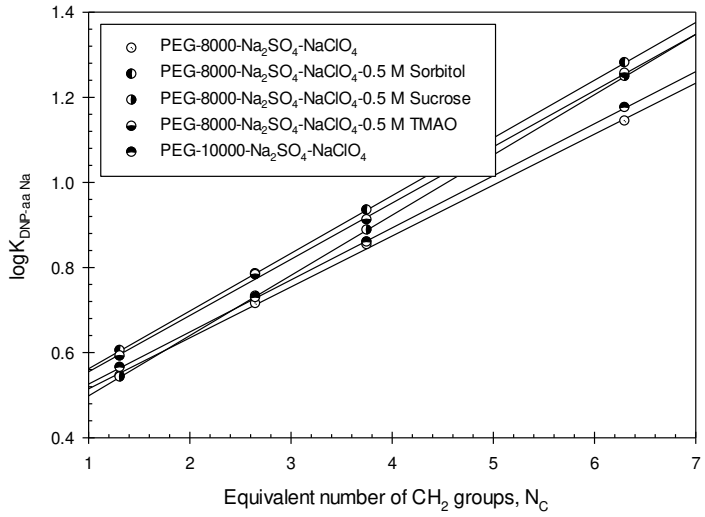


Figure S7.3. Logarithm of the partition coefficient value, $logK_{DNP\text{-}AA}$, for sodium salts of DNP-AA with aliphatic side-chains in 11.10 wt.% PEG(8K or 10K) – 6.33 wt.% Na_2SO_4 – 0.215 M $NaClO_4$ – 0.01 M NaPB, pH 6.8 and 11.10 wt.% PEG8K – 6.33 wt.% Na_2SO_4 – 0.215 M $NaClO_4$ – 0.5 M osmolyte – 0.01 M NaPB, pH 6.8 ATPSs as a function of equivalent length of the side-chain, N_C. Data from Table 7.3.

It can be seen in Fig. S7.1-S7.3 that the data in each ATPS may be described as [5], [20], [21]:

$$\log K_{DNP-AA}^{(i)} = C^{(i)} + E^{(i)} \times N_C$$

(Equation S7.1)

where K_{DNP-AA} is the partition coefficient of a DNP-amino acids Na-salt; N_c is the equivalent number of CH_2 groups in the side-chain, E and C are constants for a given i-th ATPS characterizing the difference between the relative hydrophobicity and electrostatic properties of the phases correspondingly.

As the standard free energy of transfer of a solute from the bottom phase to the top phase is described as:

$$\Delta G^0 = - RT \ln K$$

(Equation S7.2)

where R is the universal gas constant and T is the absolute temperature in Kelvin, it follows that

$$\Delta G^0(CH_2) = - RTE^*$$

(Equation S7.3)

where E^* is parameter E expressed in natural logarithm units; $\Delta G^0(CH_2)$ is the standard free energy of transfer of a methylene group from one phase to another. The $\Delta G^0(CH_2)$ values calculated from the experimental data with Eqs. S7.2 and S7.3 are listed in Tables S7.1-S7.3.

Table S7.1. Partition coefficients for organic compounds and proteins in 12 %wt. DEX – 6% wt. PEG8K – 0.01 M K/NaPB, pH 7.4 and 12 %wt. DEX – 6 %wt. PEG8K – 0.5 M osmolyte – 0.01 M K/NaPB, pH 7.4 ATPSs. Data from [20], [29].

	Partition coefficients				
COMPOUND	**w/o Osmolyte**	**0.5 M Sorbitol**	**0.5 M Sucrose**	**0.5 M TMAO**	**0.5 M Trehalose**
DNP-Ala Na	1.285±0.009	1.368±0.011	1.400±0.008	1.336±0.028	1.439±0.008
DNP-NVal Na	1.389±0.003	1.489±0.020	1.543±0.045	1.454±0.043	1.620±0.048
DNP-NLeu Na	1.445±0.008	1.627±0.033	1.661±0.029	1.562±0.010	1.765±0.037
DNP-AO Na	1.645±0.012	1.950±0.040	1.967±0.040	1.882±0.010	2.153±0.035
Benzyl alcohol	1.409±0.009	1.521±0.009	1.607±0.009	1.454±0.008	1.697±0.007
Caffeine	1.154±0.009	1.178±0.003	1.160±0.004	1.147±0.008	1.186±0.006
Coumarin	1.611±0.003	1.742±0.003	1.777±0.004	1.660±0.002	1.923±0.006
Glucoside	1.232±0.003	1.310±0.002	1.332±0.009	1.246±0.003	1.368±0.001
Methyl anthranilate	1.770±0.010	2.035±0.005	2.124±0.007	1.847±0.007	2.240±0.011
2-Phenylethanol	1.469±0.005	1.602±0.008	1.695±0.009	1.510±0.010	1.697±0.009
Phenol	1.700±0.022	2.008±0.006	2.070±0.017	1.809±0.009	2.211±0.009
Vanillin	1.709±0.009	1.823±0.009	1.969±0.005	1.761±0.005	2.105±0.006

Table S7.1. (cont.) Partition coefficients for organic compounds and proteins in 12 %wt. DEX – 6% wt. PEG8K – 0.01 M K/NaPB, pH 7.4 and 12 %wt. DEX – 6 %wt. PEG8K – 0.5 M osmolyte – 0.01 M K/NaPB, pH 7.4 ATPSs. Data from [20], [29].

	Partition coefficients				
PROTEIN	**w/o Osmolyte**	**0.5 M Sorbitol**	**0.5 M Sucrose**	**0.5 M TMAO**	**0.5 M Trehalose**
α-chymotrypsin	0.420±0.014	0.427±0.008	0.420±0.014	0.420±0.011	0.410±0.011
α-chymotrypsinogen A	1.000±0.011	1.500±0.014	1.780±0.024	1.370±0.023	1.930±0.010
Concanavalin A	0.236±0.003	0.237±0.003	0.242±0.003	0.233±0.004	0.226±0.003
Lipase	0.793±0.004	0.829±0.003	0.846±0.003	0.769±0.004	0.787±0.003
Lysozyme	0.230±0.003	0.331±0.004	0.325±0.004	0.255±0.009	0.318±0.002

B. Solute-specific coefficients determined for the indicated compounds in the presence of various ionic compositions (K/NaPB – 0.01 M sodium/potassium phosphate buffer, pH 7.4; NaPB – 0.01 M sodium phosphate buffer, pH 6.8)

Table S7.2. Solute-specific coefficients determined for the indicated compounds in the presence of various ionic compositions (0.01 M K/NaPB, pH 7.4; 0.01 M NaPB, pH 6.8)*.

COMPOUND	ATPS	S_s	A_s	B_s	C_s
DNP-Ala Na	0.01M K/NaPB[a]	-1.06±0.02	-0.77±0.06	-0.88±0.02	0.42±0.02
	0.15 M NaCl[b]	-0.47±0.05	0.4±0.1	-0.4±0.03	1.01±0.02
	0.215 M NaCl	-1.00±0.01	-0.18±0.01	0	1.19±0.03
	0.215 M $NaClO_4$	1.4±0.4	3.0±1.3	0	-0.4±0.1
	0.45 M Na_2SO_4+0.01M NaPB[c]	-0.50±0.14	0	0.52±0.08	1.39±0.03
	0.45 M Na_2SO_4+0.215 M NaCl[d]	-0.70±0.26	0	0	1.26±0.02
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	-0.42±0.07	1.6±0.19	-0.24±0.02	1.21±0.01
DNP-NVal Na	0.01M K/NaPB[a]	-1.40±0.08	-1.2±0.3	-1.11±0.08	0.59±0.09
	0.15 M NaCl[b]	-0.9±0.1	0.9±0.3	-0.82±0.05	0.99±0.03
	0.215 M NaCl	-2.09±0.04	-0.47±0.03	0	1.16±0.06
	0.215 M $NaClO_4$	3.0±0.7	7±2.3	0	-1.9±0.3
	0.45 M Na_2SO_4+0.01M NaPB[c]	1.5±0.31	0	0.80±0.18	1.67±0.06
	0.45 M Na_2SO_4+0.215 M NaCl[d]	-1.6±0.74	0	0	1.76±0.07
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	-0.9±0.27	2.1±0.71	-0.53±0.09	1.45±0.05

Table S7.2. (cont.) Solute-specific coefficients determined for the indicated compounds in the presence of various ionic compositions (0.01 M K/NaPB, pH 7.4; 0.01 M NaPB, pH 6.8)*.

COMPOUND	ATPS	S_s	A_s	B_s	C_s
DNP-NLeu Na	0.01M K/NaPB	-1.2±0.1	-1.4±0.3	-1.2±0.1	0.9±0.1
	0.15 M NaCl	-1.4±0.2	1.0±0.4	-1.14±0.09	1.06±0.05
	0.215 M NaCl	-2.71±0.05	-0.73±0.04	0	-1.19±0.08
	0.215 M $NaClO_4$	4±1	10±3.3	0	-3.1±0.4
	0.45 M Na_2SO_4+0.01M NaPB	-1.7±0.56	0	1.0±0.3	1.9±0.1
	0.45 M Na_2SO_4+0.215 M NaCl	-1.6±0.58	0	0	1.51±0.05
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	-1.8±0.23	2.2±0.61	-0.61±0.08	1.67±0.04
DNP-AO Na	0.01M K/NaPB	-1.2±0.3	0	-2.2±0.2	1.0±0.2
	0.15 M NaCl	-2.2±0.2	1.8±0.6	-2.0±0.1	1.06±0.07
	0.215 M NaCl	-4.78±0.06	-1.07±0.04	0	1.49±0.09
	0.215 M $NaClO_4$	7±1.7	16±5.6	0	-5.9±0.6
	0.45 M Na_2SO_4+0.01M NaPB	-2.8±0.73	0	2.1±0.44	2.7±0.14
	0.45 M Na_2SO_4+0.215 M NaCl	-3.5±1.3	0	0	2.2±0.12
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	-2.3±0.47	6±1.2	-1.2±0.16	2.06±0.09

Table S7.2. (cont.) Solute-specific coefficients determined for the indicated compounds in the presence of various ionic compositions (0.01 M K/NaPB, pH 7.4; 0.01 M NaPB, pH 6.8)*.

COMPOUND	ATPS	S_s	A_s	B_s	C_s
Benzyl alcohol	0.01M K/NaPB	-2.0±0.1	0	-1.39±0.08	0
	0.15 M NaCl	-2.4±0.2	0	-1.6±0.1	0
	0.215 M NaCl	-3.7±0.6	-0.67±0.09	-1.0±0.32	0
	0.215 M $NaClO_4$	-2.6±0.55	0	-2.0±0.25	0
	0.45 M Na_2SO_4+0.01M NaPB	-2.9±0.55	0	1.1±0.33	1.3±0.1
	0.45 M Na_2SO_4+0.215 M NaCl	0	5.4±0.57	-5.2±0.2	-2.91±0.09
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	-2.8±0.39	0	0	1.73±0.04
Caffeine	0.01M K/NaPB	-1.1±0.1	-1.3±0.3	-0.45±0.04	0
	0.15 M NaCl	-0.8±0.1	0	-0.8±0.1	0.13±0.03
	0.215 M NaCl	-1.2±0.24	-0.75±0.04	-0.4±0.14	0
	0.215 M $NaClO_4$	2.19±0.02	3.6±0.14	1.0±0.05	-4.2±0.09
	0.45 M Na_2SO_4+0.01M NaPB	0	0	0.3±0.1	0.72±0.05
	0.45 M Na_2SO_4+0.215 M NaCl	1.55±0.01	0	-6.01±0.01	-3.51±0.01
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	0	11±3.2	-1.8±0.32	0

Table S7.2. (cont.) Solute-specific coefficients determined for the indicated compounds in the presence of various ionic compositions (0.01 M K/NaPB, pH 7.4; 0.01 M NaPB, pH 6.8)*.

COMPOUND	ATPS	S_s	A_s	B_s	C_s
Coumarin	0.01M K/NaPB	-2.1±0.4	0	-1.6±0.3	0
	0.15 M NaCl	-2.2±0.2	0	-2.0±0.1	0.28±0.04
	0.215 M NaCl	-5.38±0.08	-1.28±0.01	-0.44±0.04	0.34±0.02
	0.215 M $NaClO_4$	5±2.2	17±7.2	0	-7.0±0.8
	0.45 M Na_2SO_4+0.01M NaPB	0	0	0	1.40±0.03
	0.45 M Na_2SO_4+0.215 M NaCl	-0.9±0.2	0	-4.9±0.25	-2.6±0.11
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	-3.9±0.8	0	0	2.29±0.09
Glucoside	0.01M K/NaPB	-1.21±0.03	-2.21±0.08	-0.28±0.02	0.68±0.02
	0.15 M NaCl	-1.3±0.1	0	-1.0±0.08	0.33±0.03
	0.215 M NaCl	-5.01±0.09	0.37±0.01	-1.86±0.05	0
	0.215 M $NaClO_4$	4±1.3	14±4.3	0	-3.9±0.5
	0.45 M Na_2SO_4+0.01M NaPB	-1.4±0.4	0	0	0.72±0.03
	0.45 M Na_2SO_4+0.215 M NaCl	-0.81±0.05	0	-6.42±0.06	-3.61±0.03
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	0	8.1±1.5	-0.7±0.26	0.8±0.15

Table S7.2. (cont.) Solute-specific coefficients determined for the indicated compounds in the presence of various ionic compositions (0.01 M K/NaPB, pH 7.4; 0.01 M NaPB, pH 6.8)*.

COMPOUND	ATPS	S_s	A_s	B_s	C_s
Methyl anthranilate	0.01M K/NaPB	-3.2±0.2	-4.0±0.4	-1.5±0.1	1.0±0.1
	0.15 M NaCl	-3.4±0.3	0	-2.7±0.2	0.23±0.06
	0.215 M NaCl	-8.4±0.07	-1.65±0.05	0	0.70±0.11
	0.215 M $NaClO_4$	7±2.8	26±9.3	0	-9±1.1
	0.45 M Na_2SO_4+0.01M NaPB	-3±0.82	0	1.7±0.49	2.1±0.16
	0.45 M Na_2SO_4+0.215 M NaCl	-1.1±0.2	0	-2.0±0.19	-0.96±0.08
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	-7.5±0.13	0	0.7±0.05	2.7±0.03
2-Phenylethanol	0.01M K/NaPB	-2.16±0.05	-2.1±0.1	-0.98±0.04	0.67±0.04
	0.15 M NaCl	-2.9±0.1	-1.1±0.4	-1.79±0.09	0.11±0.04
	0.215 M NaCl	-4.5±0.4	-0.86±0.06	-1.1±0.2	0.47±0.09
	0.215 M $NaClO_4$	4±1.4	18±4.5	0	-5.6±0.5
	0.45 M Na_2SO_4+0.01M NaPB	-2.4±0.87	0	0	1.09±0.07
	0.45 M Na_2SO_4+0.215 M NaCl	-1.5±0.34	0	-3.4±0.42	-1.9±0.19
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	-4.62±0.01	0	-0.57±0.003	1.57±0.002

Table S7.2. (cont.) Solute-specific coefficients determined for the indicated compounds in the presence of various ionic compositions (0.01 M K/NaPB, pH 7.4; 0.01 M NaPB, pH 6.8)*.

COMPOUND	ATPS	S_s	A_s	B_s	C_s
Phenol	0.01M K/NaPB	-2.9±0.4	0	-2.5±0.3	0
	0.15 M NaCl	-3.6±0.2	0	-2.4±0.1	0
	0.215 M NaCl	-8.3±0.1	-0.8±0.1	0	0.5±0.2
	0.215 M $NaClO_4$	-11±4.5	0	-32±11.8	44±18
	0.45 M Na_2SO_4+0.01M NaPB	-3.8±0.48	0	1±0.99	1.51±0.09
	0.45 M Na_2SO_4+0.215 M NaCl	-1.7±0.17	0	-2.9±0.21	-1.59±0.09
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	-5.7±0.002	0	-0.6±0.001	1.66±0.0004
Vanillin	0.01M K/NaPB	-2.5±0.3	0	-2.5±0.2	0
	0.15 M NaCl	-3.5±0.2	0	-2.6±0.1	-0.08±0.04
	0.215 M NaCl	-7.11±0.05	-0.7±0.04	0	0.95±0.08
	0.215 M $NaClO_4$	0	13±2.4	0	-3.6±0.1
	0.45 M Na_2SO_4+0.01M NaPB	-3±1.4	0	1.5±0.85	1.9±0.27
	0.45 M Na_2SO_4+0.215 M NaCl	-2.2±0.29	0	-1.5±0.35	-0.9±0.16
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	-6.7±0.67	0	0.8±0.27	2.4±0.15

Table S7.2. (cont.) Solute-specific coefficients determined for the indicated compounds in the presence of various ionic compositions (0.01 M K/NaPB, pH 7.4; 0.01 M NaPB, pH 6.8)*.

PROTEIN	ATPS	S_s	A_s	B_s	C_s
α-chymotrypsin	0.01M K/NaPB	6±0.02	0	5.88±0.02	3.01±0.02
	0.215 M NaCl	-13.1±0.36	1.5±0.25	0	13.9±0.6
	0.215 M $NaClO_4$	15±1.5	57±4.9	0	-8.3±0.6
	0.45 M Na_2SO_4+0.01M NaPB	9.9±1.1	33.2±7.7	-2.3±0.5	-3.2±0.3
	0.45 M Na_2SO_4+0.215 M NaCl	5±1.6	-15±5.6	0	-1.3±0.13
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	-0.87±0.01	-0.3±0.05	0.11±0.01	-1.97±0.004
α-chymotrypsinogen A	0.01M K/NaPB	5.05±0.03	-0.6±0.08	4.42±0.02	7.6±0.02
	0.215 M NaCl	-3.9±0.3	-5.3±0.2	0	-8.4±0.4
	0.215 M $NaClO_4$	18±1.8	62±5.5	11±1.9	-34±3
	0.45 M Na_2SO_4+0.01M NaPB	9.6±0.96	24±7.1	0	-1.2±0.21
	0.45 M Na_2SO_4+0.215 M NaCl	3.29±0.01	-10.6±0.03	0	0
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	-1.03±0.04	0	0	-1.93±0.05

Table S7.2. (cont.) Solute-specific coefficients determined for the indicated compounds in the presence of various ionic compositions (0.01 M K/NaPB, pH 7.4; 0.01 M NaPB, pH 6.8)*.

PROTEIN	ATPS	S_s	A_s	B_s	C_s
Concanavalin A	0.01M K/NaPB	9.8±0.3	0	10.1±0.2	5.1±0.2
	0.215 M NaCl	1.9±0.4	0.34±0.07	-2.4±0.2	-3.2±0.1
	0.215 M $NaClO_4$	25.6±0.8	68±5.5	0	0
	0.45 M Na_2SO_4+0.01M NaPB	2.4±1.1	0	-2.2±0.6	-1.9±0.2
	0.45 M Na_2SO_4+0.215 M NaCl	3.9±0.72	0	-4±0.87	-3.1±0.39
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	3.3±0.14	2.3±0.36	0.4±0.05	-1.7±0.03
Lipase	0.01M K/NaPB	1.2±0.5	0	2.1±0.4	1.0±0.4
	0.15 M NaCl	1.8±0.1	0.6±0.2	1.8±0.1	0.6±0.1
	0.215 M NaCl	3.5±0.2	0.43±0.03	-0.6±0.1	0
	0.215 M $NaClO_4$	6.4±0.2	26±1.2	0	0
	0.45 M Na_2SO_4+0.01M NaPB	0.8±0.28	0	-0.4±0.17	-0.48±0.05
	0.45 M Na_2SO_4+0.215 M NaCl	0.7±0.12	0	-0.8±0.14	-0.81±0.06
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	1.7±0.28	0	0.4±0.11	-0.41±0.06

Table S7.2. (cont.) Solute-specific coefficients determined for the indicated compounds in the presence of various ionic compositions (0.01 M K/NaPB, pH 7.4; 0.01 M NaPB, pH 6.8)*.

PROTEIN	ATPS	S_s	A_s	B_s	C_s
Lysozyme	0.01M K/NaPB	13±1	0	13±1	10±1
	0.15 M NaCl	-4.3±0.5	-3.7±0.9	-3.1±0.4	2.5±0.5
	0.215 M NaCl	-13.5±0.07	-2.21±0.05	0	3.2±0.1
	0.215 M $NaClO_4$	11.1±0.3	73.6±0.8	16±0.3	-48.9±0.
	0.45 M Na_2SO_4+0.01M NaPB	12±2.4	0	1.7±0.58	0
	0.45 M Na_2SO_4+0.215 M NaCl	11.6±0.25	20.4±0.85	4.5±0.18	1.91±0.09
	0.45 M Na_2SO_4+0.215 M $NaClO_4$	-7.1±0.38	-4±1	0.9±0.13	3.38±0.07

* – The polymer composition is known to be irrelevant for the solute-specific coefficients. It may be helpful to indicate that the presence of 0.45 M Na_2SO_4 denotes the solute-specific coefficients determined in PEG-Na_2SO_4 ATPSs

a – Data from [20], [29]

b – Data from [28]

c – Data from [23]

d – Data from [21]

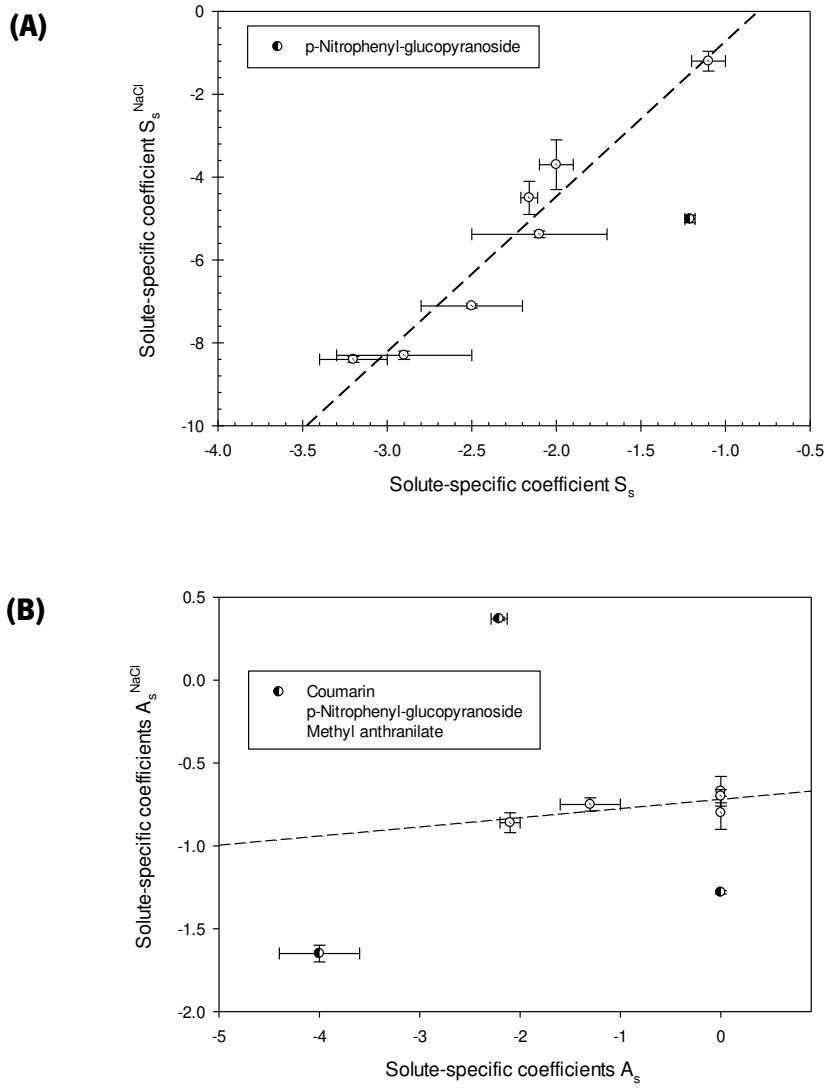
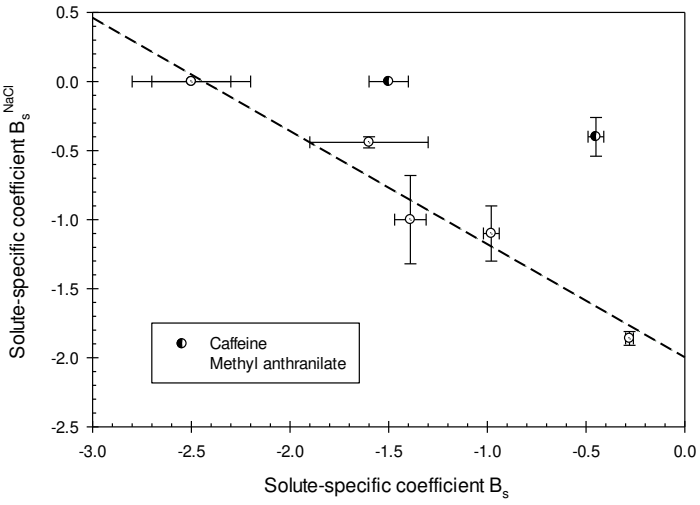


Figure S7.4. Solute-specific coefficients in the presence of 0.215 M NaCl for all organic compounds examine (except Na salts of DNP-AA) plotted against those for the same compounds in the absence of the NaCl additive. **(A)** Coefficient S_s. **(B)** Coefficient A_s.

(C)



(D)

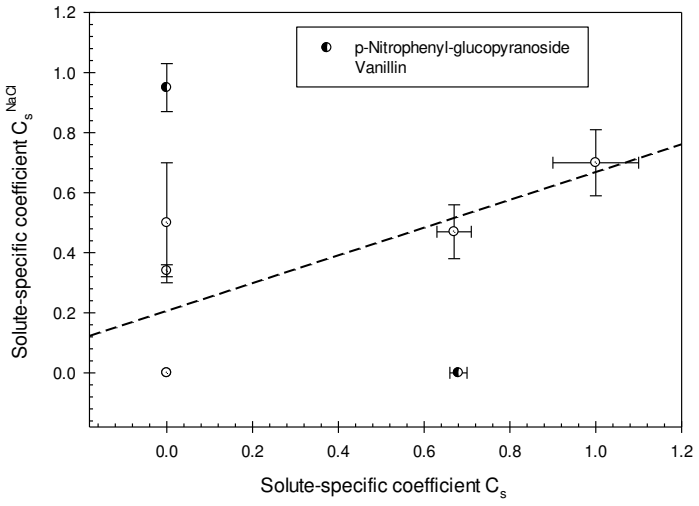


Figure S7.4. (cont.) Solute-specific coefficients in the presence of 0.215 M NaCl for all organic compounds examine (except Na salts of DNP-AA) plotted against those for the same compounds in the absence of the NaCl additive. **(C)** Coefficient B_s. **(D)** Coefficient C_s.

8.1. Main conclusions

The Aqueous Two-Phase Systems technology has been the object of intensive research in the last few decades. Indeed, their peculiar advantages, such as the innocuous and biocompatible environment for biomolecules, the low operational costs and the extreme sensitivity towards the surface properties of a diversity of solutes, make them attractive not only for separation purposes but mainly for bioanalytical applications.

Partition coefficients define the information provided by changes in the solute distribution behavior under altered partition conditions or as a response to a change in its properties. Thus, the understanding of the partitioning mechanisms is decisive for ATPSs effective utilization.

The major goal of this work was to add to the current understanding of solute partitioning in ATPSs. Thus, in Chapter 3, our results suggested that the effects of the salts additives on solute partitioning seem to be related to their influence on the water structure. These findings agree with the data previously reported on the effects of different salt additives on the solvent properties of aqueous media in different ATPSs formed by two nonionic polymers. It was shown before that the difference between the electrostatic properties of the coexisting phases may be described only considering the effect of a given salt additive on the hydrogen bonds in water. Similar effects were displayed with the salt additive concentration of ca. 0.025 to 0.1 M on the background of ~1 M Na_2SO_4 in the lower phase of the systems, which was rather surprising.

Chapter 4 presented the experimental results of the studies of partition behavior of small organic compounds and proteins in aqueous PEG–Na_2SO_4 two-phase system governed by solute-water interactions. Interestingly, the solute-solvent interactions of nonionic organic compounds and proteins in these systems differed from those in PEG–DEX systems. The experimental data obtained showed that the compound partition behavior in PEG–salt ATPSs does not depend on the molecular volume of the compound. The data obtained agree with the assumption that polar organic compounds and proteins respond to their environment in aqueous media by changing contributions of different types of solute-

water interactions in a solute- and ionic composition-specific manner. Both polar organic compounds and proteins demonstrate cooperativity of different types of solute-water interactions found to be linearly interrelated. Solvent properties of aqueous media in the coexisting phases of polymer–salt ATPSs may be quantified using the solvatochromic comparison method provided that the salt concentrations in both phases are below the level prohibiting solvatochromic measurements.

The results presented in Chapter 5 demonstrate that the addition of NaCl affected the partition behavior of small organic compounds and proteins in aqueous PEG–Na_2SO_4 two-phase systems through its influence on the solute-water interactions. The data obtained agree with the assumption that polar organic compounds and proteins respond to their environment in aqueous media by changing contributions of different types of solute-water interactions in the solute- and ionic composition-specific manner.

In Chapter 6, the data obtained were analyzed together with the results for other organic compounds and proteins reported previously, and the linear interrelationships between logarithms of partition coefficients in PEG–DEX, PEG–Na_2SO_4 and PEG–Na_2SO_4–0.215 M NaCl (all in 0.01 M K/Na- or Na-phosphate buffer, pH 7.4 or 6.8) were established. Similar relationship was found for the previously reported data for proteins in PEG–DEX, PEG600–Na_2SO_4, and PEG8K–Na_2SO_4 ATPSs. It was suggested that the linear relationships of the type established in ATPSs may be observed for biological properties of compounds as well. Also, it was concluded that the equation establishing linear interrelationships between logarithms of partition coefficients in 3 different systems seem to be rather universal. These correlations suggest that the properties and the mechanisms of phase separation in both types of polymer–polymer and polymer–salt ATPSs are of the same nature.

Chapter 7 presented the results obtained in the studies of solvent properties of aqueous PEG–DEX and PEG–Na_2SO_4 two-phase systems formed with (or without) 0.215 M NaCl and 0.215 M $NaClO_4$ in the presence (or absence) of 0.5 M sorbitol, sucrose, trehalose, and 0.5 M or 1.5 M trimethylamine N-oxide, showed that the solvent properties of the systems vary in a wide range. The differences between the solvent properties of the systems formed by polymer and salt exceed those measured in the systems formed by two polymers. The three most significant solvent properties of the systems are hydrophobic and electrostatic properties and hydrogen bonding donor acidity of the solvent media. Osmolyte additives were found to have quite significant effects on the differences between the electrostatic properties of the phases. Furthermore, analysis of the partition coefficients of 12 organic compounds and 5 proteins showed that osmolyte additives may affect the partition behavior of compounds in a compound-specific manner. The relative contributions of different types of interactions of a given compound with aqueous

media change in the presence of salt and osmolyte additives. Analysis of the ranges of partition coefficients variability in the systems utilized showed that for small organic compounds, the ranges of K-values observed in PEG–Na_2SO_4 ATPSs exceeded those determined in PEG–DEX ATPSs quite significantly. On the other hand, for proteins, the range of K-values in PEG–DEX ATPSs exceed those in PEG–Na_2SO_4 ATPSs for 3 proteins and were very similar for 2 proteins. Therefore, the ATPSs formed by two polymers can be more useful for protein analysis, while ATPSs formed by a single polymer and a salt have an advantage for protein isolation/separation.

Finally, it should be highlighted that it is more evident than ever, that ATPSs are extremely sensitive towards biomolecules surface properties. Additionally, this peculiar feature makes them a powerful tool with a high potential of application in several fields of biotechnology.

8.2. Future perspectives

ATPS partitioning was used in this work to investigate solute distribution between the coexisting aqueous phases of polymer–salt and polymer–polymer systems and to get new insights about the solute-solvent interactions that are involved in this phenomenon.

However, certain topics were not totally explored, and some new questions arouse, which would need a deeper investigation. Hence, some proposals for future work are presented ahead.

In Chapter 3, although the effects of the salt additives were shown to be related to their influence on the water structure, the limited number of salts additives examined prevents any general conclusion. Thus, it would be interesting increase the number of salts added to the system.

The results of Chapter 6 suggested that the linear relationships established in ATPSs may be observed for biological properties of compounds as well, however, we do not know yet the boundaries of its applicability to physicochemical and biological properties.

Of note, another limitation of our study is the reduced number of biomolecules (low molecular and proteins) used to create the interrelationships between the systems. It would be important to increase both the number of compounds partitioned in the systems and their variability in order to improve and better support the relationships obtained.

The establishment of the same type of correlations (Collander equation and LSER) and the analysis of the solute-solvent interactions in the same systems used but now containing other salt additives (e.g. NaSCN and NaH_2PO_4) and different salt-osmolyte combinations would be important too.

Since in this work the addition of different compounds to the systems were studied it would be interesting to evaluate the effect of new additives, such as ionic liquids, on the solvent properties of the systems and consequently on solute partitioning.

Moreover, it would be interesting to explore the solute-solvent interactions in different types of ATPSs (such as the so-called alternative ATPSs) and verify if solute partitioning is governed by the same mechanisms.

The Collander equation was initially proposed to describe solute partition in water-organic solvent systems, but this model has been effectively extended to correlate partition of unrelated compounds in two (or more) different ATPSs, supporting the idea that this model can be used to predict partitioning in ATPSs. Thus, it would be highly relevant to attempt to apply this empirical model to a real case scenario of ATPS partitioning, aiming the recovery of bioproducts from complex mixtures.

In summary, there is still a long journey to fully understand the mechanisms behind solute partitioning in ATPSs but work in this regard is currently in progress. Still, this work has added to the knowledge of solute partitioning and, corroborating previous studies, it has highlighted ATPSs partitioning as a powerful bioanalytical tool for different applications.

APPENDIX | Full papers and communications

A.1. Full papers in peer-reviewed international journals

The work developed during this PhD Thesis originated the following publications:

Nuno R. da Silva, Luisa A. Ferreira, Larissa M. Mikheeva, José A. Teixeira and Boris Y. Zaslavsky (2014). Origin of salt additive effect on solute partitioning in aqueous polyethylene glycol-8000-sodium sulfate two-phase system. *Journal of Chromatography A*, 1337, 3-8.

Nuno R. da Silva, Luisa A. Ferreira, Pedro P. Madeira, José A. Teixeira, Vladimir N. Uversky and Boris Y. Zaslavsky (2015). Analysis of partitioning of organic compounds and proteins in aqueous polyethylene glycol-sodium sulfate aqueous two-phase systems in terms of solute-solvent interactions. *Journal of Chromatography A*, 1415, 1-10.

Nuno R. da Silva, Luisa A. Ferreira, Pedro P. Madeira, José A. Teixeira, Vladimir N. Uversky and Boris Y. Zaslavsky (2015). Effect of sodium chloride on solute-solvent interactions in aqueous polyethylene glycol-sodium sulfate two-phase systems. *Journal of Chromatography A*, 1425, 51-61.

Luisa A. Ferreira, **Nuno R. da Silva**, Samarina R. Wlodarczyk, Joana A. Loureiro, Pedro P. Madeira, José A. Teixeira, Vladimir N. Uversky and Boris Y. Zaslavsky (2016). Interrelationship between partition behavior of organic compounds and proteins in aqueous dextran-polyethylene glycol and polyethylene glycol-sodium sulfate two-phase systems. *Journal of Chromatography A*, 1443, 21-25.

Nuno R. da Silva, Luisa A. Ferreira, José A. Teixeira, Vladimir N. Uversky and Boris Y. Zaslavsky (2019). Effects of sodium chloride and sodium perchlorate on properties and partition behavior of solutes in aqueous dextran-polyethylene glycol and polyethylene glycol-sodium sulfate two-phase systems. *Journal of Chromatography A*, 1583, 28-38.

A.2. Communications in conferences

The work developed during this PhD Thesis was presented in the following conference:

ORAL COMMUNICATION

Nuno R. da Silva, Pedro A. Madeira, José A. Teixeira and Boris Y. Zaslavsky. Manipulation of partition coefficient in ATPSs: understanding the addition of osmolytes and salts. 12th ESBES, European Symposium on Biochemical Engineering Sciences, Lisboa (Portugal), 9th-12th September 2018.

"The scientist trying to discover nature's secrets soon finds that only the most persistent, rigorously honest and boldly imaginative effort can win. Nature plays the perfect Sphinx and is completely adamant to every clumsy attempt to force the locks that guard her secrets. Yet to the man who finds the correct combination for one of these, i.e., the truth, she yields without the slightest resistance. Further, the devotee of science, that is, if I may change the metaphor, the man who woos nature for her secrets, must develop enormous tolerance in seeking for ideas which may please nature, and enormous patience, self-restraint and humility when his ideas over and over again are rejected by nature before he arrives at one to please her. When the scientist does finally find such an idea, there is often something very intimate in his feeling of communion with nature."

Robert S. Mulliken, Science and the Scientific Attitude, Science, 86, 65 (1937)