Universidade de Aveiro
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MEHRIBAN ALIYEVA

O EFEITO DE IÕES EM COMPOSTOS MODELO DE PROTEÍNAS EM SOLUÇÃO AQUOSA: ESTUDOS EXPERIMENTAIS E COMPUTACIONAIS

ION EFFECTS ON PROTEIN MODEL COMPOUNDS IN AQUEOUS SYSTEMS: EXPERIMENTAL AND COMPUTATIONAL STUDIES

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Engenharia Química, realizada sob a orientação científica do Professor João Manuel Costa Araújo Pereira Coutinho, Professor Catedrático do Departamento de Química da Universidade de Aveiro, e coorientação do Doutor José Richard Baptista Gomes, do Departamento de Química da Universidade de Aveiro, e coorientação do Professor Simão Pedro de Almeida Pinho, da Escola Superior de Tecnologia e Gestão do Instituto Politécnico de Bragança.

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To my parents.

o júri

Presidente

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Palavras-chave

Solubilidade, aminoácidos, peptídeos, eletrólitos, efeito salino, salting-in, salting-out, catião monovalente, catião divalente, pH, ácido, base, modelação, ePC-SAFT

Resumo

Soluções aquosas contendo eletrólitos são o ambiente natural de muitas biomoléculas, desempenhando um papel importante na regulação de sua estrutura e comportamento físico-químico, governando inúmeros processos bioquímicos. O conhecimento da solubilidade de aminoácidos (AA), peptídeos e proteínas na presença de sais é, portanto, de extrema importância do ponto de vista biológico e de relevo em muitas aplicações das indústrias alimentar e farmacêutica. Mesmo que dados relevantes sobre a solubilidade de AA em soluções aquosas de eletrólitos possam ser encontrados, as lacunas são evidentes, principalmente para AA aromáticos e aqueles que apresentam mais de um grupo amino ou carboxílico.

Esta Tese considera a solubilidade da glicina, L-leucina, L-fenilalanina e ácido L-aspártico em soluções aquosas dos sais compostos pela combinação dos catiões monovalentes (Na^+, K^+, NH_4^+) com os aniões Cl^-, NO_3^- e SCN^-, e os catiões divalentes (Mg^{2+} e Ca^{2+}) com os aniões Cl^-, NO_3^-, tendo ainda sido avaliada a solubilidade dos isómeros L e DL da valina em soluções aquosas de $NaNO_3$ e KNO_3.

Adicionalmente, o efeito salino na solubilidade dos ácido L-glutâmico, ácido L-aspártico, L-triptofano e L-tirosina, raramente encontrados na literatura, foi realizado em soluções aquosas de KCl e $(NH_4)_2SO_4$, sais em que é abundante a informação com outros aminoácidos, permitindo interpretações mais completas.

Todas as medidas foram realizadas a 298.2 K com molalidade do sal até 2 mol/kg. O método isotérmico dos frascos com agitação foi combinado com os métodos gravimétrico, espectroscopia UV, ou indíce de refração para análise da composição. Para examinar eventuais alterações na forma cristalográfica dos aminoácidos, foram realizados estudos complementares de fase sólida. Foi ainda medido o pH da solução saturada. Os catiões monovalentes não pareceram estabelecer interações relevantes com os AA e apresentam um impacto muito menor na solubilidade dos AA do que os catiões divalentes, sendo o Ca^{2+} aquele com efeito mais pronunciado. Excetuando o sulfato, os aniões seguem a série de Hofmeister. Em geral, AA com cadeias laterais apolares apresentaram solubilidade menor do que aqueles com grupos polares. O impacto de um sal com um anião orgânico (tosilato) foi também estudado, apresentando o maior efeito de salting-in entre todos os sais de sódio. Além disso, os estudos de dependência solubilidade com o pH para a glicina, diglicina e N-acetilglicina, foram realizados em água e em solução 1 molal de Na_2SO_4.

A equação de estado ePC-SAFT foi ainda aplicada na descrição das solubilidade dos AA nas misturas de água + ácido L-glutâmico/ácido L-aspártico/L-triptofano/L-tirosina + KCl/$(NH_4)_2SO_4$.

Keywords: Solubility, amino acids, peptides, electrolytes, salt effect, salting-in, salting-out, monovalent cation, divalent cation, pH, acid, base, modeling, ePC-SAFT

Abstract

Aqueous solutions containing electrolytes are the natural environment of many biomolecules, playing an important role in regulating their structure and physicochemical behaviour, governing numerous biochemical processes. The knowledge of the solubility of amino acids, peptides, and proteins in the presence of salts is therefore of utmost importance from a biological perspective in many food and pharmaceutical applications. Even if relevant data on amino acids solubility in aqueous electrolyte solutions can be found, the gaps are evident, particularly for aromatic amino acids and those presenting more than one amino or carboxylic group.

This Thesis considers the solubility of glycine, L-leucine, L-phenylalanine, and L-aspartic acid in aqueous solutions of the salts composed by combining the monovalent (Na^+, K^+, NH_4^+) cations with the Cl^-, NO_3^- and SCN^- anions and divalent (Mg^{2+} and Ca^{2+}) cations with the Cl^-, NO_3^- anions, and also the solubility of L and DL isomers of valine in aqueous $NaNO_3$ and KNO_3 solutions.

Additionally, the salt effect on the solubility of the amino acids: L-glutamic acid, L-tryptophan, and L-tyrosine, rarely found in the literature, were carried out in aqueous solutions of KCl and $(NH_4)_2SO_4$, salts for which much information can be found allowing for a better rationalization of the data.

All the measurements were performed at 298.2 K with molality up to 2 mol/kg. The isothermal shake-flask method combined with the gravimetric, UV spectroscopy, or refractive index measurements was used for compositional analysis. To examine any changes in the amino acids crystallographic form, supplementary solid phase studies were realized. The pH of the saturated solution was also measured. The monovalent cations did not seem to establish relevant interactions with the amino acids and had a much lower impact on their aqueous solubility than divalent cations, being Ca^{2+} the one showing a more pronounced effect. For all anions, except the sulfate, the solubility effect follows the Hofmeister series. In general, amino acids with apolar side chains had lower solubility than those with polar groups. After investigating the impact of inorganic salts on the solubility of amino acids, the impact of a salt with an organic anion (tosylate) also was studied, and a salting-in effect was observed. Besides, the solubility-pH dependence studies for glycine and two peptides containing this amino acid; diglycine and N-acetylglycine, were performed in water and 1 molal Na_2SO_4 solution.

The electrolyte perturbed-Chain Statistical Association Theory (ePC-SAFT) was applied to describe the AA solubility in mixtures of water + L-glutamic acid/ L-aspartic acid/ L-tryptophan/ L-tyrosine + KCl/ $(NH_4)_2SO_4$.

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Nomenclature

Abbreviations

GROMACS	A free and open-source software suite for high-performance molecular dynamics and output analysis
Calculated	Calculated
CCDC	Cambridge Crystallographic Data Centre
ePC-SAFT	Electrolyte Perturbed-Chain Statistical Association Theory
EPS	Electrostatic potential surface
Eq.	Equation
exp	Experimental
FSC	Fast Scanning Calorimetry
e.g.	For example
HPLC	High-performance liquid chromatography
Trans/cis	Isomers that have different configurations (groups permanently in different places in space)
KF	Karl-Fischer
L	Liquid state
MC	Model compound
MD	Molecular dynamic
NMR	Nuclear magnetic resonance
PC-SAFT	Perturbed-Chain Statistical Association Theory
RDF	Radial distribution function
Ref	Reference
S	Solid state
i.e.	That is
UV	Ultraviolet
XRD	X-Ray diffraction

Amino acids

Ala	Alanine
Abu	Aminobutyric acid
Ada	Aminodecanoic acid
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Cys	Cysteine
Glu	Glutamic acid
Gln	Glutamine
Gly	Glycine
His	Histidine
Ile	Isoleucine

Leu	Leucine
Lys	Lysine
Met	Methionine
Nva	Nor-valine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

Peptides

Gly-Gly	Diglycine
Gly-Ala	Glycine-alanine
NAGly	N-acetylglycine
VPGVG	Valine-proline-glycine-valine-glycine

Salts

$AlCl_3$	Aluminium chloride
$Al_2(SO_4)_3$	Aluminium sulfate
NH_4Cl	Ammonium chloride
NH_4NO_3	Ammonium nitrate
$(NH_4)_2SO_4$	Ammonium sulfate
NH_4SCN	Ammonium thiocyanate
$BaCl_2$	Barium chloride
$CaCl_2$	Calcium chloride
$Ca(NO_3)_2$	Calcium nitrate
$CuCl_2$	Copper chloride
$LiCl$	Lithium chloride
Li_2SO_4	Lithium sulfate
$MgCl_2$	Magnesium chloride
$Mg(NO_3)_2$	Magnesium nitrate
$MgSO_4$	Magnesium sulfate
$NiCl_2$	Nickel chloride
CH_3COOK	Potassium acetate
KBr	Potassium bromide
KCl	Potassium chloride
KF	Potassium fluoride
KI	Potassium iodide
KNO_3	Potassium nitrate
K_2SO_4	Potassium sulfate
KSCN	Potassium thiocyanate
NaBr	Sodium bromide
NaCl	Sodium chloride
NaF	Sodium fluoride
NaI	Sodium iodide

$NaNO_3$	Sodium nitrate
$NaClO_4$	Sodium perchlorate
NaOTs	Sodium p-toluenesulfonate
$NaSO_4$	Sodium sulfate
NaSCN	Sodium thiocyanate
$ZnCl_2$	Zinc chloride

Cations

NH_3^+RCOOH	Amino acid
NH_4^+	Ammonium
Ca^{2+}	Calcium
Cs^+	Cesium
Cdm^{2+}	Copper
Cu^{2+}	Guanidium
H^+	Hydrogen
Fe^{2+}	Iron
Li^+	Lithium
Mg^{2+}	Magnesium
Mn^{2+}	Manganese
Me^{2+}	Metal
K^+	Potassium
Rb^+	Rubidium
Na^+	Sodium
$N(CH_3)_4^+$	Tetramethylammonium
Zn^{2+}	Zinc

Anions

OAc^-/CH_3COO^-	Acetate
NH_2RCOO^-	Amino acid
Br^-	Bromide
ClO_3^-	Chlorate
Cl^-	Chloride
cit^-	Citrate
PF_6^-	Hexafluorophospate
HPO_4^{2-}	Hydrogen phosphate
OH^-	Hydroxide
I^-	Iodide
NO_3^-	Nitrate
ClO_4^-	Perchlorate
SO_4^{2-}	Sulfate
BF_4^-	Tetrafluoroborate
SCN^-	Thiocyanate

Groups

$-NH_2$	Amino group
$-CH_2C_6H_5$	Benzyl group
$-C_4H_9$	Butyl group
C=O	Carbonyl group
-COOH	Carboxyl group
$-C_3H_7$	Isopropyl group
$-CH_3$	Methyl group
$-CH_2-$	Methylene group
-SH	Thiol group
N-terminus	Amino-termins
C-terminus	Carboxyl-termins
-R	Side chain
C_t	Terminal carbon group
C_B	Other carbon atoms of the same alkyl chain
O_p	Carbonyl oxygen

Compounds

AA/NH_2RCOOH	Amino acid
C_2H_5OH	Ethanol
HCl	Hydrochloric acid
N-Boc-Pro-NHMe	N-(tert-butyloxycarbonyl) proline methyl amide
N-Boc-Pro-OMe	N-(tert-butyloxycarbonyl) proline methyl ester
N-Ac-Pro-OMe	N-acetyl proline methyl ester
HNO_3	Nitric acid
NMA	N-methyl acetamide
KOH	Potassium hydroxide
C_3H_8O	Propanol
RNA	Ribonucleic acid
NaOH	Sodium hydroxide
H_2O	Water
$NH_3^+RCOO^-$	Zwitterionic amino acid

Symbols

γ_i^L	Activity coefficient
a^{assoc}	Association attraction energy
$\frac{\varepsilon^{AiBi}}{k_B}$	Association-energy parameter
κ^{AiBi}	Association-volume parameter
ARD	Average relative deviation
k_{ij}	Binary interaction parameter

$a^{Coulomb}$	Coulomb attraction energy
α, β, γ	Different polymorphs
a^{disp}	Dispersion attraction energy
$\frac{u_i}{k_B}$	Dispersion energy parameter
u_i	Dispersive energy of component *i*
uj	Dispersive energy of component *j*
K, K_1, K_2	Equilibrium constants
a^{hc}	Hard-chain attraction energy
$\Delta_{hyd}H$	Hydration enthalpy
pI	Isoelectric point
R^2	Measurements of how closely a curve matches the data that was generated
Δh_{0i}^{SL}	Melting entalphy
T_{0i}^{SL}	Melting temperature
N	Number of association sites
m^{seg}	Number of segments
P	Pressure
u_r	Relative uncertainty
S_{AA}	Solubility of amino acid
T	Temperature
σ_i	Temperature-independent segment diameter of molecule i
$\Delta c_{p0i}^{SL}(T)$	The difference between the heat capacities of the liquid state and the solid state
$b_{c_{p0i}^{L}}$ ($b_{c_{p0i}^{S}}$)	The intercept of the liquid (solid) heat capacities
a^{res}	The residual Helmholtz energy
$a_{c_{p0i}^{L}}$ ($a_{c_{p0i}^{S}}$)	The slope of the liquid (solid) heat capacities
R	The universal gas constant
u	Uncertainty

Chapter 1 – General Introduction

This chapter includes a general introduction to the amino acids (AAs), peptides, and salts that will be studied in this work. The chemical structures of the amino acids studied are shown in this chapter and the structures of all other amino acids mentioned in this work are shown in **Appendix A**. A literature review on the solubility data related to aqueous mixtures of amino acids in the presence of electrolytes and also on the molecular dynamic simulations studies is also presented here. Besides, the solubility results of all the studied amino acids and peptides in pure water are given and a critical comparison with the literature was performed.

1.1. Amino acids, peptides and salts

Proteins are complex polymeric molecules that are present in all living organisms' cells. They consist of long polypeptide chains (quaternary structure), i.e., sequences of amino acids (the primary structure) bonded by peptide bonds that are the result of condensation reactions involving the amino terminus from one amino acid and the carboxylic terminus of another amino acid. Their folded tridimensional structures (secondary and tertiary structures) are stabilized by e.g., van der Waals contacts and sulfide hydrogen bonds within the same or between different polypeptide chains. Proteins may have multiple roles in living organisms, e.g., they can act as enzymes, hormones, antibodies, transporters, and muscle fibers, hence, being of utmost importance in many biochemical processes[1]. They require special attention because of their biological and pharmacological properties, helping to treat human diseases like diabetes, cancer, and hemophilia, among others[2–5]. Knowledge of their solubility is not only important to understand biochemical processes, but also to bioprocesses where they are used or produced, such as extraction, drying, crystallization or precipitation usually applied to separate and purify proteins[2,6]. Factors such as pH, ionic strength, temperature, and additives can change the solubility of proteins,[2,7–10] and it has been noticed that the concentration and chemical characteristics of ions present can introduce multiple and significant effects that still need to be better and more deeply understood[2,8,9,11–17]. It should be noted that among more than 300 AAs present in nature, only the L-enantiomers of just 20 common α-amino acids are normally incorporated in proteins[1] (**Figure 1. 1**), in many different combinations and sequences, leading to tridimensional structures having different properties and activities[18].

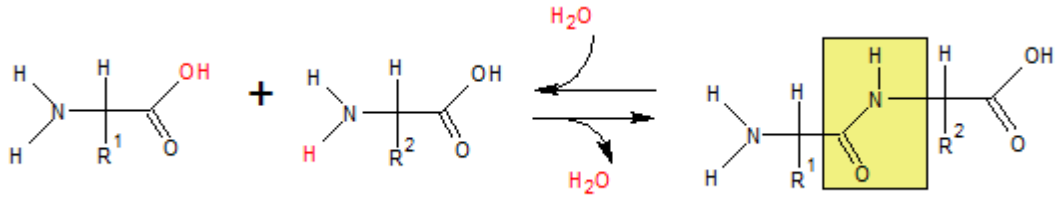

Figure 1. 1. General reaction of the synthesis of peptides[1].

Amino acids and small peptides can be used as model compounds (MC) to rationalize the salt effect on biomolecules solubility, aiming to provide insights into the behaviour of larger macromolecules. In this way, the direct study of the more complex protein/salt solutions is avoided, which has proven to be quite complex, and this option is also better from an analytical point of view. Some reasons for the complexity of the study of proteins in solution are their number, size, complex structure, global charge, and surface phenomena.

All amino acids contain at least one amine (-NH_2) and one carboxyl (-COOH) group, and a side chain (-R group) which is different for each amino acid. They can be divided in five classes, based on the properties of the R groups such as nonpolar, aliphatic; polar, uncharged; basic; acidic, and aromatic. Nonpolar aliphatic and aromatic groups are hydrophobic; polar, uncharged are more hydrophilic and positively (basic) or negatively (acid) charged groups are the most hydrophilic ones[1,19]. AAs have different biochemical properties, that vary with the different groups present in their side chains.

The amino acids chosen in this work present different polarities. Glycine (Gly), L-leucine (Leu) and L, and DL-valine (Val) have nonpolar, hydrophobic groups; L-phenylalanine (Phe), L-tryptophan (Trp) and L-tyrosine (Tyr) have nonpolar, hydrophobic aromatic side chains; while L-aspartic acid (Asp) and L-glutamic acid (Glu) have a second hydrophilic carboxyl group[1].

These amino acids are common building blocks of several relevant proteins, as reviewed in Drauz *et al.*[19]: aspartic acid in edestin, hemoglobin, and barley globulin; glutamic acid in gliadin, zein, wheat gliadin, and maize zein; glycine in gelatin and silk fibroin; L-leucine in edestin, hemoglobin, serum proteins and maize zeins; L-valine in casein, beef sinew and beef aorta; L-phenylalanine in zein, egg albumin and serum albumin; tyrosine in fibrin, silk fibroin, and papain; and tryptophan in fibrin and egg lysozyme. Besides the fact that they are building blocks, glutamate (the ionic form of Glu) plays important roles in regulating

gene expression, cell signaling, antioxidative responses, and immunity, and additionally, with glutamine, and aspartate (the ionic form of Asp), these AAs are major metabolic fuels for the small intestine. Along with glycine, they also regulate neurological function. Among essential AAs, the most importance has been placed on Leu and Trp, which are functional AAs and regulate key metabolic pathways that are needed for maintenance, growth, reproduction and immunity. Leu activates mammalian target of rapamycin to stimulate protein synthesis and inhibit proteolysis and Trp modulates neurological and immunological functions through multiple metabolites, including serotonin and melatonin[18,20].

The extension of solubility studies to peptides is a milestone in this project. In fact, to be able to take into account the interactions with the peptide bond is highly relevant, as very few experimental data and theoretical analyses are available for these compounds, and interpretations from different researchers are very contradictory. In this work, the solubility studies of two peptides, taking the simplest amino acid - glycine to form peptides: glycylglycine or diglycine (Gly-Gly) and N-acetylglycine (NAGly) were carried out at different pH conditions. Among all peptides, diglycine is the simplest peptide. The melting point of these white crystals is approximately 233 °C. Gly-Gly is used in the synthesis of more complex peptides. N-acetylglycine is a white powder with a melting point of 206 °C. It is a chemical and pharmaceutical intermediate used in many fields[21–25], such as the pharmaceutical and biological fields and the agrochemical industry. This peptide is also used as the blocking agent of the N-terminus (amino-terminus) to prepare unnatural and unusual amino acids and their analogs to modify peptides.

The chemical structures of the studied compounds are shown in **Figure 1. 2.**

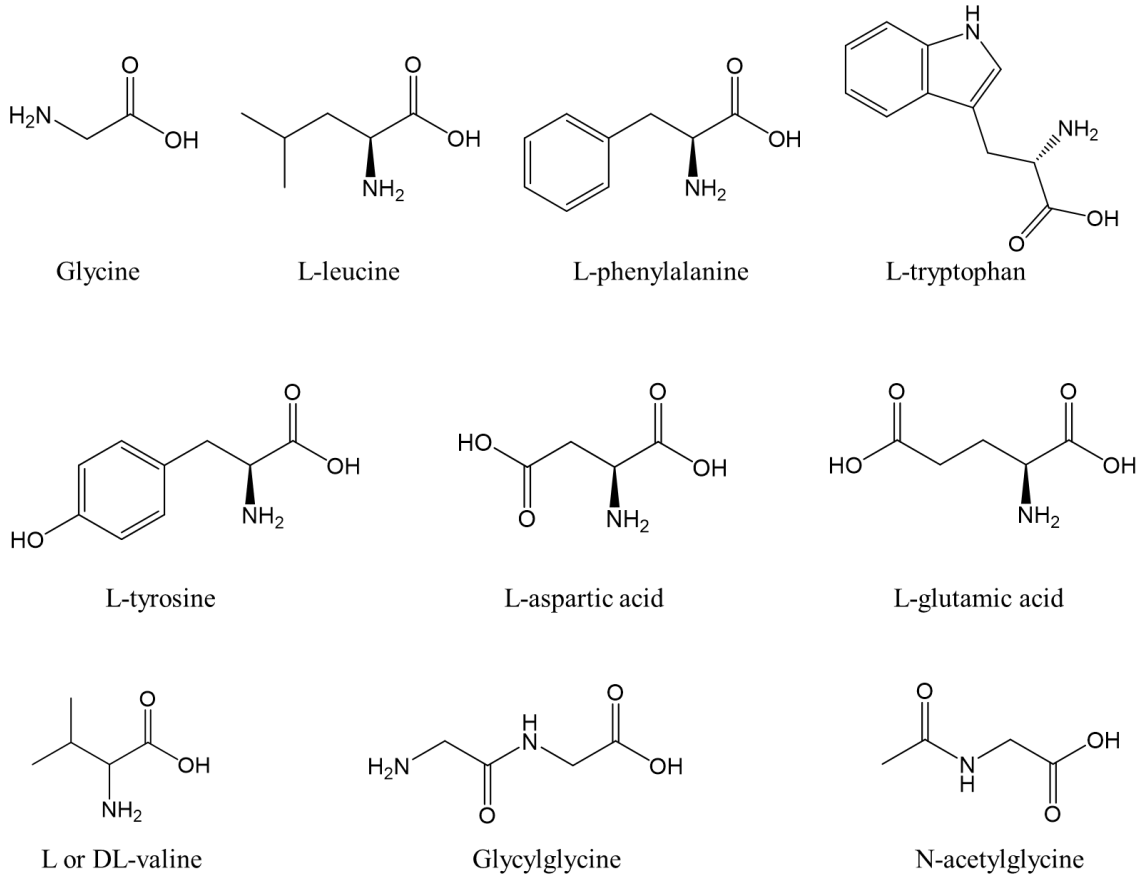

Figure 1. 2. Chemical structures of the amino acids and peptides studied in this Thesis.

As aqueous electrolyte solutions are the natural environment of many biomolecules, it is also important to improve the understanding of the behavior of those organic compounds in this environment. For example, to prepare L-aspartic acid the chemosynthesis method, enzymatic method, extraction method, and zymothechnics methods are used. The knowledge of the solubility of L-asp in these aqueous salt solutions is necessary to optimize the crystallization process. The presence of these co-solutes results in the salting-in or salting-out effect, and this happens due to a decrease and an increase in the activity coefficient of AAs with increasing salt concentration[26]. The study of the aqueous solubility of the lower molecular weight amino acids in electrolyte solutions can provide important insights regarding the molecular-level interactions associated with the ion effects affecting the behavior of these biomolecules[27]. The selected ions in **Figure 1. 3** were chosen from the Hofmeister series due to their biological relevance, while covering a wide range of aqueous solubility effects: ammonium, potassium, sodium, magnesium and calcium cations; sulfate, chloride, nitrate, and thiocyanate anions.

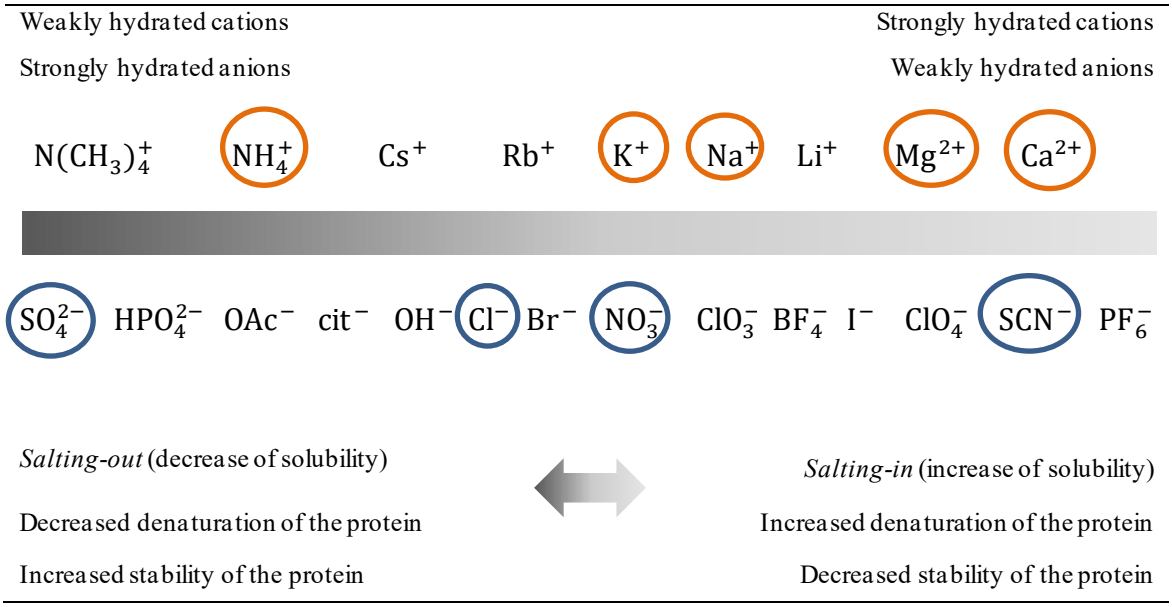

Figure 1. 3. Cations and anions ordering in a Hofmeister series[28].

Metal ions play important roles in many cellular processes[29]. Metals, such as Na^+, K^+, Mg^{2+}, and Ca^{2+} are demanding for the stability, proper folding, and functioning of RNA (ribonucleic acid) and proteins[30–33]. Divalent metal cations such as Mn^{2+} and Zn^{2+} are important in many enzymatic reactions, Cu^{2+} and Fe^{2+} are essential ions of respiration and photosynthesis[34], Ca^{2+} cation has been used in the solubilization of myofibrillar proteins with relevance in the food industry[35], and γ-glycine crystals produced from the aqueous solutions with Mg^{2+} cation showed to be effective in laser applications and fabrication of electro-optical devices[36]. Additionally, it is well established the relevance of Zn^{2+}, Mg^{2+}, and Ca^{2+} to stabilize the structure of folded proteins and, in some cases, to fix a particular physiologically active conformation of the protein[37].

Sodium p-toluenesulfonate, or sodium p-tosylate (NaOTs, **Figure 1. 4**), studied in this work as a solvent additive is a hydrotrope that helps to increase the solubility of the solute in a given solvent. Hydrotropes are compounds that are used in the pharmaceutical industry, to increase the solubility of poorly soluble drugs. This solubility enhancement technique does not require chemical modification of hydrophobic drugs, use of organic solvents, or preparation of an emulsion system[38]. They improve the solubility by forming weak interaction with solute molecules[39]. Hydrotropes consist of both hydrophobic and hydrophilic groups and an important class are ionic organic salts. The ones with the larger hydrophobic part have more effect on the solubility, while the presence of the charge on the hydrophilic part is less significant[38].

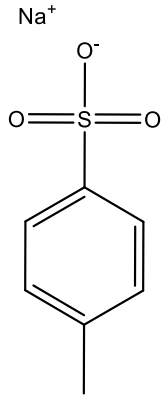

Figure 1. 4. Chemical structure of Sodium p-tosylate.

1.2. Literature review

1.2.1. Solubility data of amino acids

An extensive literature review on the solubility of AAs in electrolyte solutions was carried out and is presented in **Table 1. 1.** The AAs and salts studied in this work are highlighted with blue and bold text font and their intersection represent the studied aqueous systems, while the numbers are the references that indicate the aqueous ternary systems already published in the open literature. **Table 1. 1** considers all the systems found in the literature without establishing a quality check or analysis of their consistency. As can be observed, the most studied AAs were glycine /Gly), alanine (Ala), valine, and serine (Ser), and the salts NaCl (by far), KCl, Na_2SO_4, $NaNO_3$ and KNO_3. These are in the list of the salts selected by us which will allow to validate our methodology by direct comparison of the data and to perform a more extensive comparative analysis on the effect of each salt. Nevertheless, even if relevant data on AAs' solubility in aqueous electrolyte solutions can be found, the gaps are evident, particularly for amino acids with aromatic groups or presenting more than one amino or carboxylic group such as arginine (Arg) or L-aspartic acid, for example. It is also significant that anions in the Hofmeister series such as carbonate, perchlorate, and thiocyanate are very seldom included in this type of studies.

Table 1. 1. Literature review on salting effect on the AAs solubility in aqueous electrolyte solutions.

Salts	AAs*																
	Nonpolar, aliphatic						Polar, uncharged				Basic		Acidic		Aromatic		
	Gly	Ala	Pro	**Val**	**Leu**	Ile	Ser	Thr	Cys	Gln	Lys	His	**Asp**	**Glu**	**Phe**	**Tyr**	**Trp**
LiCl				40		40											
Li_2SO_4				40		40											
NaF	41,42	41,42		42			41,42										
NaCl	43–51	46,49,52	49, 53	46,49	44,45,54 ,55		46,52		56	57	56	57	26,57	57,58	55,59	44,45, 54,60	60
NaBr	42,61	42,61		42,61			42,61		56		56						
NaI	42,62	42,62		42			42,62										
$NaNO_3$	49,63–66	49,63,67	49, 53	49,63		68	59,63, 68								69		
Na_2SO_4	42,70–72	42,70–72		42,71, 72		73	42,73		56		56						
KF	41,42	41,42		42			41,42										
KCl	46,49– 51,70,74,75	46,49,52, 70,74	49, 53	46,49		74	46,52, 74	74					26	58			
KBr	42,61,75	42,61		42,61			42,61										
KI	42	42		42			42		56		56						
KNO_3	49,63,64	49,63,76	49, 53	49,63		68	63,68, 76										
KCH_3COO	75																
K_2SO_4	42,77	40,42,77		40,42		40, 73	42,73, 77										

Salts	AAs*																
	Nonpolar, aliphatic						Polar, uncharged				Basic		Acidic		Aromatic		
	Gly	Ala	Pro	**Val**	**Leu**	Ile	Ser	Thr	Cys	Gln	Lys	His	**Asp**	**Glu**	**Phe**	**Tyr**	**Trp**
NH_4Cl	66,78	79		79		79											
NH_4NO_3	66																
$(NH_4)_2SO_4$	6,49,66	6,49	49	49,79		6	6	6									
$MgCl_2$	47,80	79		79		79											
$MgSO_4$		79		79		79											
$CaCl_2$	42	40,42		40,42		40	42		56		56						
$BaCl_2$	42	42		42			42										
$NiCl_2$																	81
$CuCl_2$					81										81		81
$ZnCl_2$																	81
$AlCl_3$				40		40											
$Al_2(SO_4)_3$				40		40											

*Glycine (Gly); Alanine (Ala); Proline (Pro); Valine (Val); Leucine (Leu); Isoleucine (Ile); Serine (Ser); Threonine (Thr); Cysteine (Cys); Glutamine (Gln); Lysine (Lys); Histidine (His); Aspartic acid (Asp); Glutamic acid (Glu); Phenylalanine (Phe); Tyrosine (Tyr); Tryptophan (Trp). Cell colour: orange – salting-in effect; blue – salting-out effect; yellow – both salting-in and salting-out effects in the whole concentration range; grey – contradictory results reported in the literature.

Due to the large amount of solubility data of glycine in aqueous electrolyte solutions, a summary of the experimental data found is presented separately in **Table 1. 2**.

Table 1. 2. Summary of the solubility data of glycine in aqueous electrolyte solutions published in the literature.

Amino acid	Salt	*T* range/K	Salt concentration range	Reference
	NaCl	298.15	0 to 200 g/L	43
	NaCl	298.15	0, 1 and 3 mol/dm^3 of solution	44
	NaCl	298	0 to 3 mol/dm^3 of solution	45
	NaCl; KCl	298.2 to 343.2	0 to 6; 0 to 4 mol/kg of H_2O	51
	KCl; Na_2SO_4	298.15	0 to 2; 0 to 1.5 mol/kg of H_2O	70
	KCl	323.15	0 to 2 mol/kg of H_2O	74
	$(NH_4)_2SO_4$	298.15, 323.15	0 to 2 mol/kg of H_2O	6
	NaCl, KCl	298.2	0 to 1.5 mol/kg of H_2O	46
	$NaNO_3$, KNO_3	298.2	0 to 1.5 mol/kg of H_2O	63
	$MgCl_2$; $MgCl_2$+NaCl	283.15 to 333.15; 283.15 to 343.15	0 to 3.5; 0.5, 1, 1.5+0.15 to 2.5 mol/kg	47
	HCl+ $MgCl_2$	283.15 to 343.15	0.5 to 1 + 0.25, 0.5 and 0.75 mol/kg	80
	C_2H_5OH+NaCl; C_3H_8O+NaCl	283.15 to 333.15	0 to 0.1876 mol/mol of solution + 0.5, 1 and 2 mol/kg of H_2O; 0 to 0.0640 mol/mol of solution + 0.5 mol/kg of H_2O	48

	NaBr; KBr	298.15	0 to 2.5 mol/kg of H_2O	61
	NaCl, KCl; $NaNO_3$, KNO_3, $(NH_4)_2SO_4$	298.15	0, 1 and 3.01; 0, 1 and 3 mol/kg of H_2O	49
	NaCl, KCl	288.15 to 308.15	0 to 5 mol/kg of H_2O	50
	$NaNO_3$, KNO_3	288.15 to 308.15	0 to 3 mol/kg of H_2O	64
	NaF, KF	298.15	0 to 1.5 mol/kg of H_2O	41
	Na_2SO_4	298.15, 323.15	0 to 1.5 mol/kg of H_2O	71
	$NaNO_3$	288.15 to 308.15	0 to 4 mol/kg of H_2O	65
	K_2SO_4	298.15	0 to 0.65 mol/kg of H_2O	77
	KCl, KBr and CH_3COOK	298.15	0 to 4 mol/kg of solution	75
	NaF, KF, NaBr, KBr, NaI, KI, Na_2SO_4, K_2SO_4, $CaCl_2$, $BaCl_2$	298.15	0 to 1 mol/kg of H_2O	42
	NaI	298.15	0 to 2.5 mol/kg of H_2O	62
	Na_2SO_4	288.15 to 308.15	0 to 1.5 mol/kg of H_2O	72
	NH_4Cl	283.2 to 353.2	0 to 14.7885 mol/kg of H_2O	78
α-glycine	$NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl	293.15 to 343.15	0 to 10% (m_{salt}/m_{water})	66

A complete literature review on the solubility of aspartic acid, glutamic acid, tyrosine, tryptophan, leucine, phenylalanine and valine was also carried out. The experimental data are summarized in **Table 1.3**.

.**Table 1. 3.** Summary of the solubility data of aspartic acid, glutamic acid, tyrosine, tryptophan, leucine, phenylalanine and valine in aqueous electrolyte solutions, found in the literature.

Amino acid	Salt	*T* range/K	Salt concentration range	References
D-aspartic acid; L-aspartic acid	NaCl, KCl; KCl	292.8 to 344.10; 292.9 to 338.19	0 to 1.8251, 0 to 1.4360; 0 to 3.0446 mol/kg of solution	26
L-aspartic acid; L-glutamic acid	NaCl	298.15	0 to 5.556; 0.001 to 5.246 mol/kg of H_2O	57
L-glutamic acid	NaCl, KCl	298.15 to 323.15	0 to 3 mol/kg of H_2O	58
DL-tyrosine; DL-tryptophan	NaCl	298.15	0 to 4.999; 0 to 5.005 mol/L of solution	60
L-tyrosine, L-leucine	NaCl	298.15	0, 1 and 3 mol/dm^3 of solution	44
L-tyrosine, L-leucine	NaCl	298	0 to 3 mol/dm^3 of solution	45
L-tyrosine, L-leucine	NaCl	298.15	0 to 3 mol/kg of H_2O	54
tryptophan*; phenylalanine*, leucine*	$CuCl_2$, $ZnCl_2$, $NiCl_2$; $CuCl_2$	Room *T*	0 to 0.5; 0 and 0.5 mol/L of solution	81
DL-phenylalanine	$NaNO_3$	298.15	0 to 0.5 mol/kg of H_2O	69
DL-phenylalanine	NaCl	288.15 to 308.15	0 to 6 mol/kg of H_2O	59
L-phenylalanine; L-leucine	NaCl	298.15	0.101 to 3.130; 0.056 to 5.035 mol/kg of H_2O	55
L-valine	K_2SO_4; $CaCl_2$; LiCl; Li_2SO_4; $AlCl_3$; $Al_2(SO_4)_3$	298.15	0 to 0.5; 0 to 2; 0 to 2; 0 and 1; 0 and 0.5;	40

			0 and 0.5 mol/kg of H_2O	
DL-valine	NaF, KF, NaBr, KBr, NaI, KI, Na_2SO_4, K_2SO_4, $CaCl_2$, $BaCl_2$	298.15	0 to 1 mol/kg of H_2O	42
DL-valine	NaCl, KCl	298.2	0 to 1.5 mol/kg of H_2O	46
L-valine	NaCl, KCl; $NaNO_3$, KNO_3, $(NH_4)_2SO_4$	298.15	0 to 3.01; 0 to 3 mol/kg of H_2O	49
DL-valine	NaBr, KBr	298.15	0 to 2.5 mol/kg of H_2O	61
DL-valine	$NaNO_3$, KNO_3	298.2	0 to 1.5 mol/kg of H_2O	63
DL-valine	Na_2SO_4	298.15, 323.15	0 to 1.5 mol/kg of H_2O	71
DL-valine	Na_2SO_4	288.15 to 308.15	0 to 1.5 mol/kg of H_2O	72
L-valine	NH_4Cl, $(NH_4)_2SO_4$, $MgCl_2$, $MgSO_4$	298.15	0 to 2 mol/kg of H_2O	79

*isomer not specified.

In **Table 1. 4** a literature review on the solubility of amino acids not experimentally studied in this work is presented, considering only publications since 2014. Even though the salt concentration studied in some works reaches 6 mol/kg, usually the range is 0-3 mol/kg, and the measurements are mainly performed at 298 K.

Table 1. 4. Summary of the salt effect on the solubility data of amino acids not studied in this work. Data published in the literature since 2014.

Amino acid	Salt	*T* range/K	Salt concentration range	References
L-isoleucine, L-serine	Na_2SO_4, K_2SO_4	288.15 to 308.15	0 to 2.5 mol/kg of H_2O	73
L-isoleucine, L-serine	$NaNO_3$, KNO_3	288.15 to 308.15	0 to 2.5 mol/kg of H_2O	68
L-lysine, L-cysteine	NaBr, NaCl, KI, Na_2SO_4, $CaCl_2$	298.15	0 to 1 mol/kg of H_2O	56
DL-alanine, DL-serine	NaBr, KBr	298.15	0 to 2.5 mol/kg of H_2O	61

L-alanine, L-proline	NaCl, KCl; $NaNO_3$, KNO_3, $(NH_4)_2SO_4$	298.15	0, 1 and 3.01; 0, 1 and 3 mol/kg of solution	49
DL-alanine	$NaNO_3$	288.15 to 308.15	0 to 5 mol/kg of H_2O	67
DL-alanine, DL-serine	KNO_3	298.15	0 to 3 mol/kg of H_2O	76
DL-alanine, DL-norvaline, DL-serine	NaF, KF	298.15	0 to 1.5 mol/kg of H_2O	41
DL-α-aminobutyric acid	KCl	288.15 to 308.15	0 to 3.7 mol/kg of H_2O	82
DL-serine	$NaNO_3$	288.15 to 308.15	0 to 6 mol/kg of H_2O	59
L-cystine	NaCl	298.15	0 to 3 mol/kg of H_2O	54
L-proline	NaCl, $NaNO_3$, KCl, KNO_3	288.15 to 308.15	0 to 3 mol/kg of H_2O	53
DL-nor-valine	$NaNO_3$, KNO_3	288.15 to 308.15	0 to 3 mol/kg of H_2O	64
DL-alanine	Na_2SO_4	298.15 to 323.15	0 to 1.5 mol/kg of H_2O	71
DL-alanine; L-isoleucine, L-valine	K_2SO_4, $CaCl_2$; K_2SO_4, $CaCl_2$, LiCl, Li_2SO_4, $AlCl_3$, $Al_2(SO_4)_3$	298.15	0 to 0.5; 0 to 2; 1; 0.5 mol/kg of H_2O	40
DL-alanine, DL-serine	NaCl, KCl	288.15 to 308.15	0 to 3.5 mol/kg of H_2O	52
DL-nor-valine	NaCl; KCl	288.15 to 308.15	0 to 4.8 mol/kg of H_2O	83
DL-α-amino butyric acid	$NaNO_3$	288.15 to 308.15	0 to 5 mol/kg of H_2O	84
L-glutamine; L-histidine	NaCl	298.15	0 to 5.6; 0 to 5.606 mol/kg of H_2O	57
DL-alanine, DL-norvaline, DL-serine	NaI	298.15	0 to 2.5 mol/kg of H_2O	62
DL-alanine, DL-norvaline, DL-serine	K_2SO_4	298.15	0 to 0.65 mol/kg of H_2O	77

For all amino acids, their solubilities in pure water at 298.2 K were already reported by several authors, as collected in **Table 1. 5**. It should be mentioned that, in many cases, the

solid phase structure was not registered. Rowland *et al.*[85] made a summary of literature sources for solubility measurements of α-glycine in water. The minimum solubility value found was around 248 g/1000 g of H_2O. As glycine, studied in this work, in all aqueous solutions crystallized only in the hexagonal crystal system, the γ-form (section 3.5), values higher than 248 g/1000 g of H_2O[41,42,46,49,50,61–63,65,72,75,86–91] were not included in **Table 1. 5**. Nevertheless, the literature average and standard deviation were calculated for comparison purposes and are also reported in **Table 1. 5**.

Table 1. 5. Summary of the literature solubility data of the amino acids studied in this work in pure water at 298.2 K.

Amino acid	Solubility of AAs/ g of AA/1000 g of H_2O	Reference	Literature average ± standard deviation AAs/ g of AA/1000 g of H_2O
Glycine	234.350	92	236.7 ± 3.6
	235.700	70	
	234.894	93	
	242.026	78	
L-leucine	21.774	90	22.4 ± 1.1
	24.260	88	
	21.600	87	
	21.800	91	
	23.243	44	
	21.520	94	
L-phenylalanine	28.000	91	28.4 ± 0.9
	29.700*	81	
	27.800	93	
	27.900	87	
L-tryptophan	13.800	95	13.5 ± 1.21
	12.800	87	
	12.900	96	
	11.730	97	
	15.600	98	
	13.800	91	
	13.993	99	
L-tyrosine	0.451	95	0.47 ± 0.11
	0.453	88	
	0.475	87	
	0.502	100	
	0.352	97	
	0.600	99	
	0.479	86	
	0.362	44	
L-aspartic acid	5.235	26	5.2 ± 0.33
	5.000	88	
	5.393	86	
	4.950	101	
	4.992	102	
	5.744	57	
	5.090	91	
	4.925	103	
	5.733	99	

L-glutamic acid	8.500	104	8.9 ± 0.77
	8.683	105	
	8.610	101	
	8.543	106	
	10.800	91	
	8.700	103	
	8.578	107	
	8.622	58	
	9.072	57	
	8.878	99	
	9.071	97	
L-valine	58.400	91	58.4 ± 0.33
	58.458	90	
	57.893	40	
	58.576	49	
	58.449	79	
DL-valine	79.311	71	75.6 ± 6.6
	70.876	42	
	88.100	72	
	74.410	86	
	70.900	88	
	70.200	46	
	80.834	61	
	70.291	63	

*isomer not specified.

1.2.2. Solubility data of peptides

For peptides, the amount of available solubility data is much less. The review summarized in **Table 1. 6** covers all the collected information.

Table 1. 6. Summary of the salt effect on the solubility of peptides published in the literature.

Peptide	Salt	T range/K	Salt concentration range	References
Diglycine Glycyl-L-alanine	NaCl; Na_2SO_4 and $(NH_4)_2SO_4$	298.15	0.1 to 6, 0.1 to 1.0241 and 0.1 to 1.0652 mol/kg of H_2O	108
Diglycine Triglycine	NaCl	298.15	0 to 200 g/L	43
Diglycine N-acetylglycine	$(NH_4)_2SO_4$; $MgSO_4$; K_2SO_4; Na_2SO_4; $CaCl_2$; $MgCl_2$; NH_4Cl; KCl and NaCl	298.15	0 to 2 mol/kg of H_2O	109
Diglycine Triglycine Tetraglycine Cyclic diglycine	KCl; KBr and CH_3COOK	298.15	0 to 4 mol/kg of solution	75
Diglycine Triglycine	CH_6ClN_3	298.25	0 to 6 mol/L	87

Diglycine	$NaNO_3$	288.15-308.15	1 to 4 mol/kg of H_2O	65
Proline-Leucine	NaCl	288.15-313.15	0 to 1.0234 mol/kg of H_2O	110
Tryptophan-Phenylalanine; Phenylalanine-Phenylalanine; Tryptophan-Alanine-Phenylalanine	$CuCl_2$	room T	0 and 0.5 mol/L	81

To find solubility data of peptides in aqueous salt solutions is more difficult as they are more complex molecules and less commercially available. Diglycine is the most studied peptide and will be also studied in this work. Most studies were performed at 298.15 K, but in two works[65,110], it was extended from 288.15 to 313.15 K. The most studied salt concentration range is 0-2 mol/kg.

For Gly-Gly and NAGly the solubilities in pure water at 298.2 K are collected in **Table 1. 7**.

Table 1. 7. Summary of the literature solubility data of the peptides studied in this work in pure water at 298.2 K.

Amino acid	Solubility of AAs/ g of AA/1000 g of H_2O	Reference	Literature average ± standard deviation AAs/ g of AA/1000 g of H_2O
Diglycine	227.500	75	228.6 ± 2.6
	226.600	87	
	228.000	111	
	226.700	65	
	233.000	95	
	227.500	112,113	
	229.889	108	
	226.700	114	
	233.192	115	
	226.994	116	
N-acetylglycine	41.676	117	41.5 ± 0.22
	41.400	111	

To understand the effect of the different salts on the solubility of diglycine, data from different sources are compiled in **Figure 1. 5**. As can be seen in **Figure 1. 5**, with exception of potassium acetate (CH_3COOK) all the salts induce a salting-in effect on the solubility of diglycine, in the decreasing order (evaluated at 1 molal): $CaCl_2$ > $MgCl_2$ > $MgSO_4$ > $(NH_4)_2SO_4$ > Na_2SO_4 > $NaNO_3$ > NaCl > NH_4Cl > $NaNO_3$ > KBr > KCl. Salts with strongly hydrated polyvalent cations such as Ca^{2+} and Mg^{2+} show a high salting-in effect which is in

accordance with the Hofmeister series. Due to high charge density they are able to form complexes with diglycine, thus promoting a significant increase in its solubility[34]. As shown before, divalent cations present strong interactions with the COO^- groups and do not establish important interactions with the hydrophobic parts of the amino acids[40]. The monovalent cation salts with the chloride anion (NH_4Cl, NaCl and KCl) show almost the same salting-in effect. Monovalent cations with the sulfate anion such as $(NH_4)_2SO_4$, K_2SO_4, and Na_2SO_4 also show similar effects on the solubility of diglycine at low molalities. The relative solubility (ratio between the solubility of AA, expressed as the mass of the amino acid in 1 kg of water, in aqueous salt solutions to that in pure water, S/S_0) of diglycine in aqueous $MgSO_4$ solutions, however, is always the highest. For 2 molal salt concentration, the relative solubility follows the order $MgSO_4 > (NH_4)_2SO_4 > Na_2SO_4$. The Na_2SO_4 salt[109] induces a salting-out effect only when the molality is higher than 1, what happens probably because of the presence of salting-out sulfate anion that can contribute to change the solubility effect[79]. At higher molalities (up to 4 molal), there are only data for a few salts: a higher salting-in effect is obtained with $NaNO_3$, followed by KBr. Finally, CH_3COOK induces a salting-out effect in the whole molality range studied. When the effect of salts with the same cations was compared, the MgCl showed higher salting-in effect than $MgSO_4$ what follows the order in Hofmeister series for proteins. The salts with ammonium cation showed the opposite result. The salting-in effects of Na_2SO_4 and NaCl were similar. As the Cl^- and Br^- anions are next to each other in the Hofmeister series these anions with potassium cation also showed very close results. The only anion which showed the salting-out effect with potassium cation was acetate which is one the first in the list of strong salting-out anions in the Hofmeister series.

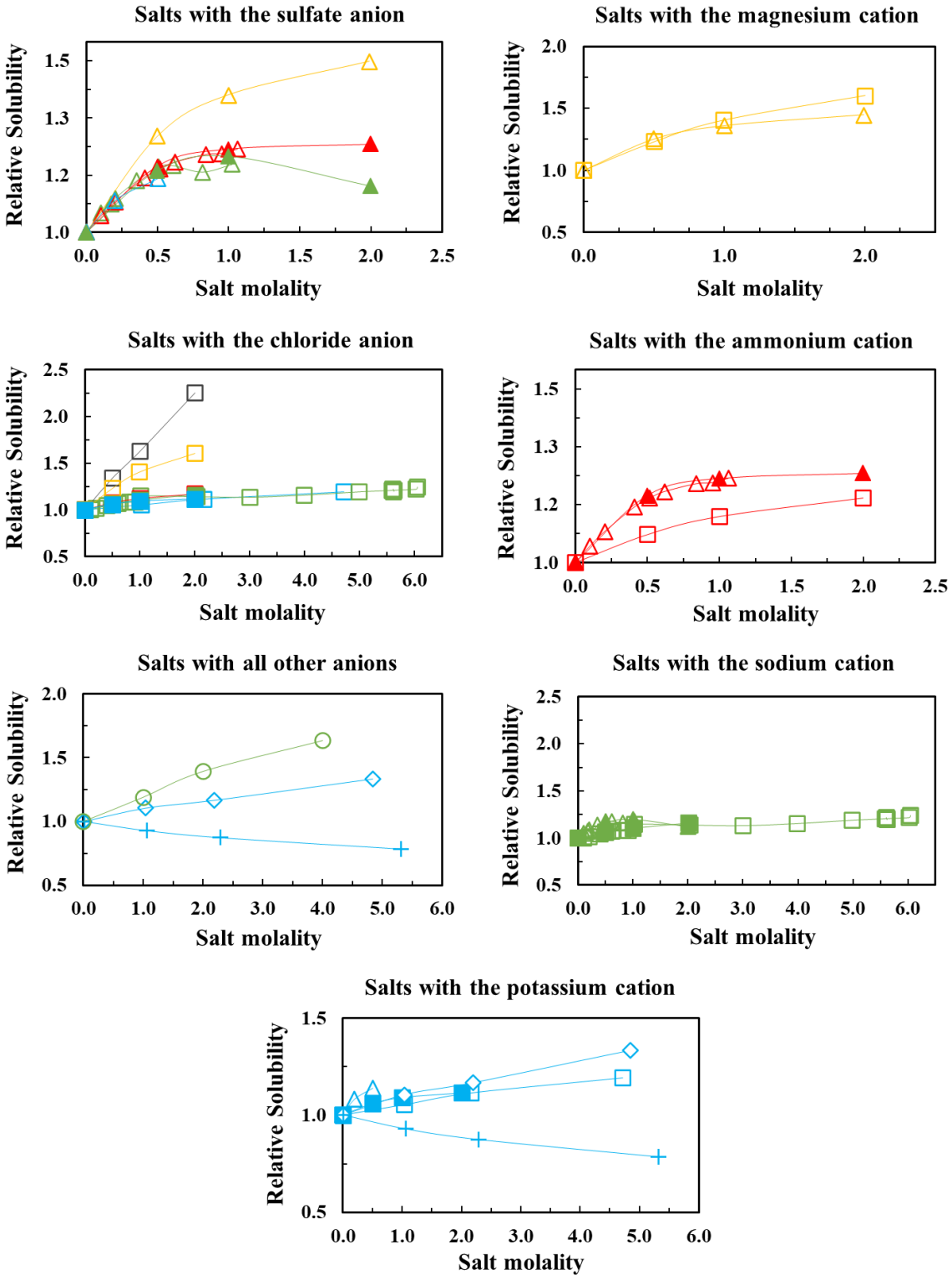

Figure 1. 5. Relative solubility of diglycine in aqueous saline solutions, at 298.2 K: □, $CaCl_2$[109]; □, $MgCl_2$[109]; □, NH_4Cl[109]; ■, KCl[109]; □, KCl[75]; ■, NaCl[109]; □, NaCl[108]; △, $MgSO_4$[109]; ▲, $(NH_4)_2SO_4$[109]; △, $(NH_4)_2SO_4$[108]; △, K_2SO_4[109]; ▲, Na_2SO_4[109]; △, Na_2SO_4[108]; ◇, KBr[75]; +, CH_3COOK[75]; ○, $NaNO_3$[65]. Lines are guide to the eyes.

Venkatesu *et al.*[75] studied the solubility of glycine peptides with different chain structures: glycine, diglycine, triglycine, tetraglycine and cyclic diglycine. As expected, the solubilities of the glycine peptides in pure water decrease from Gly (25.09 g/100 g H_2O) to tetraglycine (0.395 g/100 g H_2O). When considering the effect of the studied salts (KCl, KBr and CH_3COOK), for a given salt molality, the relative solubility increases from Gly to tetraglycine, even though the difference between glycine and diglycine is very small (**Figure 1. 6**). In the Hofmeister series for proteins (**Figure 1. 3**) the anions are in the following decreasing order of salting-out effect: $CH_3COO^- > Cl^- > Br^-$. The halogens have similar effects on the solubility of peptides. As all these anions are in the area of salting out and the salts provoke salting-in effect, perhaps the potassium cation interacts with the peptide bond, probably with the carbonyl (C=O) group.

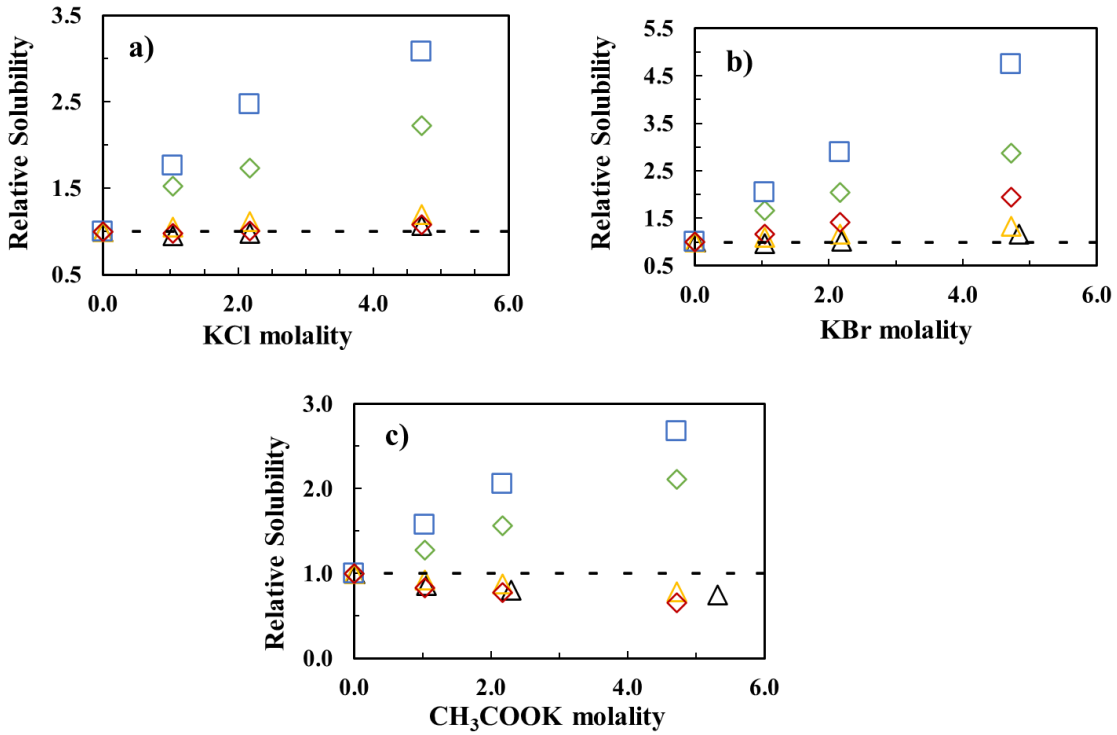

Figure 1. 6. Relative solubility of △, glycine; △, diglycine; ◇, triglycine; □, tetraglycine and ◇, cyclic diglycine in aqueous a) KCl; b) KBr; c) KCH_3COO solutions at 298.2 K. Data from Venkatesu *et al.*[75].

In all cases, a salting-in effect is observed except for CH_3COOK that induces a salting-out effect in glycine and diglycine. Cyclic diglycine is the cyclic dimer of glycine and has the structural formula shown in **Figure 1. 7**. As can be seen, it contains only two carbonyl groups and, thus, no end charge effects, and hydrogen bonding occur between peptide linkages. This

structure makes cyclic diglycine a good model compound for hydrogen bonding which takes place within the peptide backbone of a protein. The absence on this cyclic peptide of charged end groups may explain its low solubility. The relative solubility follows a similar trend to Gly and diglycine in KCl and KCH_3COO solutions, but a higher salting-in is induced by KBr solutions.

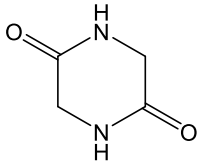

Figure 1. 7. Chemical structure of cyclic diglycine.

The diversity of peptides that can be formed by combining different amino acids units is immense, but, as mentioned before, almost no information can be found on the effect of salts on the solubility of peptides, even for dipeptides. A rare example can be found in **Figure 1. 8**.

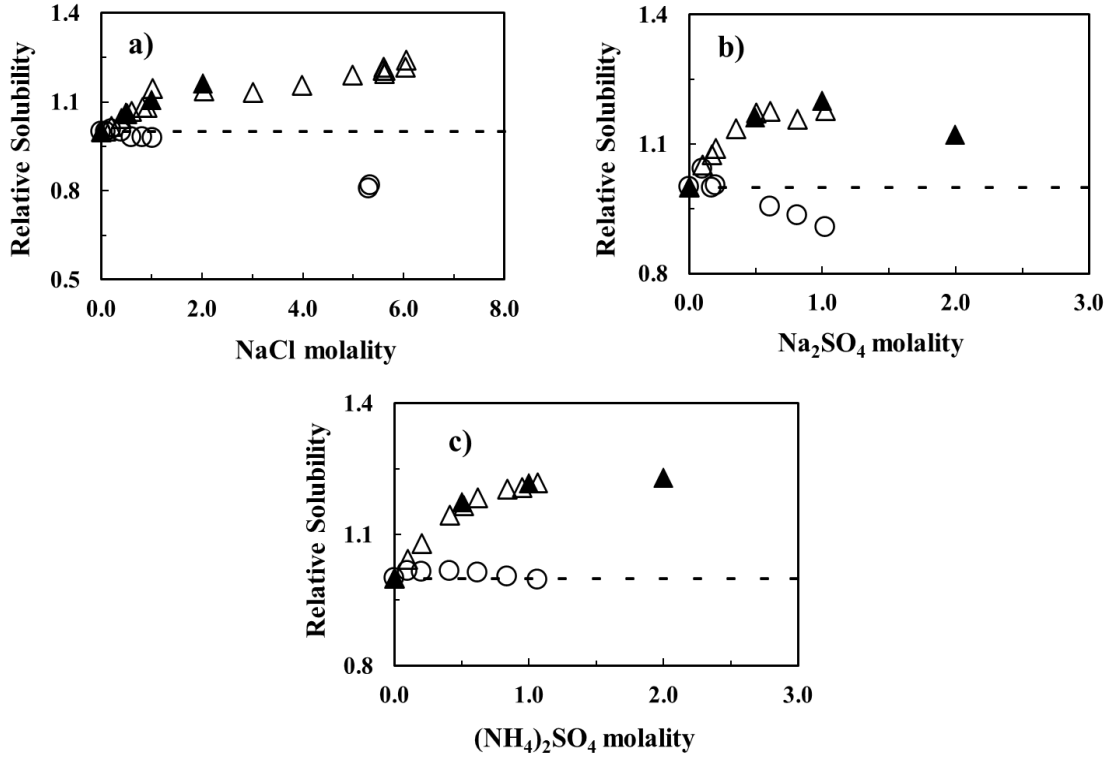

Figure 1. 8. Relative solubility of △, diglycine[108]; ▲, diglycine[109], and ○, glycyl-L-alanine[108] in aqueous a) NaCl, b) Na_2SO_4, c) $(NH_4)_2SO_4$ solutions at 298.15 K.

By comparing the solubility data of diglycine (Gly-Gly) and glycyl-L-alanine (Gly-Ala), the effect of the additional methyl group in Gly-L-Ala can be analysed. As can be seen, the

relative solubility of Gly-L-Ala is lower than diglycine in the studied systems. In all cases, the relative solubility of diglycine increases until salt concentration up to 1 molal, with similar magnitude for the three salts. After, when the concentration of Na_2SO_4 is 2 molal, the relative solubility of diglycine decreases[109]. For this peptide, the data from two different sources are consistent. On the other hand, for Gly-L-Ala, a small salting-in effect is observed at very low salt molalities, followed by a salting-out with increasing salt concentration. It is important to highlight here that one of the most relevant salting-out salts for proteins (ammonium sulfate) presents here a similar or even lower salting-out effect when compared with sodium chloride or sodium sulfate.

At saturation, as the salt concentration increases, the activity coefficient of glycine decreases which explains the salting-in effect[118]. On the other hand, the activity coefficient of glycyl-L-alanine moderately increases, and a salting-out effect is induced. Breil *et al.*[108] reported that due to an experimental error, the solubility values of glycyl-L-alanine in aqueous NaCl solution in the range of 2-5 molal were not considered.

1.2.3. Effect of pH on the solubility

The impact of the pH on the solubility of amino acids in water is very important. The pH of the environment affects the net charge of the molecule. The pH at which an amino acid, peptide or protein has a net charge equal to zero, in other words, where is equivalence between the positive and negative charges, is called the isoelectric point (pI)[119]. The solubility of proteins depends on a balance between the different interactions between proteins and the surrounding environment, being influenced by the composition of the medium[120].

When an amino acid dissolves in water at a pH close to the pI of the molecule, it turns into zwitterion, a molecular form that contains an equal number of positive and negative charges:

$$NH_2RCOOH\ (s) \overset{K}{\rightleftharpoons} NH_3^+RCOO^-\ (aq) \tag{1.1}$$

In acidic media the reversible acid-base reaction shifts from right to left (eq. 1.2) and the zwitterion accepts a proton and becomes positively charged:

$$NH_3^+RCOOH \overset{K_1}{\rightleftharpoons} H^+ + NH_3^+RCOO^- \tag{1.2}$$

In alkaline media, the zwitterion donates a proton and becomes negatively charged, due to the ionization of the amino group:

$$NH_3^+RCOO^- \overset{K_2}{\rightleftharpoons} H^+ + NH_2RCOO^- \quad (1.3)$$

K_1 and K_2 are the acid-base equilibrium constants of the two dissociation reactions[119], while K is a physical equilibrium constant related the solubilization process.

One of the first studies in the topic was on the solubility of tyrosine in aqueous HCl/NaOH solution[121]. The pH varied from 1.45 to 9.95. The lowest solubility was found at isoelectric point where pH varies between 5.1 and 5.5. The addition of acid increased the solubility and the highest solubility found was 6.3 times higher than at the isoelectric point, at pH 1.45, while the addition of base increased the solubility 13.7 times, at pH 9.95.

In 1971 Needham *et al.*[122] studied the pH effect on the solubility of glycine, L-alanine, L-valine, L-phenylalanine, and DL-aminooctanoic acid in pure water and in pure methanol at 298.15 K in the pH range between 2 and 10. The solubilities were measured using the shake flask method with further gravimetric analysis. At all pHs the solubility in water was inversely proportional to the size of the apolar part of the amino amino acids. Thus, the aqueous solubility followed the order: glycine > L-alanine > L-valine > L-phenylalanine. A low non-aqueous solubility was explained by a dominance of the charged α-amino carboxylic acid portion of the molecule. In both solvents the addition of either acid or base increased the solubility of the amino acids. In methanol it was observed a greater divergence from the isoelectric pH than in water.

In 1989 Zumstein and Rousseau[123] measured the solubility of L-isoleucine in aqueous HCl solution using high-performance liquid chromatography (HPLC) as analytical technique. It was found that when the concentration of acid was low, the total solubility of the amino acid was controlled by the zwitterionic form. Moreover, the solubility increased with the addition of acid as more of the soluble acidic form of L-isoleucine was formed by the chemical equilibrium shift. At very high HCl concentrations L-ile·HCl·H_2O was precipitated, and a salting-out effect was identified. The effect of the addition of NaCl, KCl and NH_4Cl at the solubility peak was checked in the same work. NaCl reduced the solubility of amino acid, like HCl, while KCl and NH_4Cl did not lead to much of a reduction in solubility. To get greater

yields, the authors advised to use NaCl instead of HCl as a precipitant for the recovery of L-ile from fermentation broths, because of the absence of additional liquid solvent. Following, Gatewood and Rousseau[124] studied the effect of NaOH on the solubilities of L-isoleucine, L-leucine, and L-valine at 298.15 K in the pH range 6.38 – 13.47. The solubilities of L-isoleucine and L-valine were also calculated by the Henderson-Hasselbalch equation at pHs 15.11 – 15.30, because of a negative interaction between the pH meter and sodium ions in solution at these high pH values. Increasing pH the solubilities of the amino acids were also increasing, but when the pH was higher than 12 the solubilities decreased. The reason of this decrease is again the formation of a new solid-phase sodium salt, Na·L-ile·1/2H_2O.

In the work of Carta and Tola[44,45] the solubility of different amino acids in water at various pHs and NaCl concentrations was studied at 298.15 K. Chemical analysis were carried out using spectrophotometric analyses and by determining the mass of the residue obtained by evaporating known volumes of the samples. The solubilities are given in mol/dm^3 of solution, which is not the best for modeling purposes. The addition of salt increased the solubility of L-cystine but decreased the solubilities of L-tyrosine and L-leucine. The addition of NaCl did not significantly affect the solubility of glycine. The size of the constant band around the isoelectric point is very similar for all the studied AAs.

In 1998 Pradhan and Vera[125] studied the effect of NaOH/HCl or KOH/HNO_3 on the solubility of glycine, DL-alanine, DL-valine and DL-serine at 298.2 K. The pH varied from 2 to 10 and the solubility was measured by gravimetry. It was shown that in this pH range, there is an increase in the solubility, but the impact on the solubility of DL-alanine is much smaller at higher concentrations of the acids or the bases.

Fuchs *et al.*[126] studied the solubility of DL-methionine (DL-met) in pure water as well as in aqueous electrolyte solutions at 303, 318 and 333 K in the pH range 1.5–9.5 by gravimetric measurements. For this HCl and NaOH were used. The solubility of the amino acids increased with adding acid/base and with increasing temperature. In 2009, the solubilities of DL-alanine, L-leucine, L-isoleucine, L-serine and DL-phenylalanine in water at 298.15 K were measured and quantified by ninhydrin assay with a colorimetric method[119]. The pH was controlled by adding HCl or NaOH, changing from 2 to 10. The solubility was mostly affected by the variation of pH for the DL-alanine, L-leucine, L-isoleucine and L-serine. The solubility of these amino acids increased as the pH deviated from the isoelectric point.

The solubility of glycine and its oligopeptides was studied at various pH in water, an aqueous solution of ethanol, NaCl, or PEG 600 by Lu *et al.*[43]. Moving from the isoelectric point increases the solubility, while the addition of cosolvents affects differently the solubility. The addition of ethanol and PEG 600 generally decreases the solubility of glycine and its oligopeptides, but the addition of NaCl increases the solubilities of diglycine and triglycine.

The effect of pH (from 2 to 10) on the solubilities of L-asparagine, L-glutamine, L-tyrosine, L-aspartic acid, and L-glutamic acid in water at 298.2 K was studied in the work of Lee *et al.*[103]. To reach the needed pH deionized water was mixed with HCl or NaOH and the ternary solution was prepared by adding a small excess of AA. The solubility in water studied by ninhydrin assay using a calorimetric method, followed the order: L-gln > L-asn > L-glu > L-asp > L-tyr, and the solubility of AAs was found the lowest at the isoelectric point.

In the work by Franco *et al.*[127] a new model to describe the solubility of amino acids and peptides as a function of pH was successfully applied. The model combines chemical and physical equilibrium, being the activity coefficients are calculated by an extended Pitzer model. The calculated data was compared with the experimental solubility data collected from the literature for DL-alanine, L-leucine, L-isoleucine, L-serine, L-tyrosine, DL-phenylalanine, glycine and its oligopeptides at 298.2 K, and for DL-methionine at 303 K.

The influence of the pH and ionic liquids (IL) on the solubility of L-alanine and L-glutamic acid was also studied by Voges *et al.*[128], with measurements carried out at 303.2 K and using UV spectroscopy combined with gravimetry as analytical techniques. Additionally, PC-SAFT was applied to represent the solubility. The calculated solubility was found constant between pH 4 and 8 and increased just when pH was higher or lower. On the other hand, the calculated solubility of L-glutamic acid was in an agreement with the experimental data up to pH 6.2. While the calculated solubility of L-glutamic acid was monotonically increasing with increasing pH, the experimental solubility was constant between pH 6.2 and 8.2 and increased when pH was higher than 8.2. At pH 6.2 the solid phase changed from L-glutamic acid to L-glutamate monohydrate.

The solubility data of DL-alanine, L-valine, L-isoleucine, and L-leucine as a function of pH were found in different works. The data found for DL-alanine, L-valine, and L-isoleucine were similar. The consistency of the data between different sources can be observed in **Figure 1. 9** for DL-alanine. However, the data found for L-leucine in the work of Tseng et al.[119]

was different from the results of Gatewood and Rousseau[124]. Although the solubility of L-alanine was similar to the solubility of DL-alanine at high pHs, the data found at the isoelectric point and low pHs showed lower solubility of L-alanine than DL-alanine. The difference can be also verified in the case of phenylalanine over the whole pH range, where the L isomer shows higher solubility than DL-phenylalanine with a greater divergence from the isoelectric point.

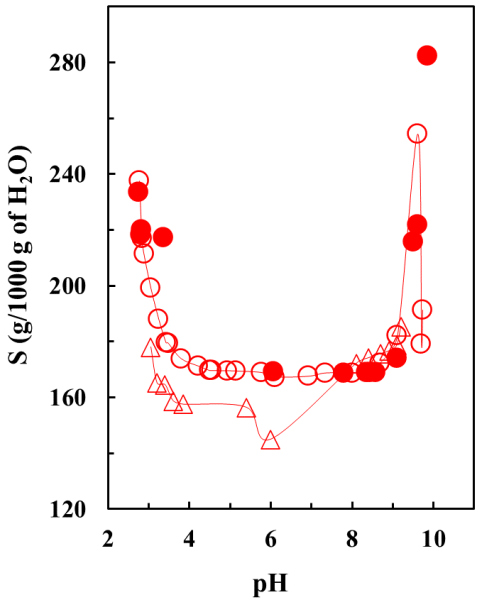

Figure 1. 9. Solubility of ●, DL-alanine [125]; ○, DL-alanine [119]; △, L-alanine [122] in water as function of pH at 298.2 K.

1.2.4. Molecular dynamics

The molecular dynamics (MD) simulation method is a very useful tool to understand the molecular-level mechanisms related to the interactions in condensed phase systems as in aqueous solutions of salts and amino acids or peptides. Despite all efforts, the effect of the nature and concentration of ions has not yet been well explained which hinders the understanding of many biochemical processes, including biochemical disorders related with human diseases[3–5,19]. After many studies, different effects of ions with different charges on the AA solubility were observed, some of which follow the Hofmeister series. In a series of recent papers[27,40,79], the effect of different salts on the solubility of alanine, valine, isoleucine, 2-aminodecanoic acid (Ada) was studied and molecular dynamics simulations were performed to assist the interpretation of the experimental data. The chemical structures of these amino acids are shown in **Figure 1. 10**.

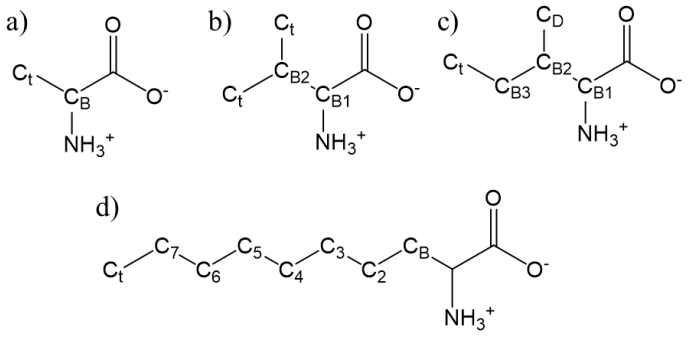

Figure 1. 10. Structure and atom labelling of a) alanine (Ala), b) valine (Val), c) isoleucine (Ile) and d) 2-aminodecanoic acid (Ada).

In all these works, molecular dynamics studies were carried out for the zwitterionic forms (pH = 7) of amino acids, using the GROMACS computer code. The isothermal-isobaric conditions were fixed for the simulations, at T = 298.15 K and p = 1 bar. The Lennard-Jones potential model was used to calculate the intermolecular interaction energy between pairs of neighboring atoms, while the point-charge Coulomb potential model was used for the electrostatic interactions. Radial distribution functions (RDFs) were calculated for selected ions around a particular group of the studied amino acids. Tomé *et al.*[27] performed MD simulations for alanine, valine, isoleucine and 2-aminodecanoic acid in aqueous NaCl, KCl, $NaNO_3$, $NaClO_4$ and Na_2SO_4 solutions. A small but noticeable decrease of the intensity of the RDF peak of the water oxygens around the terminal carbon atom was observed from Na_2SO_4 to NaCl/$NaNO_3$ to $NaClO_4$. This means the amino acids were more solvated by water in the presence of the sulfate anion, than in the presence of perchlorate, that induces a salting-in effect. The calculated RDFs show that the divalent sulfate ion interacts with the positively charged group of amino acids; a salting-in effect was observed for small amino acids like glycine, but a salting-out effect was found for amino acids with large nonpolar side chains. Interestingly, ClO_4^- is able to bind the nonpolar part of amino acids with large nonpolar moieties and show salting-in effect. The NO_3^- and Cl^- anions do not change significantly the solubility of amino acids, but the first interacts with the apolar part and increases the solubility more noticeable if the nonpolar group is large, while the second shows a slight salting-out effect as it does not interact with apolar parts. To see the effect of salt concentration on the solubility of amino acids, MD calculations were performed for alanine in aqueous KCl solution at three concentrations. The RDFs showed how the interaction of salt ions with

charged and nonpolar moieties of the amino acid decreased while the salt concentration increases.

The specific effects of cations can be found in another work by Tomé *et al.*[79], in which results from MD simulations for alanine, valine and isoleucine in aqueous $MgCl_2$, $MgSO_4$, NH_4Cl or $(NH_4)_2SO_4$ solutions are reported. It was shown that sulfate anion induces a salting-out effect in all the studied amino acids regardless of the cations, as expected from the Hofmeister series for proteins. From the RDFs, it was observed that the peaks of NH_4^+, Na^+ and K^+ around the terminal carbon atom of the amino acid side chain (Ct, **Figure 1. 10**) occur at a larger distance and these cations do not interact with the hydrophobic groups of the amino acids. The low charge density ammonium cation shows stronger interaction (through a combination of ion-induced dipole and dispersion interactions, promoting a smaller stabilization of the amino acids in water) than the sodium and potassium ions, which do not interact with the hydrophobic groups of alanine, valine or isoleucine. KCl showed higher salting-out effect than NaCl because of weaker interactions of K^+ with the nonpolar groups of alanine. The salts containing Mg^{2+} showed strong salting-in effect because of strong interaction with the COO^- groups of the amino acids and the formation of complexes. A comparison of simulation data obtained for Val in aqueous NH_4Cl and $(NH_4)_2SO_4$ or $MgCl_2$ and $MgSO_4$ solutions showed that the presence of salting-out anion decreases the effect of salting-in cation. In the case of $(NH_4)_2SO_4$, salting-out effect induced by the sulfate anion even changes the solubility effect. So, salts containing the strong salting-out sulfate anion, induce a salting-out effect on the solubility of amino acids, in the cases where cations have a small affinity to the amino acid. The less salting-in effect of $MgSO_4$ with valine than in the case of $MgCl_2$ can be explained in terms of the cation-anion competition. It was concluded that cations causing the salting-in effect to have different molecular mechanisms compared to the anions causing salting-in effect. Moreover, if anions interact with nonpolar groups of the biomolecules, salting-in cations interact with the charged end groups, especially with the carboxylate group.

Tomé *et al.*[40] also studied the effect of polyvalent and monovalent cations on the solubility of alanine, valine and isoleucine in aqueous LiCl, Li_2SO_4, K_2SO_4, $CaCl_2$, $AlCl_3$ or $Al_2(SO_4)_3$ solutions. From the radial distribution functions (both the intensity and position of the peaks), showing the interactions of C_t and C_b (other carbon atoms of the same alkyl chain) atoms of isoleucine with cations, they concluded that the interactions only occur at a second

solvation layer with polyvalent Ca^{2+} and Al^{3+} cations and not in the presence of the monovalent cations as Li^{+} or K^{+}. The strongest peak was observed for $AlCl_3$ and the weakest for $Al_2(SO_4)_3$. The peak of $CaCl_2$ occurred for longer distance than $AlCl_3$ and $Al_2(SO_4)$, and the peak of the interaction of polyvalent cations with C_b atom was more intense than with C_t atom. It was proposed that it happened because C_b atom is closer to the COO^- group. Also, the interaction of divalent and trivalent cations with the negatively charged group of isoleucine was stronger than the interaction of monovalent cations. The strength of the interaction between the oxygen atoms of the carboxyl group of isoleucine and the cations followed the order $AlCl_3 > Al_2(SO_4)_3 > CaCl_2$. Besides the negatively charged group, Al^{3+} could also bind with the amino groups of amino acids, which also explains the strong salting-in effects. Monovalent cations could not form very soluble charged complexes with the biomolecules as polyvalent cations probably because their interaction with water was stronger. But as can be seen (**Table 1. 8**) the divalent cations had large energies of hydration and should form hydration complexes which would contribute to the dehydration of the amino acids and lead to salting-out what did not happen. The aluminum and calcium cations showed salting-in effect.

Table 1. 8. The enthalpies of hydration of the ions[40].

Ion	$\Delta_{hyd}H$ / kJ mol^{-1}
Cl^-	-367
SO_4^{2-}	-1035
K^+	-334
Li^+	-531
Ca^{2+}	-1602
Al^{3+}	-4715

Single charged cations interact weakly with charged groups of amino acids and do not interact with their nonpolar parts.

When making the transition from the molecular dynamics' studies of amino acids to peptides, a careful validation step of the peptide bond is required. One of the few works studying the ion effect on the solubility of peptides was presented by Paterová *et al.*[129]. In that work, triglycine was studied in aqueous solutions of NaCl, NaBr, NaI, NaSCN or Na_2SO_4. The RDF showed the interaction of these anions with the regions around each of the three backbone CH_2 groups of capped and uncapped triglycine. The first region contained the N-terminus and the third one contained the C-terminus (carboxyl-terminus). With the capped

triglycine the total affinities for the anions followed the order $SO_4^{2-} < Cl^- < Br^- < I^- < SCN^-$ as the Hofmeister series for proteins. It was reported that in the case of capped triglycine, only weakly hydrated anions (iodide and thiocyanate) interacted with the peptide bond and strongly hydrated ones (like sulfate) did not show this interaction. The Cl^- and Br^- anions did not show preferential binding to the peptide, so they acted as Hofmeister-neutral ions. Compared to others, I^- had some interaction with the backbone CH_2 groups. Close to the N-terminus, SCN^- bound to the backbone CH_2 group even stronger. At the same concentrations SCN^- showed stronger salting-in effect than I^-. With the uncapped triglycine affinity of anions followed the order $SO_4^{2-} > Cl^- > Br^- > SCN^- \approx I^-$, as the Hofmeister series in reverse order, except the thiocyanate anion. MD simulations predicted that it had a stronger affinity than the halide anions. The importance of the structure and size of the biomolecules in the interactions with ions was also highlighted. So weakly hydrated anions should interact less with small peptides like triglycine compared to proteins because the former are completely surrounded by water.

Santosh *et al.*[34] described MD simulations of diglycine in aqueous solutions with the six transition metal ions Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}. The temperature and pressure were kept constant at 298.15 K and 1 atm, respectively. The analysis of results showed that there was no direct ions-dipeptide interaction. The divalent ions kept their hydration shell consisting of six water molecules and interacted with the dipeptide mostly as Me^{2+}-$6H_2O$ complexes. This hydration shell was broken only by Cl^- when the salt concentrations were high. It is important to mention that the dipeptide was not modelled as a zwitterion. The divalent cations did not show any interaction with the NH_2 group of peptide. The stronger binding of small ions (Cu^{2+}, Zn^{2+}) than larger ions (Fe^{2+}, Mn^{2+}) to the carbonyl oxygen and hydroxyl oxygen of dipeptide was observed. That interaction was mediated by water.

Bröhl *et al.*[130] studied three proline-based peptide models in aqueous sodium salts (NaSCN, Na_2SO_4 and NaOAc). The peptide models used in their work were (S)-N-acetyl proline methyl ester (N-Ac-Pro-OMe), (S)-N-(tert-butyloxycarbonyl) proline methyl ester (N-Boc-Pro-OMe), and (S)-N-(tert-butyloxycarbonyl) proline methyl amide (N-Boc-Pro-NHMe). The chemical structures of these peptide models are shown in **Figure 1. 11**.

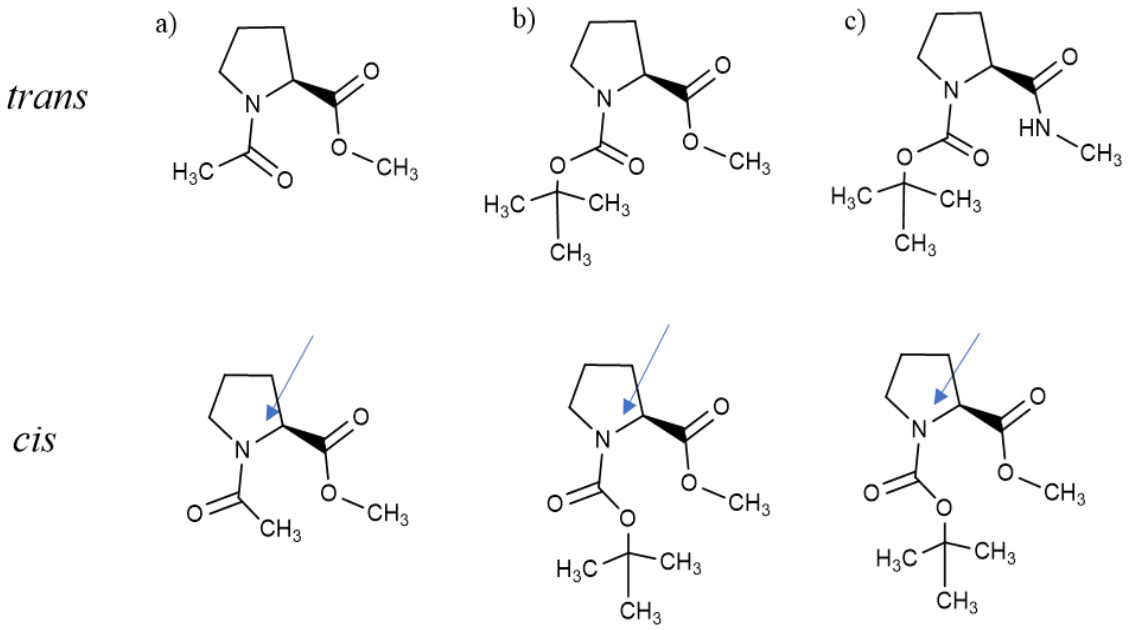

Figure 1. 11. Chemical structures of the peptide models: a) N-Ac-Pro-OMe, b) N-Boc-Pro-OMe and c) N-Boc-Pro-NHMe.

The electrostatic potential surfaces (EPS) for *cis* and *trans* conformers of all three peptide models were calculated. The results showed stronger interaction of the anions with the "back" side of the *cis* conformers (blue arrows in **Figure 1. 11**). However, as EPS were calculated for a vacuum environment, these authors performed additional MD simulations for each conformer under isothermal-isobaric conditions to understand the effect of the solvent. The anion interaction with the *cis* conformers was more noticeable from spatial and radial distribution functions of SCN^- around N-Ac-Pro-OMe, AcO^- around N-Boc-Pro-OMe, and SCN^- around N-Boc-Pro-NHMe. These studies showed that it happens because of orientation of both carbonyl groups of each *cis* conformer that is different in the *trans* conformers and allows anions to bond. The exception was the interaction of N-Boc-Pro-NHMe with SCN^- solution, which showed a strong interaction with both the *trans* and *cis* conformers, and N-Boc-Pro-NHMe with AcO^-, where a slightly stronger interaction with the *trans* conformer was observed. The authors explained that this interaction may have happened because the force field used in this work ignored polarizability, as it could show problems with the acetate ion force field. The simulations showed the weak interaction of cations (Na^+) with the peptide model (with the amide groups), in agreement with the theory that anion-amide interactions are stronger[131].

In 2016, Balos *et al.*[132] used the rotational mobility of a model amide in aqueous solution to estimate the interactions of the amide group with different anions. The N-methylacetamide (NMA, **Figure 1. 12**) aqueous solutions in the presence of inorganic salts (the potassium salts of Cl^-, NO_3^-, Br^-, I^-, and SCN^-) were used.

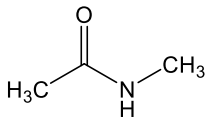

Figure 1. 12. Chemical structure of N-methylacetamide (NMA).

Even if NMA and its rotational dynamics were different from a protein, according to the authors it is an ideal model to study interactions with the amide group. The studies showed that the rotational mobility of NMA was not affected by the salts such as KCl and KNO_3 and was only affected by denaturants such as KI and KSCN. The results of this work were compared with the results of the previous work[133,134], where it was studied the interaction of the guanidium cation (Gdm^+) with amide groups, using NMA and compared with other cations such K^+, Na^+, Li^+, and Mg^{2+}. As a result, it was found that even if the Gdm^+ interacts with the amide (as Na^+), this interaction is weaker, compared to Li^+ and Mg^{2+}, while no interaction was observed for K^+. The comparison of two works showed that some anion-amide interactions are weaker than cation-amide interactions. For example, the strongly denaturing Li^+ cation had stronger effect on the mobility of NMA than SCN^- anion. The results showed that monovalent cations decrease the rotational mobility around twice more than monovalent anions, when both of them are located at the end in the Hofmeister series. The fact that the interactions of the cations with the C=O of NMA group are stronger than the interactions of anions with the N-H group was also observed in other work[129,135–137].

In the work of Hladiková *et al.*[135], three modifications of the triglycine molecule (uncapped, half-capped (with the N-terminus terminated by an acetyl group), and fully capped (with the N-terminus terminated by an acetyl group and the C-terminus by an N methylamide group) were studied. Their structures are shown in **Figure 1. 13**.

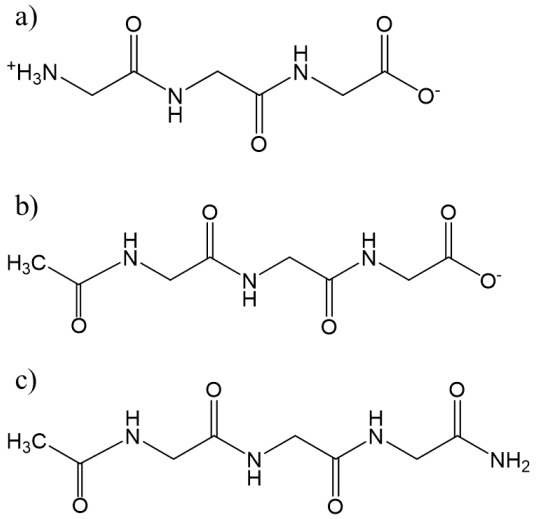

Figure 1. 13. Chemical structures of a) uncapped, b) half-capped and c) capped triglycine molecule.

Each of them was solvated in NaF, NaCl, NaBr, NaI, NaSCN, $NaClO_4$ and Na_2SO_4 aqueous solutions. The salting-out constants were calculated and compared with the experimental values found by Nandi and Robinson[17], where they measured the salting-out constants of short olygoglycines and for this they capped both the C- and N-termini. It was noticeable that the simulations of the half-capped system were quantitatively similar with the experiment, even though for the other two variants some differences were patent. These results suggest that Nandi and Robinson[17] failed in capping C-terminus. The half-capped triglycine was negatively charged and had only the N-terminus neutralized, and so anions had to be repelled from it and sodium attracted. The strongly hydrated anions (SO_4^{2-} and F^-) dominated over Na^+ cation, thus leading to a positive salting-out constant. The weakly hydrated anions (I^-, ClO_4^-, SCN^-) were also attracted to the peptide backbone but led to negative salting-out constants. So, in the case of half-capped triglycine molecule, a salting-in or salting-out effect is the result of interactions of both cations and anions with the backbone and the C-terminus and cannot be explained simply in terms of only ion-backbone interactions. To see the Hofmeister effects of ions on the peptide backbone, the capped triglycine should be studied. The sodium cations and strongly hydrated anions weakly interacted with the capped triglycine backbone and led to close to zero positive salting-out constant, what again showed that in the work of Nandi and Robinson[17] the triglycine was not fully capped. The weakly hydrated anions were attracted to the backbone (to the site with the amide NH group) and led to negative salting-out constants. In the case of uncapped triglycine, the strongly hydrated ions led

to large salting-out constants, implying a salting-in effect. The weakly hydrated anions also led to negative salting-out coefficients, as a result of additional attraction to the backbone. Thus, there was no Hofmeister ordering for uncapped triglycine. In total, simulations and measurements showed stronger ion interactions with the charged C-terminus of an aqueous peptide.

Finally, Rembert *et al.*[138] showed the interactions of SCN^-, I^-, SO_4^{2-} and Cl^- anions (all with the sodium cation) with a model system containing a pentameric amino acid repeating unit, $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Pro)_{120}$ (uncharged 600-residue elastin-like polypeptide). This a very interesting work, as the peptide studied has a very high molecular weight. The charges of the N-terminus (by acetylating) and C-terminus (by methylating) were removed. The isothermal-isobaric conditions were used in the simulations. Anions such as SCN^- and I^- (large, soft, denaturating anions) interacted with the polypeptide backbone through a hybrid binding site that consists of the amide nitrogen and the adjacent α-carbon (thus showing salting-in effect) and did not interact with the hydrophobic side chains. The Cl^- anion interacted weakly with the amide nitrogen/α-carbon, but the SO_4^{2-} presented no binding with the backbone and hydrophobic side chains of the polypeptide. The Na^+ cation bound only to the C=O sides of the peptide bonds and not to CH_n groups.

1.3. Objectives and summary of the measurements

The study of solubilities of amino acids in aqueous electrolyte solutions was a main objective of this work. And although much data on amino acid solubility in aqueous salt solutions can already be found, some measurements were carried out for the first time in this Thesis, which opened the opportunity to contribute significantly for new knowledge on the solvation science area. The goal was to select ions from the Hofmeister series covering a wide range in the series to check their effects on the solubilities of AAs with different polarity. Additionally, the objectives were also to model the solubility data AAs using the ePC-SAFT, and to check the effect of pH on the solubility of AA and peptides.

In line with the work on the topic being developed in our group[6,27,40,70,74,79], this Thesis considers the solubility of L-aspartic acid, L-phenylalanine, glycine, and L-leucine in an aqueous inorganic solution containing the anions chloride, nitrate or thiocyanate, and the cations sodium, potassium or ammonium, and also magnesium or calcium cations, with chloride or nitrate anions. The solubility measurements are provided for L-glutamic acid, L-aspartic

acid, L-tryptophan and L-tyrosine in aqueous KCl and $(NH_4)_2SO_4$ solutions as well. Along with this, the studies were carried out for Asp in aqueous $(NH_4)_2SO_4$ solution and for L and DL isomers of valine in aqueous $NaNO_3$ and KNO_3 solutions. The effect of the salt with the organic anion (Na-tosylate) on the solubility of glycine, L-leucine, L-phenylalanine and L-aspartic acid were also studied. The pH of all the saturated solutions was monitored, and the solid-phase studies of the AAs as received from the supplier and solids settled in equilibrium with the saturated solution were provided. The solubility data of Asp, Glu, Trp and Tyr in aqueous KCl and $(NH_4)_2SO_4$ solutions were modeled using ePC-SAFT. The solubility measurements of glycine, diglycine and N-acetylglycine were checked in water and aqueous Na_2SO_4 solution with an addition of NaOH or H_2SO_4. The solubility measurements of L-aspartic acid, L-leucine, L-phenylalanine in aqueous solutions of KCl, $NaNO_3$, KNO_3, NH_4Cl, NH_4NO_3, $MgCl_2$, $CaCl_2$; L-glutamic acid in aqueous $(NH_4)_2SO_4$ solution; L-tryptophan and L-tyrosine in aqueous KCl and $(NH_4)_2SO_4$ solutions; all the solubility measurements in aqueous solutions of $Mg(NO_3)_2$, $Ca(NO_3)_2$, NaSCN, KSCN and NH_4SCN; and the pH influence on the solubility of glycine in aqueous Na_2SO_4 solution, diglycine, and N-acetylglycine in water and aqueous Na_2SO_4 solution were measured for the first time in this work.

Chapter 2 – Materials and methodology

Herewith it is included a description of the chemicals used and methodologies for solubility and pH measurements, solid – phase studies and thermodynamic modelling. To assist the discussion of the results, the chemical species speciation as a function of pH is presented in **Appendix B**.

2.1. Chemicals and experimental methods

2.1.1. Chemicals

The source, CAS and purity of the used chemicals are given in **Table 2. 1.** All the AAs were used without further purification and stored in a desiccator to maintain the AAs dry. The non-hydrated salts were dried in the oven at 343.15 K for at least 24 h and, before use, cooled in the desiccator with silica gel. The solvents were prepared using deionized water (resistivity of 18.2 MΩ·cm, no particles with size ≥ 0.22 μm, and total organic carbon < 5 ppb).

Table 2. 1. Source and mass fraction purity of the compounds.

Chemicals	Supplier	CAS	Mass fraction purity
Glycine (Gly)	Merck	56-40-6	≥ 0.997
L-leucine (Leu)	Merck	61-90-5	≥ 0.990
L-phenylalanine (Phe)	Merck	63-91-2	≥ 0.990
L-tryptophan (Trp)	Affymetrix	73-22-3	0.985-1.015
L-tyrosine (Tyr)	Merck	60-18-4	≥ 0.990
L-aspartic acid (Asp)	Alfa Aesar	56-84-8	≥ 0.980
L-glutamic acid (Glu)	Alfa Aesar	56-86-0	> 0.990
L-valine (Val)	Alfa Aesar	72-18-4	0.990
DL-valine (Val)	Alfa Aesar	516-06-3	0.990
Diglycine (Gly-Gly)	Sigma-Aldrich	556-50-3	≥ 0.990
N-acetylglycine (NAGly)	Acros Organics	543-24-8	0.990
Sodium chloride	Fluka	7647-14-5	≥ 0.995
Sodium nitrate	PanReac	7631-99-4	≥ 0.990
Sodium thiocyanate	Acros Organics	540-72-7	> 0.980
Potassium chloride	PanReac	7447-40-7	≥ 0.990
Potassium nitrate	PanReac	7757-79-1	≥ 0.990

Potassium thiocyanate	Acros Organics	330-20-0	> 0.990
Ammonium chloride	PanReac	12125-02-9	≥ 0.995
Ammonium nitrate	PanReac	6484-52-2	≥ 0.990
Ammonium thiocyanate	Acros Organics	1762-95-4	> 0.990
Ammonium sulfate	PanReac	7783-20-2	0.990
Magnesium chloride hexa-hydrate	PanReac	7791-18-6	≥ 0.990
Magnesium nitrate hexahy-drate	PanReac	13446-18-9	≥ 0.980
Calcium chloride dihydrate	Fluka	10035-04-8	≥ 0.990
Calcium nitrate tetrahydrate	Alfa Aesar	13477-34-4	≥ 0.990
Sodium p-toluenesulfonate	Acros Organics	657-84-1	≥ 0.975

2.1.2. Solubility experiments

The solubility was measured using the traditional shake flask method with analysis of the composition of the saturated solutions carried out by gravimetry, UV spectroscopy, or measuring the refractive index. The experimental setup is presented in **Figure 2. 1**. It includes the thermostat water bath that allows for maintaining the solutions at a constant temperature (±0.1 K), being stirred on the magnetic stirring plates.

The water + salt solutions (0.5, 1, or 2 molal) were prepared by gravimetry (Denver Instrument, ± 0.0001 g) while the ternary systems were prepared by adding AA (in slight excess to the expected saturation limit) into the equilibrium cell and a known amount of aqueous salt solution. To measure the solubilities of glycine, diglycine, and N-acetylglycine at different pH the solvent was prepared by adding an acid or a base to water. 1 molal solution of Na_2SO_4 was prepared by adding the needed amount of salt into the water + acid/base mixture. In these measurements, H_2SO_4 or NaOH was used. The ternary solutions were placed into the water bath at 298.2 K and mixed with a magnetic stirrer for around 30 hours to reach equilibrium. The speed of the mixing was maintained between 500-700 rpm in all the experiments. After, the mixing was stopped, and the solutions were left to rest for at least 12 hours to settle the undissolved particles before sampling. Four samples (approximately 2-4 cm^3) were collected from the saturated ternary system into the glass vessels with the known

masses using preheated plastic syringes with syringe filters (0.45 mm pore diameter, **Figure 2. 1**), to prevent precipitation of solutes in solutions.

Finally, the composition of the samples was measured using the methods described in the next section.

Figure 2. 1. The thermostat water bath, pH meter and all the necessary materials.

2.1.2.1. Chemical analysis methods

2.1.2.1.1. Gravimetry

The glass vessels with the samples were immediately weighed. The drying followed two steps; first, the vessels were placed in the fume hood to evaporate water. After forming crystals (**Figure 2. 2**), they were dried in the drying stove at 343.15 K. Before weighing the samples, they were first cooled in the desiccator with silica gel. The process was repeated every week until crystals were dried entirely, and a constant mass value was achieved. After calculating the difference in the masses, the amount of evaporated water was found and using the knowledge of the initial concentration of salt, the mass of the salt was determined. The remaining mass was the amount of amino acid dissolved in the aqueous salt solution. Each solubility value is an average of at least three different measurements[70].

Figure 2. 2. The glass vessels with formed crystals.

2.1.2.1.2. Refractive index measurement

This method was used to find the solubilities of the AAs dissolved in aqueous solutions of hydrated salts, thiocyanates and Na-tosylate, and also to find the effect of pH on the solubilities of glycine, dipeptide and N-acetylglycine in water and in 1 molal Na_2SO_4 aqueous solution.

The refractive index was measured (at 298.2 K) in a digital refractometer (Abbemat 500, Anton Paar, **Figure 2. 3**) with a reproducibility within ± 0.00002. The measuring range is between 1.30-1.72 nD, in a temperature range between 10 and 85°C.

Figure 2. 3. Refractometer (Abbemat 500, Anton Paar).

The calibration curve ($R^2 > 0.904$), relating the amino acid concentration (in g kg^{-1} of solution) and the refractive index, was built by weighting five standard solutions of known AA composition at a fixed salt concentration (**Appendix D: Figure D 1**). The refractive index was measured (at 298.2 K) in a digital refractometer (Abbemat 500, Anton Paar) with a reproducibility within ± 0.00002.

The glass vessels with the samples were weighed, and if needed, diluted with a weighed amount of (water + salt) solution in order to get refractive indexes within the calibration curve range. Before each set, the refractive index of pure water was verified. Finally, the refractive indexes of each solution were determined twice, by placing about 1 ml of sample with a micropipette on the refractometer prism, and the solubility value was calculated from the calibration curve. At least three independent values are used to find the final average solubility. It is very important to inform that the exactly same aqueous salt solution (0.5, 1, or 2 molal) initially prepared is used to find the calibration curve, to prepare the saturated solution, and also to dilute (when needed) the saturated solution before the refractive index measurement.

2.1.2.1.3. UV spectroscopy method

The determination of the concentration of the AA with the lowest solubility (L-tyrosine) in the saturated ternary systems was carried out by UV spectroscopy (Jasco V-730 spectrometer, **Figure 2. 4**) with wide wavelength range – 190 to 1100 nm. To do so, calibration curves for this amino acid were obtained at 274 nm wavelength ($R^2 = 0.9998$). The samples were prepared by diluting the binary (AA+water) solution, with a known concentration, with water and thus seven standard solutions were used to build the calibration curve. For the measurement of absorbance, liquid samples were held in a cuvette. This UV spectrophotometer with a double beam optical system requires the use of a reference (water) and a sample. First, a baseline correction was done by placing water in both cuvettes and after the absorbance of the samples were measured twice. The measurements were started with the sample with the lowest concentration.

The middle area in the calibration curve was chosen and the mass fraction was registered. The calculated amount of water was added to the four glass vessels with the samples and the absorbance of each sample was measured. The equation of the calibration curve was used to

calculate the mass fractions of each solution and thus the solubility was found. The average of the solubilities values was calculated.

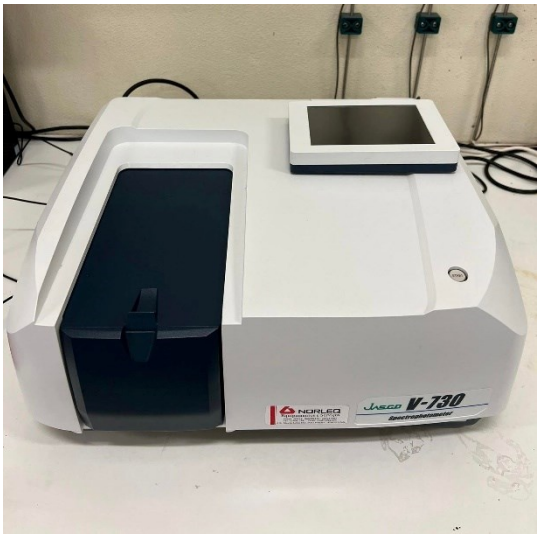

Figure 2. 4. Jasco V-730 UV-Visible spectrophotometer.

2.1.3. Karl Fischer titration

The water content of the hydrated salts (magnesium chloride hexahydrate, magnesium nitrate hexahydrate, calcium chloride dihydrate, calcium nitrate tetrahydrate) was determined by Karl-Fischer (KF) titration, and that value was considered for the salt molality determinations. For this, 831 KF Coulometer, Metrohm model (**Figure 2. 5**) was used. The method consists of titrating the sample of salt diluted in methanol, with the KF reagent, which is a solution containing iodine, sulfur dioxide and a base as buffer. According to the reaction that occurs, iodide reacts quantitatively with water.

Liquid samples were added with the aid of a 5 ml syringe with a long needle. Around 0.2 ml of a sample were injected. The actual sample weight was determined by weighing the syringe before and after injection. Starting with methanol, for each sample, at least three measurements were carried out. And from additional calculations, the amount of water presented in the studied salt was determined. The end point of the titration can be detected visually by the changing of color caused by iodine.

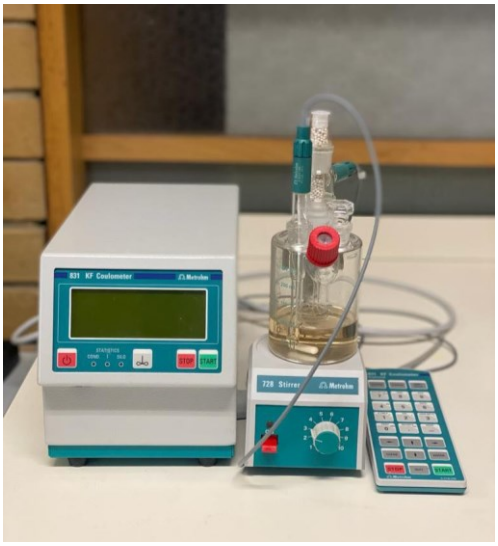

Figure 2. 5. 831 KF Coulometer, Metrohm.

2.1.4. pH measurement

As pH influences the protonation of the chemical species in solution[2], this parameter was also taken into account. Thus, after each measurement the pH of solution was measured by using a pH meter (WTW inoLab pH level 1) and pH-electrode (WTW SenTix 41) or a pH meter (Nahita Model 903, **Figure 2. 1**) and pH-electrode (METTLER TOLEDO InLab Ultra-Micro-ISM) for studies in aqueous solutions containing divalent cations and thiocyanates. A pH calibration was provided at 298.2 K by measuring a pH of buffer solutions.

2.1.5. Solid phase studies

The pure solid compounds as received from the supplier as well as solids settled in equilibrium with the saturated solution, after vacuum filtration and drying, were analyzed by powder and single crystal X-ray diffraction (XRD).

Powder XRD data were collected on a X'Pert MPD Philips diffractometer, using Cu-Kα radiation (λ = 1.5406 Å), with a curved graphite monochromator, a set incident area of 10 mm^2, and a flat plate sample holder, in a Bragg–Brentano para-focusing optics configuration. Intensity data were collected by the step counting method (step 0.02° and time 5s) in the range $5^o < 2\theta < 50^o$.

The cell parameters of suitable crystals of selected L-aspartic acid, L-phenylalanine, glycine, and L-leucine from supply as well the samples obtained after crystallization with the different salts, were determined on a Bruker D8 QUEST diffractometer equipped with a Photon

100 area detector, with monochromated Mo-Kα radiation (λ = 0.71073 Å) and operating at 150(2) K. The selected crystals analyzed were put at 40 mm from the photon 100 detector and the spots were measured using different counting times (varying from 5 to 30 s).

2.1.6. Thermodynamic modeling

The thermodynamic modeling of these complex mixtures is essential to cover all the salt/amino acid combinations, temperature and salt concentration conditions while reducing the experimental data needed. Recently, the melting properties of twenty amino acids were measured by Fast Scanning Calorimetry, and the Perturbed-Chain Statistical Association Theory (PC-SAFT) was successfully applied to the prediction of the solubility of 15 amino acids in water (including the ones studied in this work) in a wide temperature range[99]. Thus, to account for the presence of the salts, the electrolyte Perturbed-Chain Statistical Association Theory (ePC-SAFT)[139,140] was used in this work to describe the solid-liquid equilibria of the ternary mixtures water + amino acid + salt. The model has been already applied to a wide range of mixtures of amino acids and peptides, water, organic solvents, and electrolytes, being a practical tool for designing processes involving complex multicomponent systems[49,141,142].

The solid-liquid equilibrium relation between solid AA and the saturated liquid aqueous phase was applied according to Prausnitz[120]. Among others, this relation assumes pure solid AA phase, i.e., no mixed crystals, and allows determining the AA solubility x_i^L as:

$$ln\left(x_{\mathrm{i}}^{\mathrm{L}} \cdot \gamma_{\mathrm{i}}^{\mathrm{L}}\right) = \frac{\Delta h_{0\mathrm{i}}^{\mathrm{SL}}}{R \cdot T_{0\mathrm{i}}^{\mathrm{SL}}}\left(1 - \frac{T_{0\mathrm{i}}^{\mathrm{SL}}}{T}\right) - \frac{1}{R \cdot T}\int_{T_{0\mathrm{i}}^{\mathrm{SL}}}^{T} \Delta c_{\mathrm{p0i}}^{\mathrm{SL}}(T)\,dT + \frac{1}{R}\int_{T_{0\mathrm{i}}^{\mathrm{SL}}}^{T} \frac{\Delta c_{\mathrm{p0i}}^{\mathrm{SL}}(T)}{T}\,dT \qquad (2.1)$$

$$\Delta c_{\mathrm{p0i}}^{\mathrm{SL}}(T) = \left(a_{c_{\mathrm{p0i}}^{\mathrm{L}}} - a_{c_{\mathrm{p0i}}^{\mathrm{S}}}\right) \cdot T + \left(b_{c_{\mathrm{p0i}}^{\mathrm{L}}} - b_{c_{\mathrm{p0i}}^{\mathrm{S}}}\right) = \Delta a^{L-S} \cdot T + \Delta b^{L-S} \qquad (2.2)$$

In Eq. (2.1), $\gamma_{\mathrm{i}}^{\mathrm{L}}$ and R are the activity coefficient of AA in the saturated solution and the universal gas constant, respectively, and T is the temperature of the system. The melting properties are $T_{0\mathrm{i}}^{\mathrm{SL}}$ (melting temperature), $\Delta h_{0\mathrm{i}}^{\mathrm{SL}}$ (melting enthalpy at $T_{0\mathrm{i}}^{\mathrm{SL}}$), and $\Delta c_{\mathrm{p0i}}^{\mathrm{SL}}(T)$, which is the difference between the heat capacities of the liquid state (L) and the solid state (S) of the pure AA, denoted as '*0i.*' In Eq. (2.2), $\Delta c_{\mathrm{p0i}}^{\mathrm{SL}}(T)$ was assumed to change linearly with temperature, and $a_{c_{\mathrm{p0i}}^{\mathrm{L}}}$ ($a_{c_{\mathrm{p0i}}^{\mathrm{S}}}$) and $b_{c_{\mathrm{p0i}}^{\mathrm{L}}}$ ($b_{c_{\mathrm{p0i}}^{\mathrm{S}}}$) are the slope and the intercept of the liquid (solid) heat capacities, respectively.

In a previous work[143], the melting properties of several AA were measured with Fast Scanning Calorimetry (FSC), applying heating rates up to 20 000 $K \cdot s^{-1}$. These experimentally determined melting properties are required as an input for the thermodynamic framework PC-SAFT to predict the aqueous AA solubility using Eq. (2.1). The activity coefficients required in Eq. (2.1) were calculated using the revised ePC-SAFT, which combines the classical PC-SAFT[139] and the Debye-Hückel theory to account for the interactions among ions. The revised ePC-SAFT assumes the residual Helmholtz energy (a^{res}) as the sum of independent contributions accounting for hard-chain (a^{hc}), dispersion (a^{disp}), association (a^{assoc}), and Coulomb ($a^{Coulomb}$) attraction energies according to Eq. (2.3):

$$a^{res} = a^{hc} + a^{disp} + a^{assoc} + a^{Coulomb} \tag{2.3}$$

In this work, polar interactions were not accounted for and the standard Berthelot-Lorentz combining rules were applied for segment diameter and dispersion energy, while the Wolbach and Sandler mixing rules[144] were applied for the association parameters. An advanced model for electrolyte solutions[145] is available, which was not used in the present work as the conditions here focus on concentrated aqueous electrolyte solutions, and a deep study of new binary parameters within the new advanced model is not yet available. The dielectric constant is required for ePC-SAFT. The temperature-dependent function presented by Cameretti *et al.*[140] has been applied, representing the relative permittivity of pure water, assumed independent of the salt in the solution, in consistency with the modeling procedure when the ion parameters were fitted. The pure-component parameters of the AA and water are summarized in **Table 2. 2**, while the ion parameters are given in **Table 2. 3**.

Table 2. 2. PC-SAFT pure-component parameters of water and AA, the binary interaction parameters between AA and water, and melting properties from Do *et al.*[99]

	m_i^{seg}	σ_i	$\frac{u_i}{k_B}$	$\frac{\varepsilon^{AiBi}}{k_B}$	κ^{AiBi}	N	k_{ij}	T_{0i}^{SL}	Δh_{0i}^{SL}	Δa^{L-S}	Δb^{L-S}
	[−]	[Å]	[K]	[K]	[−]	[−]	[10^{-2}]	[K]	[kJ·mol^{-1}]	[J·mol^{-1}·K^{-2}]	[J·mol^{-1}·K^{-1}]
H_2O	1.2047	[a]	353.94	2425.67	0.045	-	-	-	-	-	-
Gly[c]	4.850	2.327	216.96	2598.060	0.039	2	-5.85[c]	569	24.96	-0.041	41.648
Ala[c]	5.465	2.522	287.59	3176.600	0.082	2	-6.12[c]	608	25.99	-0.057	39.923
Val[b]	7.485	2.589	306.41	3183.800	0.039	2	-7.57[b]	529	46.72	-0.102	73.915
Leu[b]	8.304	2.700	330.00	3600.000	0.020	2	-6.39[b]	518	49.09	-0.052	47.3
Ile[b]	8.241	2.586	281.88	2207.529	0.001	2	-8.75[b]	595	47.11	-0.053	51.604
Pro[b]	6.981	2.548	289.72	5527.750	0.036	2	-6.99[b]	527	16.30[e]	-0.060[e]	40.417[e]
Ser[b]	7.024	2.284	236.92	2671.930	0.039	3	-2.57[b]	519	32.98	-0.079	90.29
Thr[b]	6.329	2.606	325.37	2519.410	0.039	3	-2.78[b]	587	36.64	-0.027	78.257
Asp[d]	5.827	2.522	287.63	2544.234	0.041	3	1.45[d]	610	35.73	-0.221	176.159
Glu[d]	6.831	2.560	227.19	2544.234	0.041	3	-4.45[d]	566	48.24	-0.16	115.101
Phe[d]	9.310	2.690	391.83	3206.094	0.010	2	-5.18[d]	579	60.66	-0.139	265.092
Tyr[b]	8.139	2.280	289.37	2500.000	0.040	3	0.0227	678	49.77	-0.017	74.282
Trp[d]	10.577	2.825	260.64	2563.249	0.024	3	-7.68	620	65.55	-0.407	273.799

[a]Temperature-dependent segment diameter $\boldsymbol{\sigma}$ = 2.7927+10.11 exp(-0.01775T)-1.417 exp(-0.01146T) and parameters from Cameretti and Sadowski[146]; [b]pure-component parameters from Held *et al.*[147]; [c]pure-component parameters from Chua *et al.*[143]; [d]pure-component parameters from Do *et al.*[99]; [e]determined in this work.

Table 2. 3. PC-SAFT pure-component parameters of ions, and k_{ij} between ion and water and between anion and cation. All parameters inherited from Held *et al.*[141].

	m_{ion}^{seg}	σ_{ion}	$\frac{u_{ion}}{k_B}$	k_{ij} ion-water	k_{ij} anion-cation
	[−]	[Å]	[K]	[−]	[−]
K^+	1.000	3.3417	200.00	0.20	0.064 (K^+-Cl^-)
NH_4^+	1.000	3.5740	230.00	0.064	-1.000 (NH_4^+- SO_4^{2-})
SO_4^{2-}	1.000	2.6491	80.00	0.25	-1.000 (NH_4^+- SO_4^{2-})
Cl^-	1.000	2.7560	170.00	-0.25	0.064 (K^+-Cl^-)

These parameters were published in the literature and were inherited from the previous works, including the melting properties. An exception are the values of Δh_{0i}^{SL}, Δa^{L-S} and Δb^{L-S} of L-proline presented in **Table 2. 2**. These were determined in this work based on FSC (fast scanning calorimeter) data from Do *et al.*[99], as the fine-tuned values were not available in that publication (relatively large experimental uncertainties). According to the procedure previously followed[99], these melting properties were fitted to the solubility data of L-proline in water using the FSC data[99] as starting values and allowing the fine-tuned values only to vary within the FSC-determined uncertainty.

Chapter 3 – Solubilities of amino acids in aqueous solutions of salts composed of monovalent cations.

This chapter includes the measurement of the solubility of glycine, L-leucine, L-phenylalanine, and L-aspartic acid in aqueous salt solutions with monovalent cations, such as Na^+, K^+, and NH_4^+, and Cl^- and NO_3^- anions at 298.2 K. Whenever possible, a comparison of the results with literature was performed and the consistency analysis were provided. Effect of the structures of the AAs on their solubility was designed by studying the relative solubility of different AAs in aqueous solutions of the same salt. The pH of the saturated solutions and the results of the solid phase studies are also included.

I declare that I have provided all the solubility measurements of AAs in aqueous salt solutions and monitored the pH of the saturated solution and wrote the paper. The solid-phase studies were performed by Paula Brandão from CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal.

3.1. Experimental data and analysis

The solubilities of L-aspartic acid, L-phenylalanine, L-leucine, and glycine in the aqueous NaCl, $NaNO_3$, KCl, KNO_3, NH_4Cl, and NH_4NO_3 solutions, at various salt molalities at 298.2 K, are given in **Table 3. 1**. The maximum coefficient of variation (standard deviation/average*100) is 6.9% (system water/ammonium chloride/aspartic acid, 2 mol·kg^{-1} salt concentration), being lower than 1% in about 83% of cases.

Table 3. 1. Solubilities (g of AA/1000 g of H_2O) of the amino acids in aqueous solutions of salts at different molalities at 298.2 K and p = 0.1 MPa (standard deviation between brackets).[a]

Salts	Electrolyte molality (mol/kg of H_2O)	S_{AA} (g of AA/1000 g of H_2O)			
		Glycine	L-leucine	L-phenylalanine	L-aspartic acid
No salt	0.000	238.332 (0.127)	21.544 (0.070)	28.347 (0.083)	5.140 (0.031)
NaCl	0.500	244.619 (0.874)	20.464 (0.047)	30.192 (0.093)	6.330 (0.178)
	1.000	252.847 (0.452)	19.674 (0.057)	25.783 (0.159)	7.155 (0.274)
	2.000	263.862 (0.229)	17.160 (0.205)	22.198 (0.245)	8.076 (0.122)
$NaNO_3$	0.500	256.830 (0.429)	22.977 (0.094)	30.983 (0.046)	6.452 (0.027)
	1.000	278.033 (0.329)	23.080 (0.088)	32.165 (0.041)	7.613 (0.009)

	2.000	305.871	22.300	32.506	9.607
		(0.493)	(0.158)	(0.043)	(0.047)
KCl	0.500	243.174	21.236	28.115	6.343
		(0.108)	(0.177)	(0.046)	(0.043)
	1.000	247.229	19.899	26.501	8.045
		(0.240)	(0.048)	(0.149)	(0.177)
	2.000	252.281	17.440	23.181	9.318
		(0.370)	(0.145)	(0.432)	(0.048)
KNO_3	0.500	253.178	22.894	31.397	6.732
		(0.281)	(0.120)	(0.084)	(0.062)
	1.000	265.954	22.615	32.187	7.938
		(0.270)	(0.110)	(0.155)	(0.081)
	2.000	284.801	22.588	31.266	10.780
		(0.310)	(0.296)	(0.185)	(0.414)
NH_4Cl	0.500	245.097	22.591	29.763	6.002
		(0.199)	(0.034)	(0.060)	(0.082)
	1.000	252.599	22.250	29.887	6.777
		(0.342)	(0.093)	(0.043)	(0.063)
	2.000	264.662	21.407	29.297	6.871
		(0.364)	(0.061)	(0.825)	(0.474)
NH_4NO_3	0.500	256.108	24.545	33.639	6.652
		(0.606)	(0.129)	(0.206)	(0.034)
	1.000	274.534	26.586	37.468	7.835
		(0.209)	(0.106)	(0.087)	(0.038)
	2.000	305.613	29.014	43.279	9.910
		(0.355)	(0.077)	(0.108)	(0.089)

[a] u(salt molality) = 0.001 mol·kg^{-1}, $u_r(S_{AA})$ = 0.05, u (T) = 0.1 K and u_r(p) =0.05.

The solubility in pure water at 298 K has already been reported by several authors and is collected in **Table 1. 5**. As can be seen, the values measured in this work generally agree with the literature. The absolute solubility values in all aqueous systems follow the rank Gly > Phe > Leu > Asp, which matches the solubility in pure water. **Figure 3. 1** shows the relative solubilities (ratio between the solubility of AA, expressed as the mass of the amino acid in 1 kg of water, in aqueous salt solutions to that in pure water) of all the studied AAs in different aqueous salt solutions at 298.2 K. The results show the relevance of the AAs structural features (**Figure 1. 2**). L-Aspartic acid, the most polar, shows salting-in in all the salt solutions. Leu, the most apolar hydrophobic AA in this study, generally presents the weakest salting-in effect or a salting-out, even if in most of the salt solutions, a very similar behavior is observed between Leu and Phe. The relative solubilities in all the aqueous salt solutions follow the order Asp > Gly > Phe ≅ Leu, excepting the ammonium nitrate solution, where the ranking is Asp > Phe > Leu > Gly. Potassium chloride induces a salting-out over the

whole salt concentration range in the solutions with Phe and Leu. The presence of sodium chloride leads to a salting-out effect in the solution with Leu. Besides, the addition of $NaNO_3$ causes an increase in the solubility of Phe. In all other cases, the solubility varies non-monotonically with salt molality.

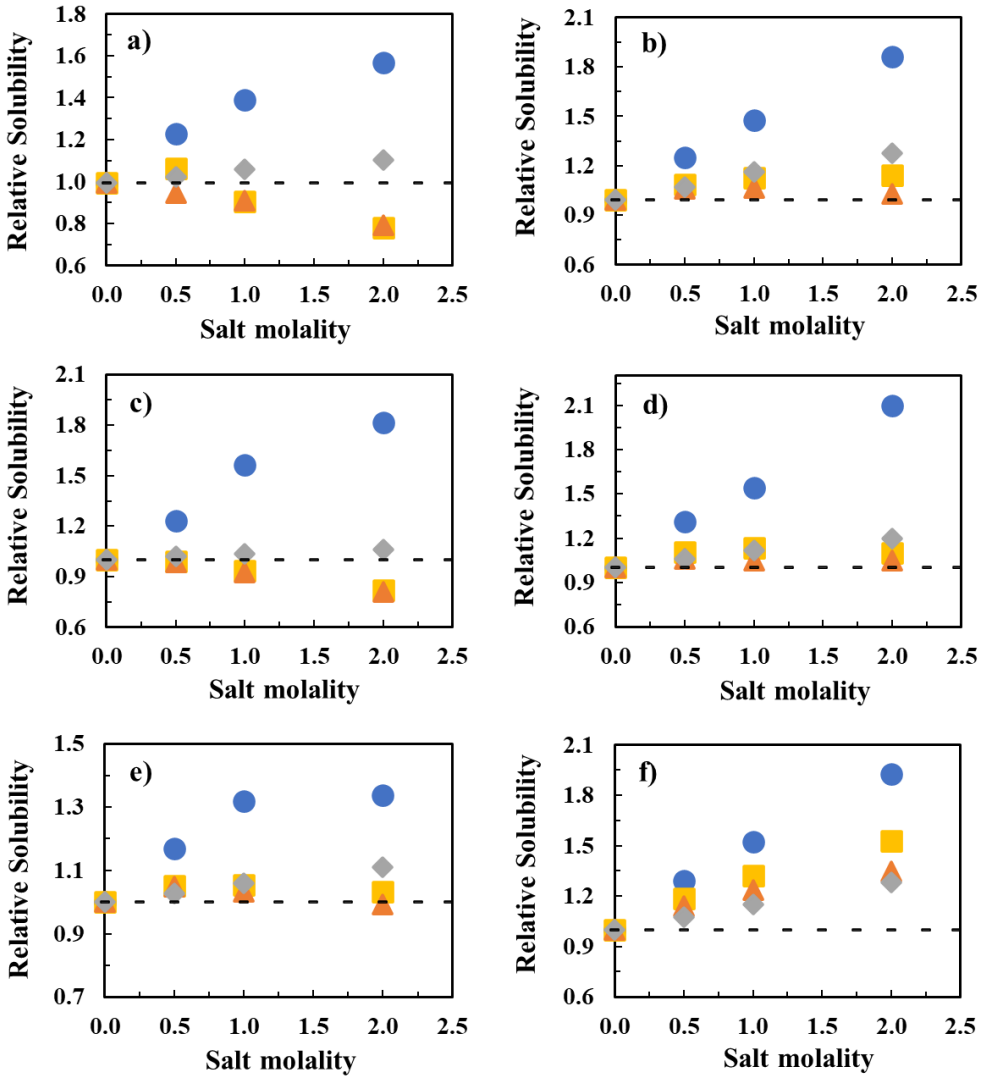

Figure 3. 1. Relative solubility of ◆, glycine; ▲, L-leucine; ■, L-phenylalanine, and ●, L-aspartic acid in aqueous a) NaCl; b) $NaNO_3$; c) KCl; d) KNO_3; e) NH_4Cl, and f) NH_4NO_3 solutions with different molalities at 298.2 K.

The effect of each salt on the relative solubility of the studied amino acids is shown in **Figure 3. 2**. The solubilities of all the AAs in aqueous salt solutions, with the nitrate anions, are higher than in the solutions containing chloride anions (for the same cation). This is supported by the molecular dynamics simulations reported by Tomé *et al.*[27]. It was concluded that the Cl^- anion has as much lower effect on the solubility of AAs, and that it does not

interact with the apolar groups of AAs. Considering the NO_3^- anion, it interacts more significantly with the hydrophobic groups of the AAs and thus contributes to increase the AA solubility. The effect of cations on the solubility of AAs was also studied in another work by the same author[40]. It was observed that monovalent cations, and particularly alkali cations, do not interact with the hydrophobic groups, and unlike the divalent cations, which interact strongly with the carboxylate groups of the AAs, for the monovalent cations, this binding is less favorable. The ammonium cation shows stronger interaction (through a combination of ion-induced dipole and dispersion interactions, promoting stabilization of the amino acids in water) than the sodium and potassium ions[79]. It is worth mentioning that only for Asp, which presents two carboxylate groups, the ammonium nitrate salt does not present the highest salting-in effect compared to the nitrate salts of the alkali cations. The relative solubility of Phe and Leu follows the same order $NH_4NO_3 > KNO_3 \approx NaNO_3 \approx NH_4Cl > KCl \approx NaCl$ (evaluated at 2 molal). The relative solubility of Gly in the aqueous salt solutions with the nitrate and chloride anions follow the same ranking $K^+ > NH_4^+ > Na^+$, while for Asp the order is found as $KNO_3 > NH_4NO_3 \approx NaNO_3 \approx KCl > NaCl > NH_4Cl$ (evaluated at 2 molal).

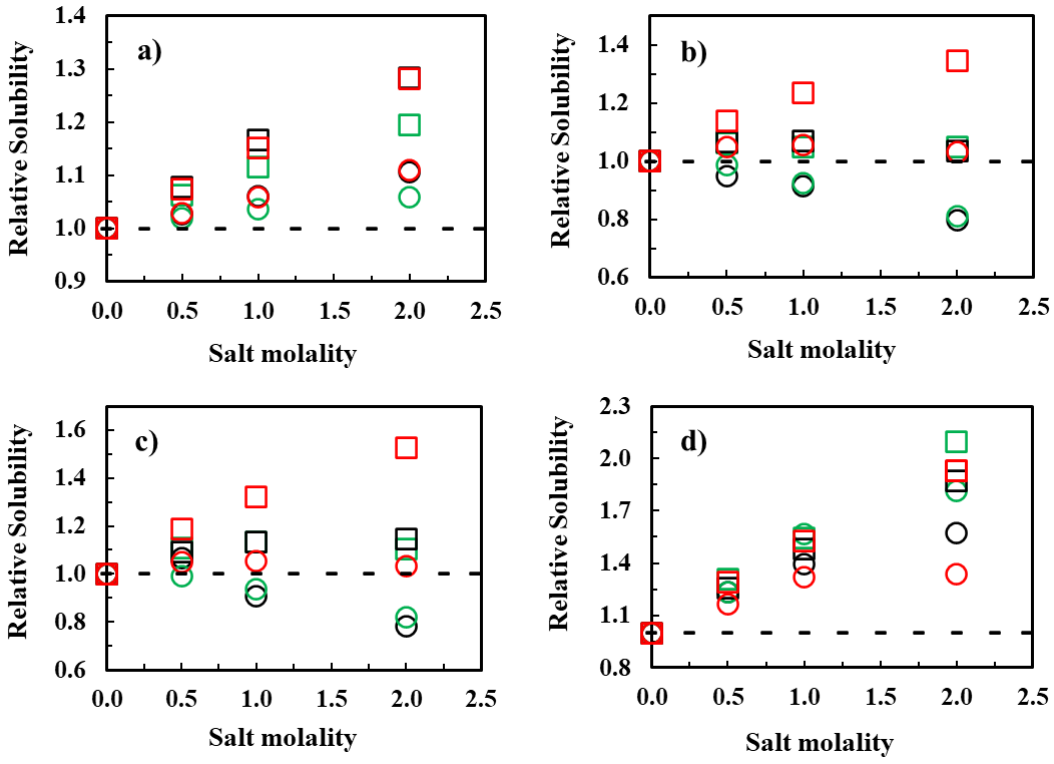

Figure 3. 2. Relative solubility of a) glycine; b) L-leucine; c) L-phenylalanine, and d) L-aspartic acid in aqueous ○, NaCl; □, $NaNO_3$; ○, KCl; □, KNO_3; ○, NH_4Cl, and □, NH_4NO_3 solutions with different molalities at 298.2 K.

As shown in **Table 1. 1**, Gly is the most studied AA. For certain aqueous electrolyte solvents, there are even AA solubility data from different researchers, allowing to check the reliability of the data measure in this work. The solubility data of Gly in aqueous KCl solution obtained in this work is consistent (**Figure 2.3b**) with the data of Ferreira *et al.*[70], that checked their different results to those by Khoshkbarchi and Vera[46], proving the reliability of their data recurring to experimental and theoretical approaches, and are also in agreement to Roy *et al.*[50]. The results of Held *et al.*[49] differed from the results of this work. Similar differences were observed when the results in aqueous NaCl solutions were compared (**Figure 3. 3a**), particularly the small salting-out at low salt molalities that was not observed in this work. The only work found with solubility data of Gly in aqueous NH_4Cl solution was the work by Zeng and Li[78], in which the results were similar to the results from this work. Concerning the nitrate salts (**Figure 3. 3d** and **e**), the relative solubility of Gly in aqueous $NaNO_3$ and KNO_3 solutions from this work is close to the data from Roy *et al.*[64], Held *et al.*[49] or Talukdar *et al.*[65], but quite different from Pradhan and Vera[63], which shows much higher solubilities. The reason can be related to a particular detail of their experimental procedure; Pradhan and Vera dried their samples in the oven for 48 h, just at 308 K, and then weighted them, which most certainly was not enough to remove all water retained by the AA and salt crystals. It is worth mentioning that there are also other factors affecting solubility in this kind of study. Gly polymorphism is well known,[148–150] and the polymorph transformation of Gly in aqueous solution can often occur[80,151–154]. For instance, Yang *et al.*[153] studied the transformation of the α form to the γ form, which happens after 20 h. The presence of salts and the stirring speed, as well as the temperature, time and pH clearly affect the transformation rate. Kitamura[155] developed important studies about strategies to control the crystallization of polymorphs, where these factors are also mentioned. Unfortunately, the solubility of Gly in NH_4NO_3 was presented just in one work[66] and not at 298.2 K. For some systems, not all solubility data found in the literature could be included due to the impossibility to accurately convert data from the units presented in these studies to those used here. For clarity, the relative solubility of glycine[50,75] in aqueous KCl solutions is not shown after 3 molal concentration.

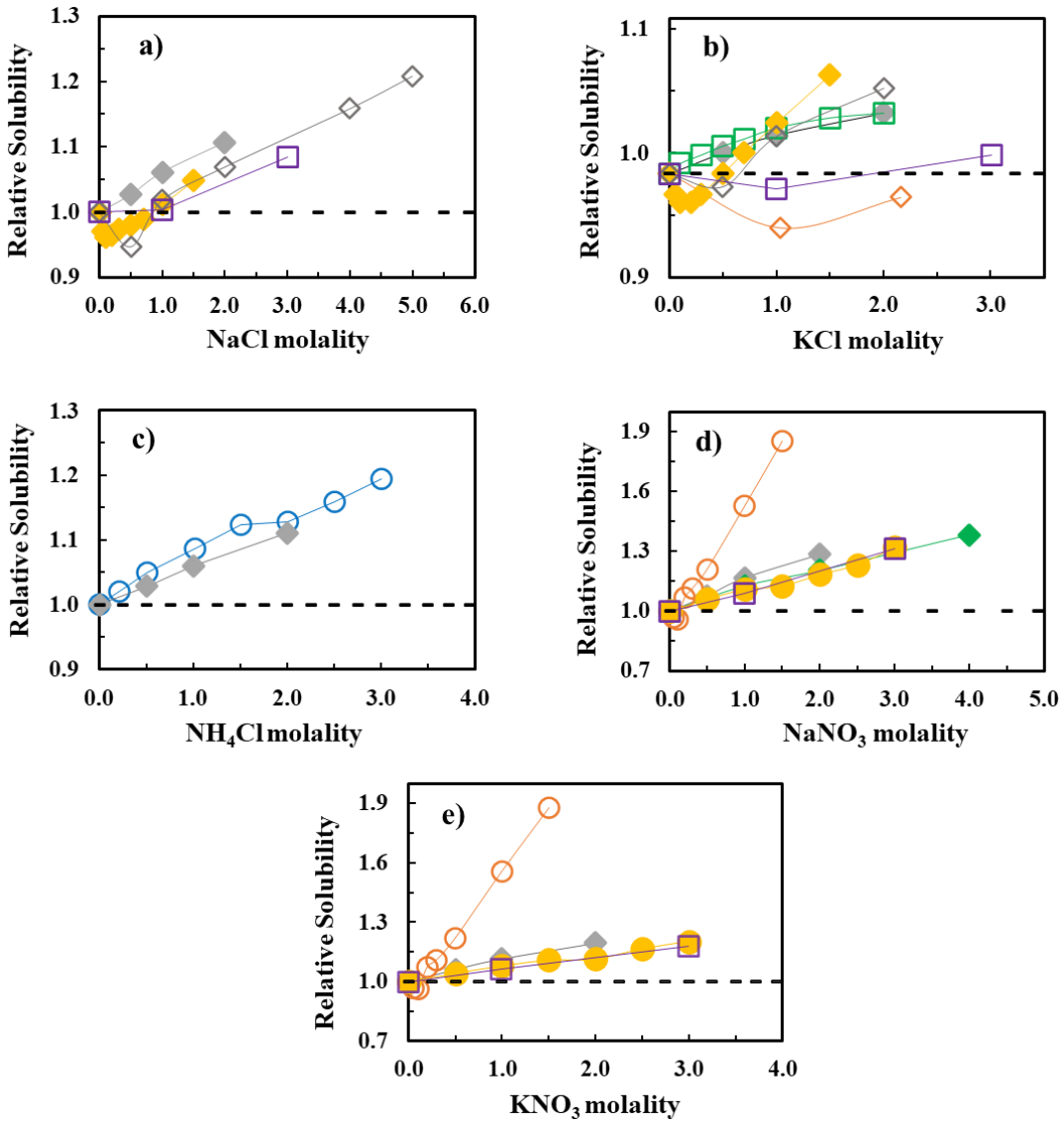

Figure 3. 3. Relative solubility of glycine in aqueous salt solutions at 298.2 K: ◆, (this work); ◇, Roy *et al.*[50]; ◆, Khoshkbarchi and Vera[46]; ◇, Venkatesu *et al.*[75]; □, Ferreira *et al.*[70]; ○, Pradhan and Vera[63]; ●, Roy *et al.*[64]; ◆, Talukdar *et al.*[65]; □, Held *et al.*[49]; ○, Zeng and Li[78]. Lines are a guide to the eyes.

As can be observed from **Table 1. 1**, for L-aspartic acid, not many solubility measurements were reported so far. Therefore, the results could be compared only in aqueous NaCl solutions, presented in **Figure 3. 4a**. Our data are very similar to Bretti *et al.*[57] and slightly higher than Wang *et al.*,[26] at higher molalities. However, as the solubility studies in aqueous KCl solution were carried out only for the D-isomer of this AA[26], no comparison could be provided. For Phe or Leu, data were also found in aqueous NaCl solutions[55]. A very good agreement is observed for Leu (**Figure 3. 4c**), as well for Phe (**Figure 3. 4b**). However, in their

work, Bretti *et al.*[55] indicate the L-phenylalanine solubility in water at 298.15 K to be 13.6 g/1000 g of H_2O, while the literature values range from 25.2 to 30.6 g/1000 g of H_2O.

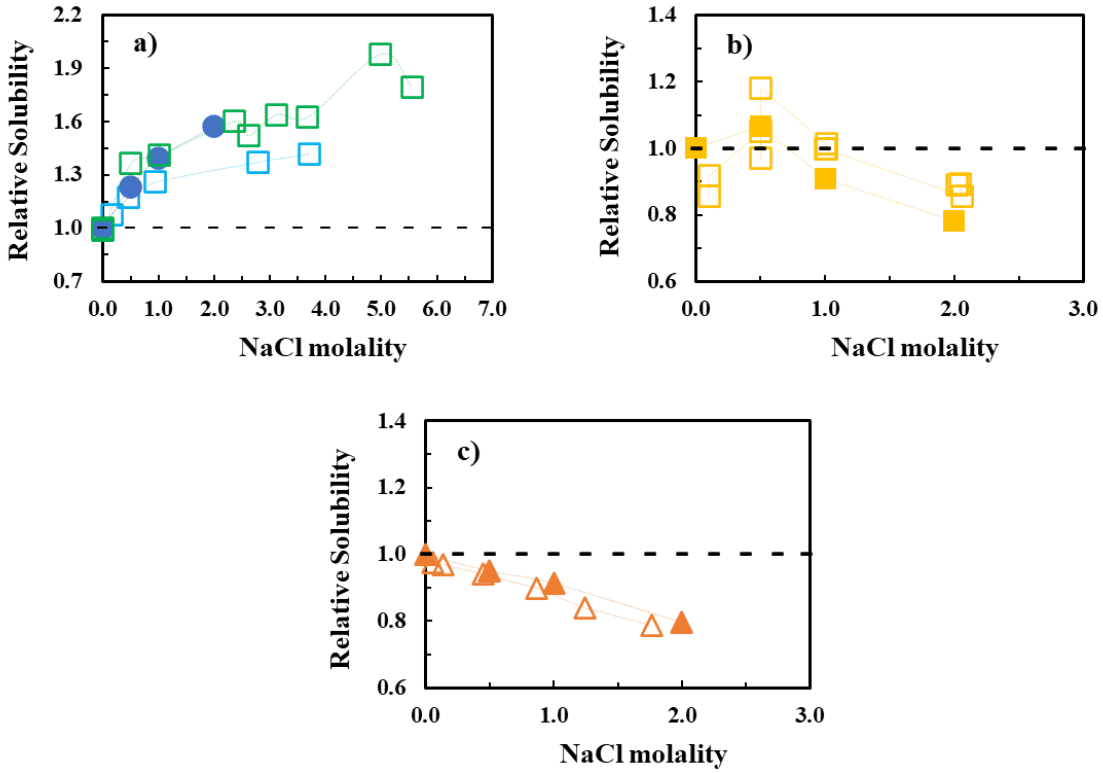

Figure 3. 4. Relative solubility of a) L-aspartic acid: ●, (this work); □, Bretti *et al.*[57]; □, Wang *et al.*[26]; b) L-phenylalanine: ■, (this work); □, Bretti *et al.*[55], and c) L-leucine: ▲, (this work); △, Bretti *et al.*[55] in aqueous salt solutions at 298.2 K. Lines are a guide to the eyes.

3.2. Ion effects on AA solubility

3.2.1. Consistent results

Considering all the experimental solubility data from this work and the literature, a set of patterns can be identified. To carry this analysis, the information available was first divided into salts containing the monovalent K^+, Na^+, or NH_4^+ cations, and evaluating the effect of the anions, since the impact of these cations on the solubility is minor compared to the anions. As shown in **Figure 3. 5** (the same color indicates the same anion, and it is limited to 3.0 salt molality for better reading), all salts induce a mild salting-in impact on Gly, except for KCH_3COO. This anion is a salting-out agent in the list of salts in the Hofmeister series. The effect of the studied salts is seen in a narrow range of the relative solubility of Gly, and some differences are most probably within the experimental error but give at least a qualitative view. The results of some works could not be included as they were found in

different units, or the solubility measurements were not provided at 298.2 K, or another isomer was studied. In addition, not all data available were included, as a consistency analysis was first carried out for a reliable interpretation (**Section 3.2.3.**). On the other hand, only the data here measured (and compared in **Figure 3. 3**) were included for the salts studied experimentally in this work. It can be concluded that in the case of glycine the salts with the nitrate anion present the highest salting-in effect (evaluated above 1 molal), followed by sulfate, iodide, bromide, chloride, and fluoride anion.

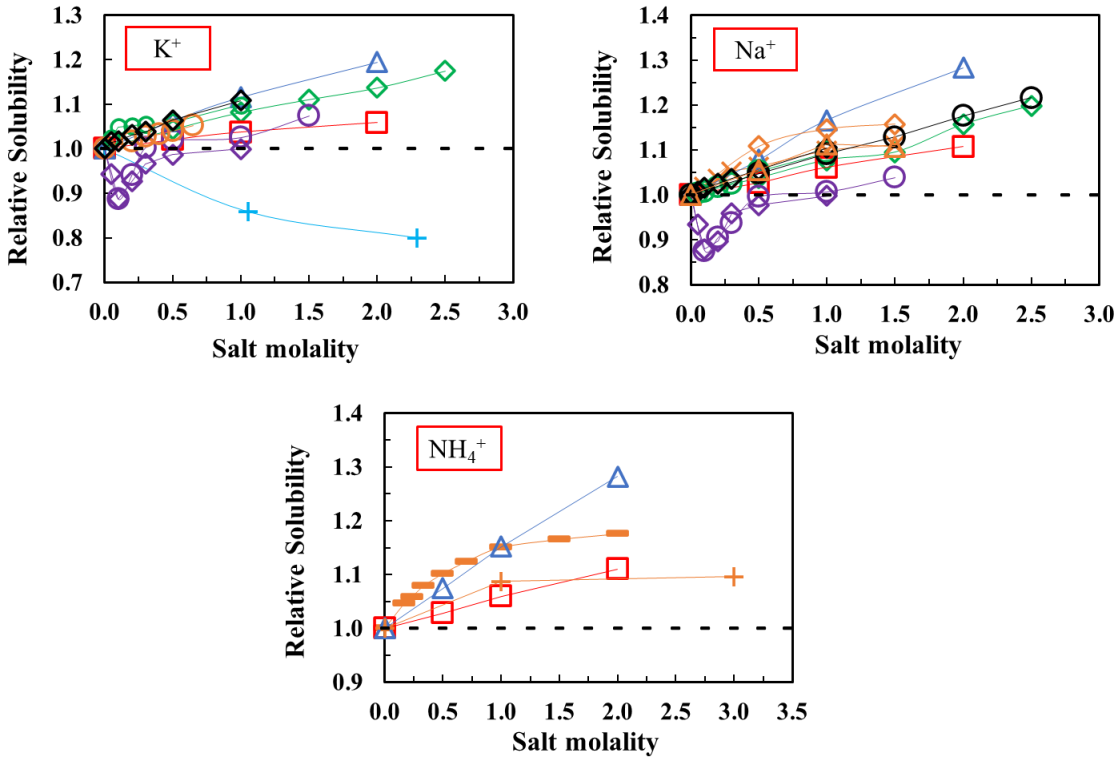

Figure 3. 5. Ion effects on the relative solubility of glycine in aqueous salt solutions at 298.2 K: △, KNO_3 (this work); □, KCl (this work); ◇, KBr[61]; ○, KBr[42]; ◇, KI[42]; ○, K_2SO_4[77]; ○, KF[41]; ◇, KF[42]; +, KCH_3COO[75]; △, $NaNO_3$ (this work); □, NaCl (this work); ◇, NaI[42]; ○, NaI[62]; x, Na_2SO_4[70]; ◇, Na_2SO_4[71]; △, Na_2SO_4[72]; ○, NaF[41]; ◇, NaF[42]; ○, NaBr[42]; ◇, NaBr[61]; △, NH_4NO_3 (this work); □, NH_4Cl (this work); —, $(NH_4)_2SO_4$[6]; +, $(NH_4)_2SO_4$[49]. Lines are a guide to the eyes.

For alanine, the diversity of experimental data published is also significant, allowing to see the effect of the additional methyl group in the amino acid side chain compared to Gly (the structures of all amino acids focused in this work are presented in **Appendix A**). **Figure 3. 6** shows the relative solubility of DL-alanine in aqueous salt solutions with Na^+, K^+, or NH_4^+ cations. In comparison with Gly, the relative solubility of DL-alanine is lower in most of the aqueous electrolyte solutions, and a salting-out effect was observed, confirming the

molecular dynamics results showing that the monovalent cations do not interact with hydrophobic moieties of the AAs. This change to Gly was more noticeable when DL-alanine was dissolved in aqueous KCl, NaCl, Na_2SO_4, NaF, $(NH_4)_2SO_4$, and K_2SO_4 solutions. Considering molecular dynamics studies[27], it was found that the SO_4^{2-} can interact only with small amino acids like glycine inducing salting-in effect, while it does not interact with large nonpolar moieties and therefore shows a salting-out effect, also supporting the experimental observations. Consistently to glycine, the salts with the nitrate anions presented the highest salting-in effect (evaluated at 1.5 molal). Even if two independent and consistent sets of data are available, the salting-in magnitude seems too large, raising some doubts on the data quality. After nitrate, the ranking follows iodide ≈ bromide, chloride, and sulfate anions.

The relative solubility of DL-alanine is higher in aqueous salt solutions with the bromide and iodide anions compared to glycine. Moreover, the salts with the fluoride anion induce a different effect on the solubility of DL-alanine, a salting-in effect with the potassium cation, and a salting-out effect with the sodium cation. These differences with the potassium and sodium cations, and halogens, also open some questions about the quality of the results, stressing the need to have a structural analysis of the solid phase, identifying the AA crystallographic form in the electrolyte solutions, and perform a comparison to the original form of the supplier. The solubility of DL-alanine in the salt solutions with the ammonium cations is much less studied. In solutions of ammonium chloride or ammonium sulfate, the relative solubility is lower than for glycine. At higher molalities, the sulfate anion induces a salting-out effect with DL-alanine, confirming the trend mentioned before.

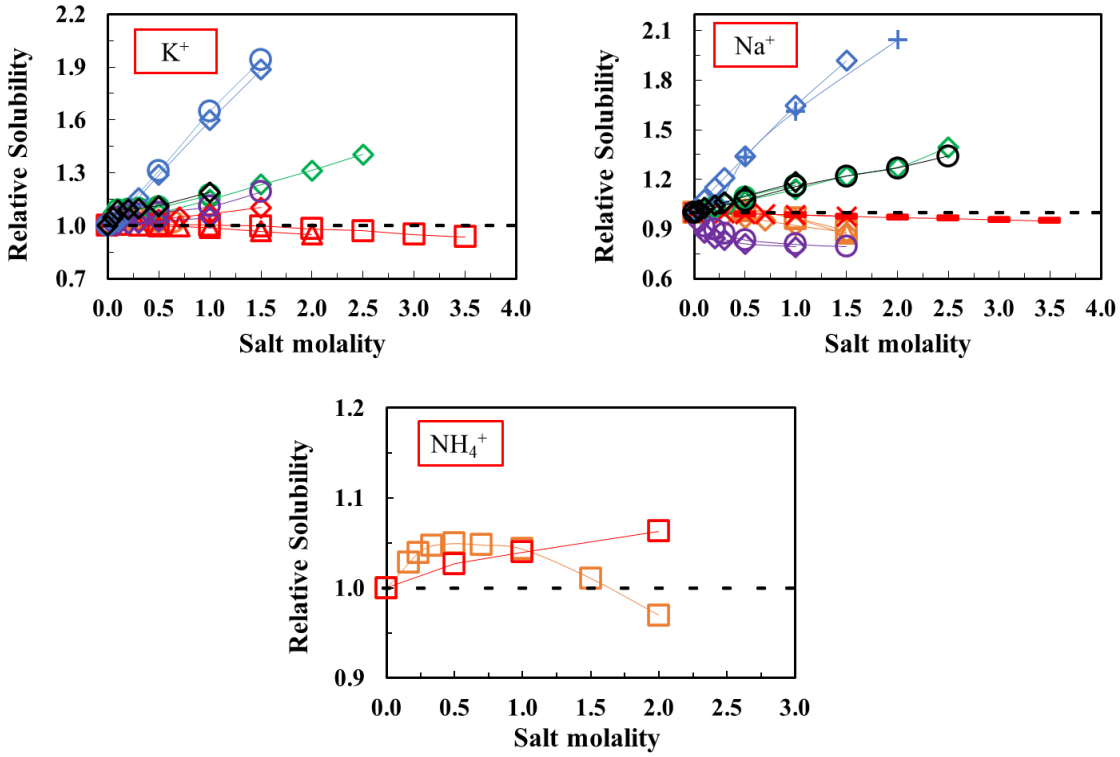

Figure 3. 6. Ion effects on the relative solubility of DL-alanine in aqueous salt solutions at 298.2 K: ○, K_2SO_4[77]; +, K_2SO_4[40]; ○, KBr[42]; ◇, KBr[61]; ◇, KI[42]; △, KCl[70]; ◇, KCl[46]; ○, KCl[52]; ○, KF[41]; ◇, KF[42]; ◇, KNO_3[63]; ○, KNO_3[76]; □, Na_2SO_4[70]; ◇, Na_2SO_4[71]; △, Na_2SO_4[72]; ○, NaBr[42]; ◇, NaBr[61]; ○, NaI[62]; ◇, NaI[42]; ×, NaCl[46]; —, NaCl[52]; ◇, NaF[42]; ○, NaF[41]; ◇, $NaNO_3$[63]; +, $NaNO_3$[67]; □, $(NH_4)_2SO_4$[6]; □, NH_4Cl[79]. Lines are a guide to the eyes.

3.2.2. Contradictory results

The same type of analysis, as in the previous section, has been attempted for valine. However, some sets of published data (referenced in **Table 1. 1**) do not follow the expected trends. Where sometimes a very mild salting-in is expected, a strong effect stands out. In aqueous Na_2SO_4 solutions, all the AAs, except glycine (**Figure 3. 7**), show a salting-out effect, and the ranking is DL-alanine < DL-valine < L-isoleucine, which is in agreement with the expected order, also derived from molecular dynamics studies. In the case of $NaNO_3$ solutions, even if nitrate has a more salting-in character than sulfate, DL-valine and DL-alanine systems show a salting-in effect that seems too intense. The rank is DL-valine > DL-alanine > glycine > L-leucine > L-isoleucine, which is not consistent as DL-valine and DL-alanine are AAs with larger apolar groups than glycine, and it would be expected to have valine positioned somewhere in the middle between alanine and leucine, as the size of the hydrocarbon chain is the main effect on the solubility change. This analysis sheds some light

on the importance of checking the data consistency before drawing general rules concerning the impact of the different anions on AA solubility.

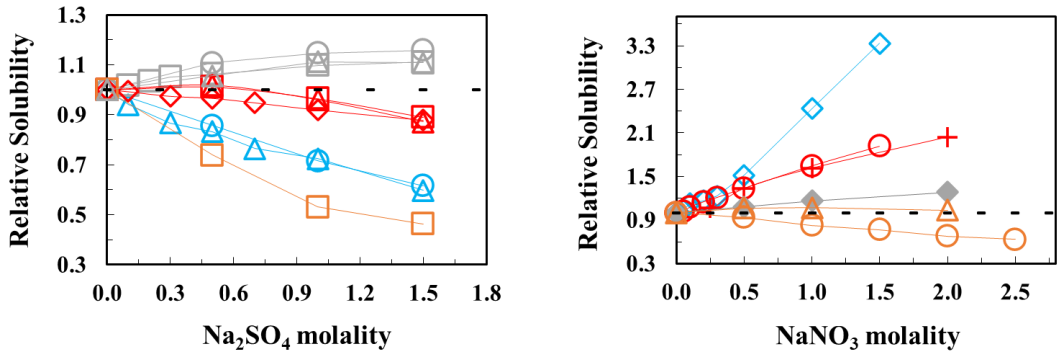

Figure 3. 7. Relative solubility of glycine: ◇, Ferreira *et al.*[70]; □, Roy *et al.*[71]; △, Ramasami[72]; ◆, (this work); DL-alanine: ◇, Roy *et al.*[71]; □, Ferreira *et al.*[70]; △, Ramasami[72]; ○, Pradhan and Vera[63]; +, Roy *et al.*[67]; DL-valine: △, Roy *et al.*[71]; ○, Ramasami[72]; ◇, Pradhan and Vera[63]; L-isoleucine: □, Chowdurry *et al.*[73]; ○, Chowdurry *et al.*[68], and L-leucine: ▲, (this work) in aqueous Na_2SO_4 and $NaNO_3$ solutions. Lines are a guide to the eyes.

3.2.3. Consistency analysis

For many aqueous salt solutions, more than one independent set of data are available. Thereby, the consistency analyses were carried out. **Figure 3. 8** presents all the solubility results of glycine in aqueous K_2SO_4, Na_2SO_4 and KBr solutions, found in the literature. One set of data in aqueous K_2SO_4 and Na_2SO_4 was the result of El-Dossoki[42]. Despite the fact that, in this work the salts also induced the same effect on the solubility of glycine, a big difference in the magnitude can be clearly seen. The solubility data obtained by Venkatesu *et al.*[75] in aqueous KBr solution also differed from the results of two other works. In this work a small salting-out effect was observed at low molality.

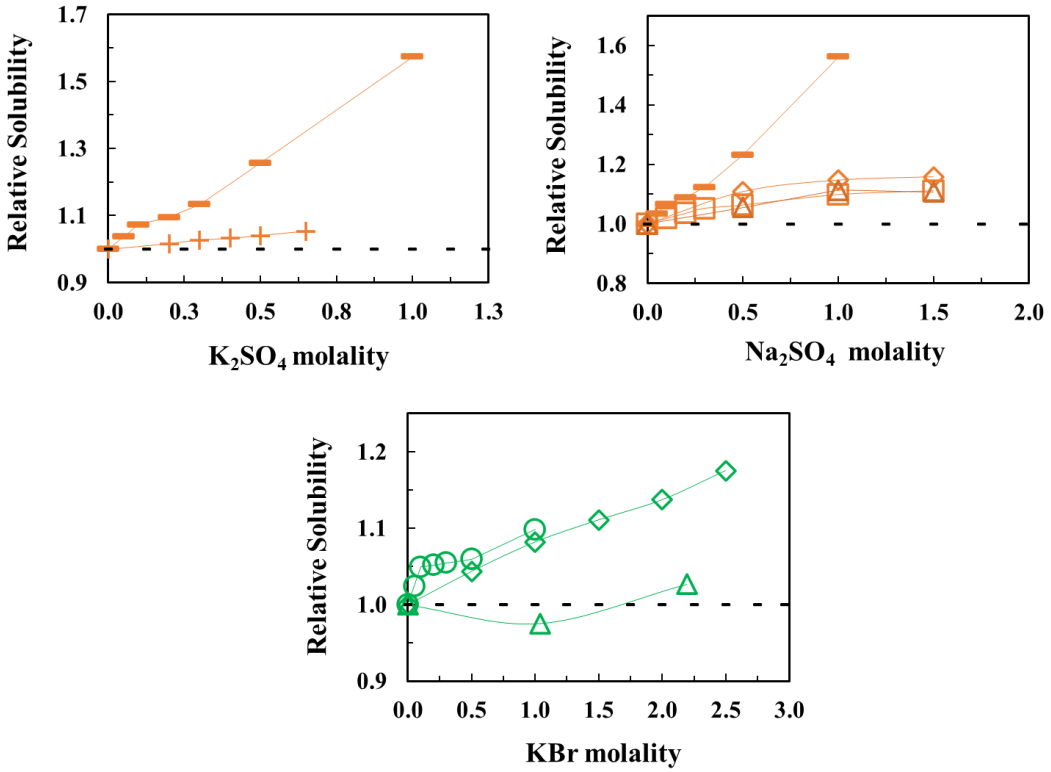

Figure 3. 8. Relative solubility of glycine in aqueous K_2SO_4, Na_2SO_4 and KBr solutions: +, Hossain *et al.*[77]; —, El-Dossoki[42]; □, Ferreira *et al.*[70]; △, Ramasami[72]; ◇, Roy *et al.*[71]; ◇, Guin *et al.*[61]; ○, El-Dossoki[42]; △, Venkatesu *et al.*[75]. Lines are a guide to the eyes.

Solubility data of DL-alanine and DL-valine in aqueous K_2SO_4 and Na_2SO_4 solutions were found in different works and showed in **Figure 3. 9**. Except the work of El-Dossoki[42], all other results are similar and consistent, and that specific set are not included in the prsesent analysis.

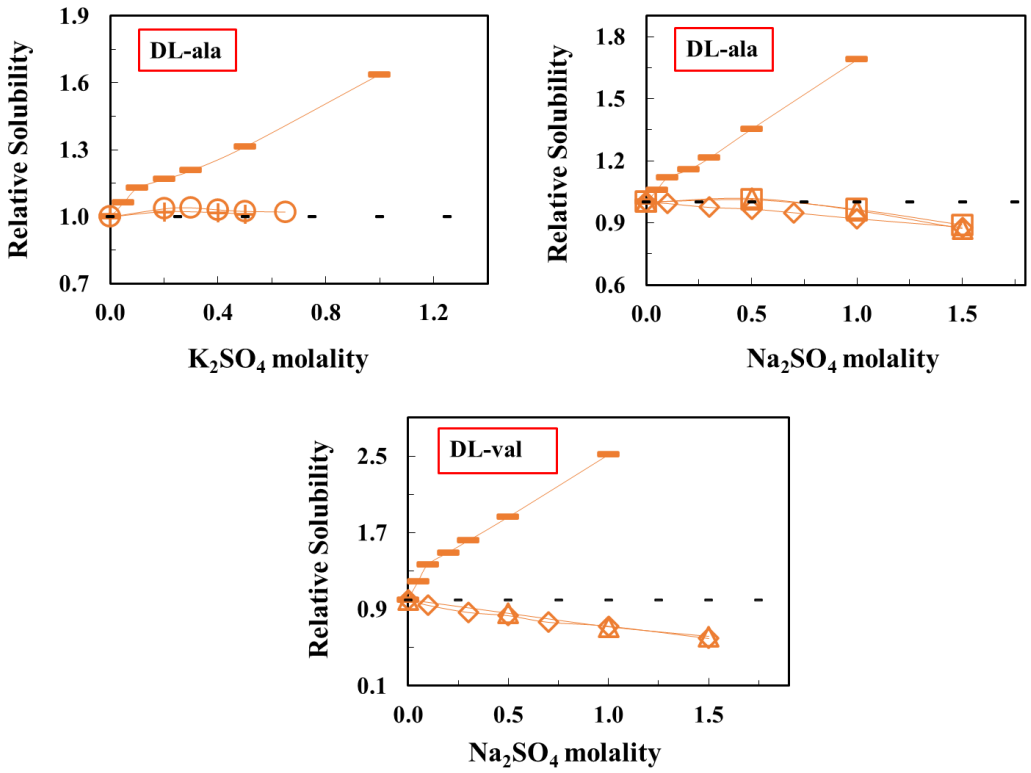

Figure 3. 9. Relative solubility of DL-alanine and DL-valine in aqueous K_2SO_4 and Na_2SO_4 solutions: ○, Hossain *et al.*[77]; —, El-Dossoki[42]; □, Ferreira *et al.*[70]; △, Ramasami[72]; ◇, Roy *et al.*[71]; +, Tomé et al.[40]. Lines are a guide to the eyes.

3.2.4. Data checking – Solubilities of L and DL-valine.

To check the effect of $NaNO_3$ on the solubility of valine, as well as the impact of optical isomerism, the solubility measurements of L ad DL-valine were carried out in aqueous $NaNO_3$ and also in KNO_3 solutions at various salt molalities and 298.2 K and the data is presented in **Table 3. 2**. The maximum coefficient of variation (standard deviation/average*100) is 1.8% (system water/sodium nitrate/DL-valine, 0.5 mol·kg^{-1} salt concentration), being lower than 1% in all other cases. The absolute solubility of DL-valine in both aqueous solutions is at least 15 g/1000g of water higher than L-valine.

Table 3. 2. Solubilities (g of AA/1000 g of H_2O) of L-valine and DL-valine in aqueous $NaNO_3$ and KNO_3 solutions at different molalities at 298.2 K (standard deviation between brackets) and p = 0.1 MPa.[a]

Salts	Electrolyte molality (mol/kg of H_2O)	S_{AA} (g of AA/1000 g of H_2O)	
		L-valine	DL-valine
No salt	0.000	62.400 (0.253)	76.171 (0.300)
$NaNO_3$	0.500	63.634 (0.302)	79.750 (1.422)
	1.000	63.783 (0.542)	80.079 (0.677)
	2.000	62.187 (0.564)	78.476 (0.688)
KNO_3	0.500	64.803 (0.075)	79.823 (0.048)
	1.000	64.844 (0.098)	81.193 (0.294)
	2.000	62.116 (0.554)	77.472 (0.279)

[a] u(salt molality) = 0.001 mol·kg^{-1}, $u_r(S_{AA})$ = 0.05, u (T) = 0.1 K and $u_r(p)$ =0.05.

The solubility of DL-valine in pure water measured in this work at 298 K agree with the literature, but for L-valine a difference to the literature average close to 4 g per 1000 g of water was consistently found (**Table 1. 5**). This can be due to the differences in the solid phase structures, which were hardly ever registered in the compiled studies.

Figure 3. 10 shows the relative solubility for the two (L- and DL-) forms of valine at 298.2 K. Despite the differences in the absolute values of the solubility of each isomer, it is clear the same relative solubility changes for the L-isomer and racemic mixture in both aqueous nitrate solutions, showing that the optical isomerism does not play a role in the impact of salts on the AA solubility.

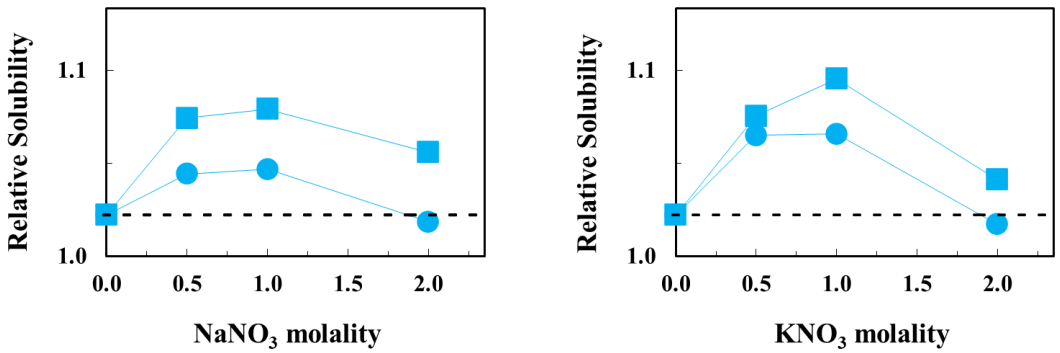

Figure 3. 10. Relative solubility of ●, L-valine and ■, DL-valine in aqueous salt solutions. Lines are a guide to the eyes.

The results obtained were also compared with the literature data as presented in **Figure 3. 7**. Similarly, to **Figure 3. 10**, it is shown the very small effect of monovalent cations on the

solubility of the AA. The NO_3^- anion interacts with the hydrophobic groups of the AA leading to a small salting-in effect for valine, in good agreement with the data by Held *et al.*[49], and highlighting the very unexpected salt effect reported by Pradhan and Vera[63].

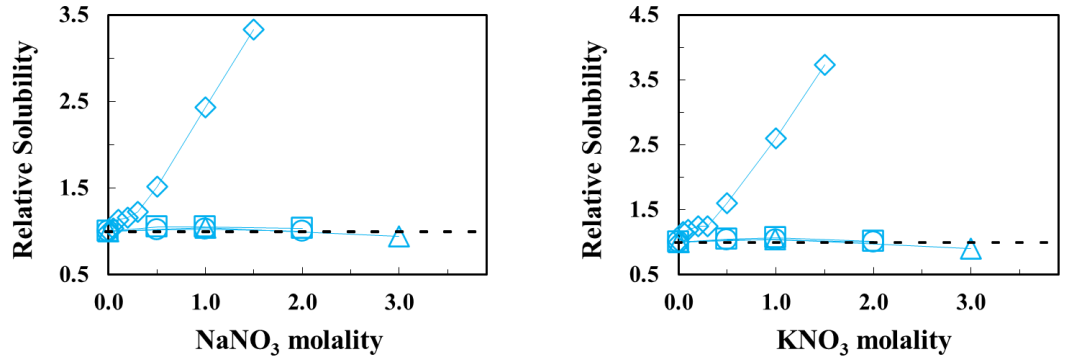

Figure 3. 11. Relative solubility of L-valine: ○, (this work); △, Held *et al.*[49], and DL-valine: □, (this work), ◇, Pradhan and Vera[63] in aqueous salt solutions. Lines are a guide to the eyes.

Using the new data, the importance of the AA structure is analyzed in both aqueous electrolyte solutions. The order in both salt solutions is the same. As can be seen in **Figure 3. 12**, valine and L-alanine shows a very slight salting-in effect with the magnitude lower than glycine and very similar to L-leucine. It would be expected to have DL-alanine positioned next to L-alanine with a much weaker salting-in effect.

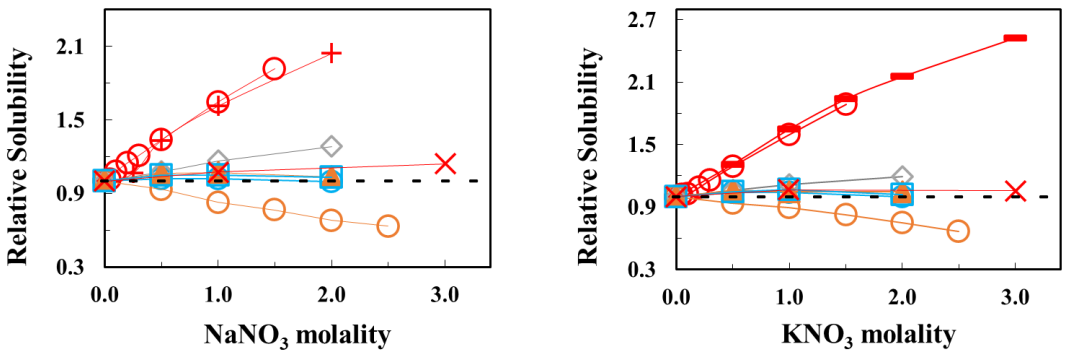

Figure 3. 12. Relative solubility of glycine: ◇, (this work); DL-alanine: ○, Pradhan and Vera[63]; +, Roy *et al.*[67]; —, Hossain *et al.*[76]; L-alanine: ×, Held *et al.*[49]; L-valine: ○, (this work); DL-valine: □, (this work); L-isoleucine: ○, Chowdurry *et al.*[68], and L-leucine: ▲, (this work) in aqueous $NaNO_3$ and KNO_3 solutions. Lines are a guide to the eyes.

3.3. Effect of the AA structure

For a given salt, its impact on the AA solubility can be explored in terms of the structural features of AA. **Figure 3. 13** illustrates the relative solubility of different AAs in aqueous NaCl solutions (by far the most studied aqueous salt solution), highlighting the relevance of the functional group on the relative solubility change. The AA showing the highest salting-in effect is L-glutamine (L-Gln), whose structure is similar to glutamic acid (Glu), where one -COOH group is replaced by an amide group. The presence of these two -COOH groups in L-aspartic acid contributes to the high salting-in effect, while L-glutamic acid[57], with an additional CH_2 group, still presents an evident salting-in, but with a smaller magnitude. Between these two AAs, the polar hydrophilic -OH group of DL-serine increases the polarity of the hydrocarbon backbone, increasing its solubility[156], while the relative solubility of L-cysteine (L-Cys) is higher than DL-serine. L-cysteine possesses an -SH group, probably contributing greatly to the salting-in magnitude, and it has been proven that in aqueous salt solutions, L-cysteine forms soluble complexes with the salts inducing the salting-in effect[56]. Next to serine is histidine (His), an alkaline AA, which has an imidazole side chain. Glycine, the simplest AA, and L-proline (L-Pro), which has a cyclic structure with the NH group in the cycle, show almost no salting-in effect, and DL and L-alanine show the smallest salting-out effect. The addition of successive CH_2 groups to alanine induces stronger salting-out in DL and L-valine, DL-nor-valine (DL-Nva), and L-leucine. L-phenylalanine, an aromatic AA, shows a hybrid behavior. Though L-lysine (L-Lys) has an additional amino group, it also contains the longer alkyl side chain, conferring a more hydrophobic character to the structure, resulting in the highest salting-out effect. Finally, although DL-tryptophan (DL-Trp) has a polar NH group in its cyclic structure and DL-tyrosine (DL-Tyr), a polar OH group, both also present large aromatic hydrophobic groups in their structures, thus being salted-out in aqueous NaCl solution, with DL-tryptophan showing a hybrid behavior.

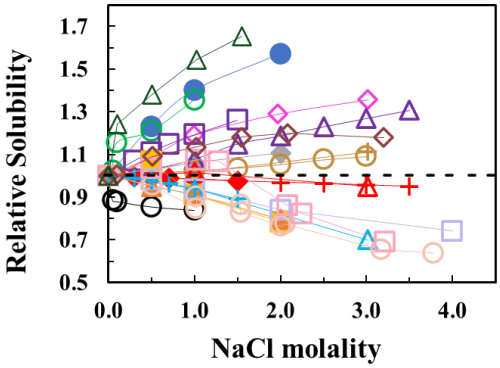

Figure 3. 13. Relative solubility of different AAs in aqueous NaCl solution at 298.2 K: ●, L-aspartic acid (this work); ■, L-phenylalanine (this work); ▲, L-leucine (this work); ◆, glycine (this work); ◇, L-glutamic acid[57]; ◆, DL-alanine[46]; +, DL-alanine[52]; △, L-alanine[49]; □, DL-serine[46]; △, DL-serine[52]; ○, L-cysteine[56]; △, L-glutamine[57]; ◇, L-histidine[57]; △, L-valine[49]; ; +, DL-valine[46]; □, DL-nor-valine[83]; ○, L-proline[53]; +, L-proline[49]; ○, DL-tyrosine[60]; □, DL-tryptophan[60]; ○, L-lysine[56]. Lines are a guide to the eyes.

The solubility dependence of AAs in the aqueous KCl solutions can be observed in **Figure 3. 14**. In good agrement with the NaCl analysis, L-aspartic acid presents the highest salting-in effect, glycine and L-proline a very weak salting-in effect, DL and L-alanine a very slight salting-out effect, L-valine, L-phenylalanine, and L-leucine showing also a salting-out effect. For the same reasons as expressed before, the data available for DL-valine[46] and DL-nor-valine[83], or L-glutamic acid[58] were not considered in the discussion. The very similar results obtained for DL-serine by Khoskhbarchi and Vera[46] and Roy *et al.*[52] indicate a much stronger salting-in behavior in KCl solutions if compared to NaCl solutions, raising some doubts not only from the fundamental interpretation derived from the physical-chemistry of the solutions, and molecular simulation, but also because for L-serine, Ferreira *et al.*[74] found a salting-in of similar magnitude to the one they found for DL-serine in aqueous NaCl solutions. Compared to serine, threonine has an additional aliphatic CH_3 group, which causes a very relevant reduction in the salting-in effect observed in serine. DL-α-aminobutyric acid (DL-α-Abu) is the AA presenting the highest salting-out, unexpectedely stronger then L-leucine.

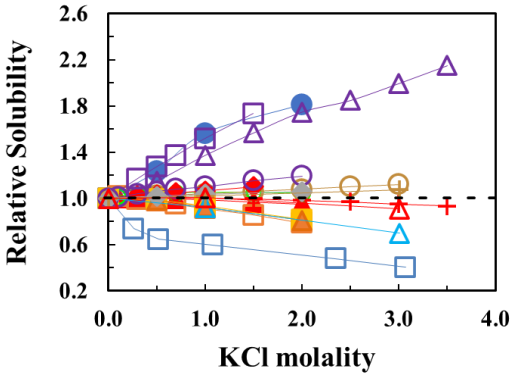

Figure 3. 14. Relative solubility of different AAs in aqueous KCl solution at 298.2 K: ●, L-aspartic acid (this work); ■, L-phenylalanine (this work); ▲, L-leucine (this work); ◆, glycine (this work); ▲, DL-alanine[70]; ◆, DL-alanine[46]; +, DL-alanine[52]; △, L-alanine[49]; □, L-isoleucine[74]; □, DL-serine[46]; △, DL-serine[52]; ○, L-serine[74]; ○, L-threonine[74]; △, L-valine[49]; □, DL-α-aminobutyric acid[82], and ○, L-proline[53]; +, L-proline[49]. Lines are a guide to the eyes.

Figure 3. 15 shows the relative solubility in aqueous $NaNO_3$ solutions, at 298.2 K. Excepting DL-nor-valine and L-proline, which in these aqueous solutions show similar salting magnitude as in the chloride salt solutions, an increase in the relative solubility was observed for all other amino acids, confirming the higher salting-in character of the nitrate anion in comparison to chloride. The results show a massive increase in the relative solubility of DL-alanine, which is too large in comparison with the L-isomer. The same was observed in the case of the serine isomers (**Figure 3. 17)**, and only the data of L-serine is left in **Figure 3. 15**. Considering the results of L-isomers, it can be concluded that after aspartic acid, the AA with the highest salting-in effect is L-serine. With the addition of methyl groups to glycine, the relative solubilities of AAs with larger apolar groups decreases, and it follows: Gly > L-Ala > L-Val ≈ DL-val ≈ L-Leu > DL-nor-Val > L-Ile. DL-α-aminobutyric acid, which has one more methyl group than alanine, seems to be in the right place compared to the results of alanine, but like in KCl aqueous solutions, show an unexpected salting magnitude considering the results of other non-polar aliphatic AAs. L-Phe with an aromatic group showed the highest salting-in effect among the AAs with apolar groups.

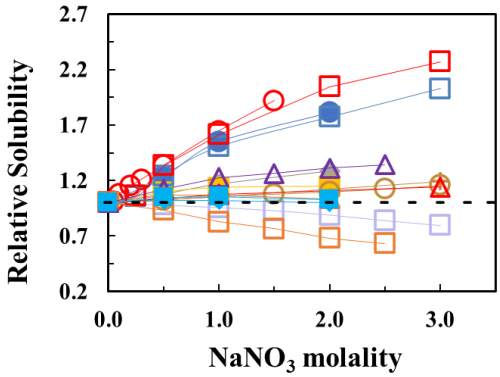

Figure 3. 15. Relative solubility of different AAs in aqueous $NaNO_3$ solution at 298.2 K: ●, L-aspartic acid (this work); ■, L-phenylalanine (this work); ▲, L-leucine (this work); ◆, glycine (this work); ●, L-valine (this work); ■, DL-valine (this work); □, L-isoleucine[68]; ○, DL-alanine[63]; □, DL-alanine[67]; △, L-alanine[49]; □, DL-nor-valine[64]; △, L-serine[68]; ○, L-proline[53]; +, L-proline[49] and □, DL-α-aminobutyric acid[84]. Lines are a guide to the eyes.

The dependence of the relative solubilities of AAs on the molality of aqueous KNO_3 solution is shown in **Figure 3. 16**. The similarities found with the $NaNO_3$ solutions again reaffirm how monovalent cations do not significantly affect the solubility of the amino acids. In both solutions with the nitrate anion, all the AAs with polar and apolar functional groups follow the same order with very similar magnitudes. Compared to KCl, the relative solubilities of all the AAs increased, and even alanine, leucine, phenylalanine, and valine which suffer salting-out in solutions with the chloride anions, show slight salting-in results in these nitrate solutions.

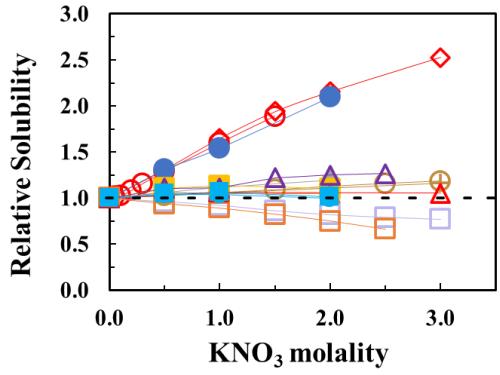

Figure 3. 16. Relative solubility of different AAs in aqueous KNO_3 solution at 298.2 K: ●, L-aspartic acid (this work); ■, L-phenylalanine (this work); ▲, L-leucine (this work); ◆, glycine (this work); ●, L-valine (this work); ■, DL-valine (this work); □, L-isoleucine[68]; ◇, DL-alanine[76]; ○, DL-alanine[63]; △, L-alanine[49], △, L-serine[68]; ○, L-proline[53]; +, L-proline[49]; and □, DL-nor-valine[64]. Lines are a guide to the eyes.

3.3.1. Consistency analysis

Figure 3. 17 presents the relative solubilities of DL-serine and L-serine in aqueous $NaNO_3$ and KNO_3 solutions at 298.2 K. Even though two independent and similar sets of data were found, the salting-in magnitude of the DL-isomer seems, in comparison to the L-serine, is too large. As a result, they were not included in **Figure 3. 15** and **Figure 3. 16**.

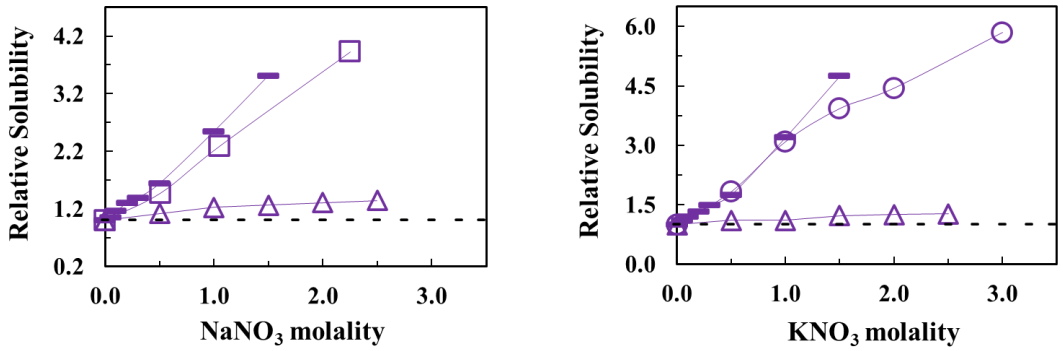

Figure 3. 17. Relative solubility of DL-serine: —, Pradhan and Vera[63]; □, Mondal *et al.*[59]; ○, Hossain *et al.*[76]; and △, L-serine: Chowdhury *et al.*[68] in aqueous $NaNO_3$ and KNO_3 solutions. Lines are a guide to the eyes.

The available data for glycine, DL-alanine, DL-serine, DL-valine, and L-isoleucine, shown in **Figure 3. 18**, confirm the difficulty to establish, with enough confidence, a rationale about the effect when changing the cation while fixing the anion (NaF/KF) or the opposite (NaF/NaBr). The results in aqueous K_2SO_4 solution also present a different order than **Figure 3. 7**, showing that the potassium cation, unlike the sodium cation, induces a salting-in effect with alanine and valine, that sheds some light on the importance of checking the data consistency before drawing general rules concerning the impact of the different anions on AA solubility, as monovalent cations do not interact with the amino acids and have a low impact on their solubility.

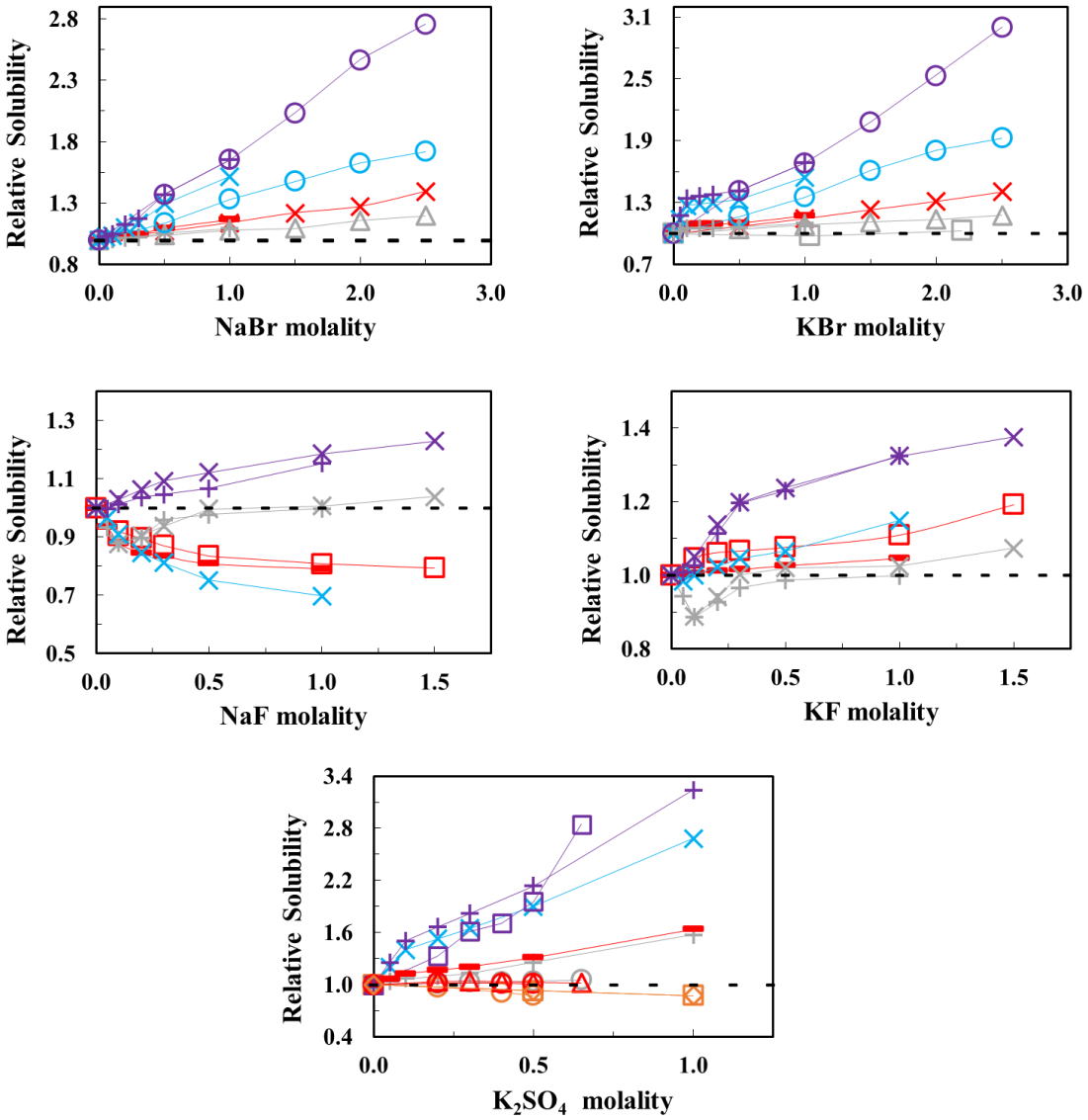

Figure 3. 18. Relative solubility of ×, DL-alanine[61]; —, DL-alanine[42]; □, DL-alanine[41]; ○, DL-alanine[79]; △, DL-alanine[77]; □, Glycine[75]; △, Glycine[61]; ×, Glycine[41]; ○, Glycine[77]; +, Glycine[42]; ×, DL-valine[42]; ○, DL-valine[61]; □, L-isoleucine[57]; ◇, L-isoleucine[73]; ○, L-isoleucine[40]; ○, DL-serine[61]; +, DL-serine[42]; ×, DL-serine[41]; □, DL-serine[77] in aqueous salt solutions at 298.2 K. Lines are a guide to the eyes.

3.4. The pH of the saturated solutions

Besides solubility, the pH of the saturated solutions was also measured at 298.2 K and is reported in **Table 3. 3**. It is possible to conclude that all amino acids are in the zwitterionic form (dipolar ions) in the saturated solutions, as can be seen in the microspecies distribution as a function of pH presented in **Appendix B**.

Table 3. 3. pH range of saturated solutions of L-aspartic acid, glycine, L-phenylalanine, or L-leucine in the studied aqueous electrolyte solutions at 298.2 K.

	pH range in the ternary solution					
AAs	NaCl	$NaNO_3$	KCl	KNO_3	NH_4Cl	NH_4NO_3
Gly	5.97*-7.17	5.97*-6.30	5.97*-6.26	5.97*-6.33	5.97*-6.22	5.97*-6.17
Leu	5.77-5.98*	5.98*-6.09	5.75-5.98*	5.83-5.98*	5.56-5.98*	5.67-5.98*
Phe	5.48*-6.07	5.48*-5.66	5.48*-6.25	5.48*-5.60	5.42-5.48*	5.44-5.48*
Asp	2.76-2.77*	2.77*-2.81	2.77*-2.97	2.77*-3.11	2.77*-3.02	2.77*-3.18
L-val	–	5.97*-6.28	–	5.97*-6.36	–	–
DL-val	–	5.97*-6.12	–	5.97*-6.20	–	–

*Saturated solution in water[1]. $u\,(pH) = 0.05$, $u\,(T) = 0.1$ K.

3.5. The solid phase studies

The solid phase studies are a strategy to examine any eventual changes in the crystallographic form of the amino acids, that might impact the salting effects. So, the solids from the supplier were analyzed by single crystal and powder diffraction. All the AA from supplier were analyzed and the information about the structure, cell parameters, CCDC code, and references relative to the crystal forms found is presented in **Table 3. 4**.

Table 3. 4. Cell parameters for Glycine, L-aspartic acid, L-phenylalanine and L-leucine from supplier determined by single crystal X-ray diffraction and comparison with published data in CCDC Cambridge database.

Amino acid	Crystal form	CCDC code	Reference
Glycine	Monoclinic P (α-glycine) a=5.10 Å; b=11.99 Å; c=5.45 Å; □= 111.53°	1416373	Jiang *et al.*[157]
	Hexagonal P (γ-glycine) a=b=7.00 Å; c=5.48 Å	1416374	Jiang *et al.*[157]
L-Aspartic acid	Monoclinic P a=5.11 Å; b=6.92 Å; c=7.60 Å; □= 100.38°	652520	Bendeif and Jelsch[158]
L-Phenylalanine	Monoclinic P a=8.80 Å; b=6.06 Å; c=31.38 Å; □= 97.36°	1012155	Ihlefeldt *et al.*[159]
L-Leucine	Monoclinic P a=9.63 Å; b=5.34 Å; c=14.65 Å; □= 94.09°	1508364	Binns *et al.*[160]
L-valine		1208818	Dalhus and Gör-bitz[161]
DL-valine		1279575	Flaig *et al.*[162]

Glycine from the supplier presents a mixture of two phases, a monoclinic corresponding to the α-form and a hexagonal corresponding to γ-form (**Appendix C**: **Figure C 1**), both already described in the literature. The solid samples from the suppliers of L-valine, and DL-valine did not allow the cell parameters determination by single crystal X-ray diffraction. Thus, these samples were analyzed by powder X-ray diffraction showing all the same powder patterns as the samples with CCDC deposition numbers 1208818[161] and 1279575[162] respectively (**Appendix C**: **Figure C 6** and **Figure C 7**). All the other amino acids from supplier show a single phase and are also well characterized in the literature (**Appendix C**: **Figure C 3**, **Figure C 4**, **Figure C 5**). In all aqueous solutions of the six electrolytes, the glycine samples (**Appendix C**: **Figure C 2**) crystallized only in the hexagonal crystal system, the γ-form. The crystalline form of the other amino acids (**Appendix C**: **Figure C 3**, **Figure C 4**, **Figure C 5**, **Figure C 6** and **Figure C 7**) investigated in some of the electrolyte solutions did not change compared to the structure found in the original solid from the supplier.

Chapter 4 – Solubilities of amino acids in aqueous solutions of salts composed of di-valent cations.

This chapter includes the measurement of the solubility of glycine, L-leucine, L-phenylalanine, and L-aspartic acid in aqueous salt solutions with divalent cations, such as Mg^{2+} and Ca^{2+}, and Cl^- and NO_3^- anions at 298.2 K. A comparison of the results with literature was carried out when it was possible. The relative solubility results were compared with the ones in aqueous solutions of salts with the monovalent cations. Effect of the structures of the AAs on their solubility was designed by studying the relative solubility of different AAs in aqueous solutions of the same salt. The pH of the saturated solutions and the results of the solid phase studies are also included.

I declare that I have provided all the solubility measurements of AAs in aqueous salt solutions and monitored the pH of the saturated solution and wrote the paper. The solid-phase studies were performed by Paula Brandão from CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal.

4.1. Experimental data and analysis

Table 4. 1 compiles the measured solubilities along with the standard deviation (in brackets) of L-aspartic acid, L-phenylalanine, L-leucine, and glycine in the aqueous $MgCl_2$, $Mg(NO_3)_2$, $CaCl_2$, and $Ca(NO_3)_2$ solutions with a salt molality of 0, 0.5, 1.0 and 2.0 at 298.2 K. In all the studied systems the absolute solubility follows the order Gly > Phe > Leu > Asp, as in pure water. The maximum coefficient of variation (standard deviation/average*100) is 3.5% (system water/calcium nitrate/aspartic acid, 1 mol·kg^{-1} salt concentration), being lower than 1% in about 79% of cases.

Table 4. 1. Solubility of the amino acids (g of AA/1000 g of H_2O), (standard deviation in brackets) in aqueous solutions of salts at different molalities, T = 298.2 K and p = 0.1 MPa.[a]

Salts	Electrolyte molality (mol/kg of H_2O)	S_{AA} (g of AA/1000 g of H_2O			
		Glycine	L-leucine	L-phenylalanine	L-aspartic acid
No salt	0.000	238.332 (0.127)	21.544 (0.070)	28.347 (0.083)	5.140 (0.031)
$MgCl_2$	0.500	272.843 (0.143)	24.939 (0.082)	32.632 (0.043)	6.722 (0.051)
	1.000	293.517 (0.340)	24.465 (0.049)	33.012 (0.138)	7.917 (0.172)
	2.000	370.316 (0.394)	28.497 (0.119)	34.289 (0.202)	10.300 (0.143)

$Mg(NO_3)_2$	0.500	303.191	29.895	42.916	8.649
		(0.168)	(0.076)	(0.066)	(0.051)
	1.000	355.417	36.981	54.307	11.443
		(0.052)	(0.226)	(0.456)	(0.328)
	2.000	519.400	51.925	76.838	18.414
		(0.153)	(0.146)	(0.184)	(0.380)
$CaCl_2$	0.500	294.982	26.275	34.812	8.421
		(0.985)	(0.080)	(0.164)	(0.128)
	1.000	354.148	29.801	39.420	9.951
		(0.700)	(0.064)	(0.767)	(0.077)
	2.000	489.824	35.734	44.668	15.497
		(0.650)	(0.385)	(0.081)	(0.189)
$Ca(NO_3)_2$	0.500	316.463	30.768	44.111	9.492
		(0.312)	(0.429)	(0.143)	(0.010)
	1.000	393.988	38.170	57.899	13.418
		(0.378)	(0.169)	(0.352)	(0.470)
	2.000	578.196	54.255	90.694	23.830
		(0.094)	(0.391)	(0.458)	(0.451)

[a] u(salt molality) = 0.001 mol·kg^{-1}, $u_r(S_{AA})$ = 0.05, u (T) = 0.1 K and $u_r(p)$ =0.05.

Figure 4. 1 presents the relative solubilities of glycine, L-leucine, L-phenylalanine, and L-aspartic acid in aqueous $MgCl_2$, $Mg(NO_3)_2$, $CaCl_2$ and $Ca(NO_3)_2$ solutions. All the salts with divalent cations induce a salting-in effect over the whole salt concentration range for all the AA. L-aspartic acid, the most polar AA due to the presence of a second negatively charged hydrophilic carboxyl group ($-CH_2COO^-$) in the side chain, presents the highest salting-in magnitude in all the aqueous salt solutions studied, which can attain a relative solubility of 4.6 in a 2 mol/kg calcium nitrate aqueous solution. The ranking differs mostly with the anion type, the relative solubilities in aqueous $MgCl_2$ and $CaCl_2$ solutions follow the order Asp > Gly > Leu ≅ Phe, while in the aqueous $Mg(NO_3)_2$ and $Ca(NO_3)_2$ solutions, the ranking is Asp > Phe > Leu ≅ Gly. The main difference is connected to the relative position of Phe and Gly, which confirms that nitrate anions are more effective in interacting with the apolar moieties of the AA, increasing the solubility of phenylalanine, and even leucine, with a larger apolar side chain, when compared to glycine.

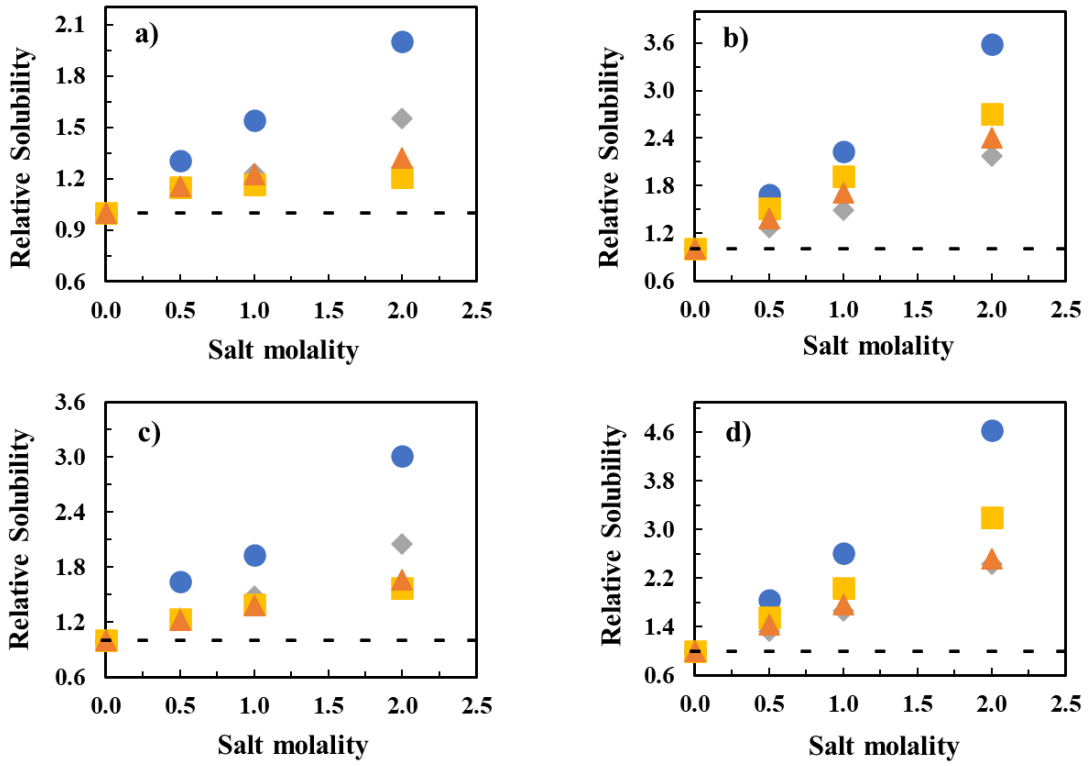

Figure 4. 1. Relative solubility of ◆, glycine; ▲, L-leucine; ■, L-phenylalanine, and ●, L-aspartic acid in aqueous a) $MgCl_2$; b) $Mg(NO_3)_2$; c) $CaCl_2$; and d) $Ca(NO_3)_2$ solutions with different molalities at 298.2 K.

Figure 4. 2 shows the effect of all the studied salts on the relative solubility of each amino acid. As observed previously with the monovalent cations, comparing salt solutions with the same divalent cation, the effect of nitrate anion on the relative solubilities of all the AA is higher than with chloride anions. In fact, using MD simulations, Tomé *et al.*[27] showed that interaction of the NO_3^- anion with the hydrophobic groups of the AAs is more significant than with chloride, causing a larger solubility increase. In the Hofmeister series[28], these anions are close to each other, but the nitrate anion is more to the right side, i.e., to where salting-in anions are located. Maintaining the anion and changing the cation, the Ca^{2+} cation induces a salting-in effect with a higher magnitude than the Mg^{2+}, again in consistency with the Hofmeister series where Ca^{2+} is the strongest salting-in cation. MD in systems with isoleucine as model AA showed very strong binding of the polyvalent cations to the carboxylate $(COO)^-$ group of the amino acid. As demonstrated in several works,[37,163,164] this type of interaction leads to the formation of stable complexes between the biomolecules and the divalent cations. However, the magnitude of the peaks in the radial distribution functions, or the distance of its appearance, is not totally conclusive since the Ca^{2+} cation presents very similar

values compared to Mg^{2+}. Nevertheless, both divalent cations present much stronger interaction than monovalent cations, but in both cases, interactions with the hydrophobic parts of the AA are not significant.

Globally, glycine shows a salting-in effect with close magnitudes in $Ca(NO_3)_2$, $Mg(NO_3)_2$, and $CaCl_2$ solutions, being that magnitude much lower in aqueous $MgCl_2$ solution (evaluated at 2 mol/kg). L-leucine and L-phenylalanine present a salting-in effect with similar magnitudes in both nitrate solutions and much lower, even if with similar magnitudes, in the solutions with the chloride anions. All the salts induce a salting-in effect in L-aspartic acid, being, among the studied AA, the one showing the larger change in solubility induced by all four salts studied.

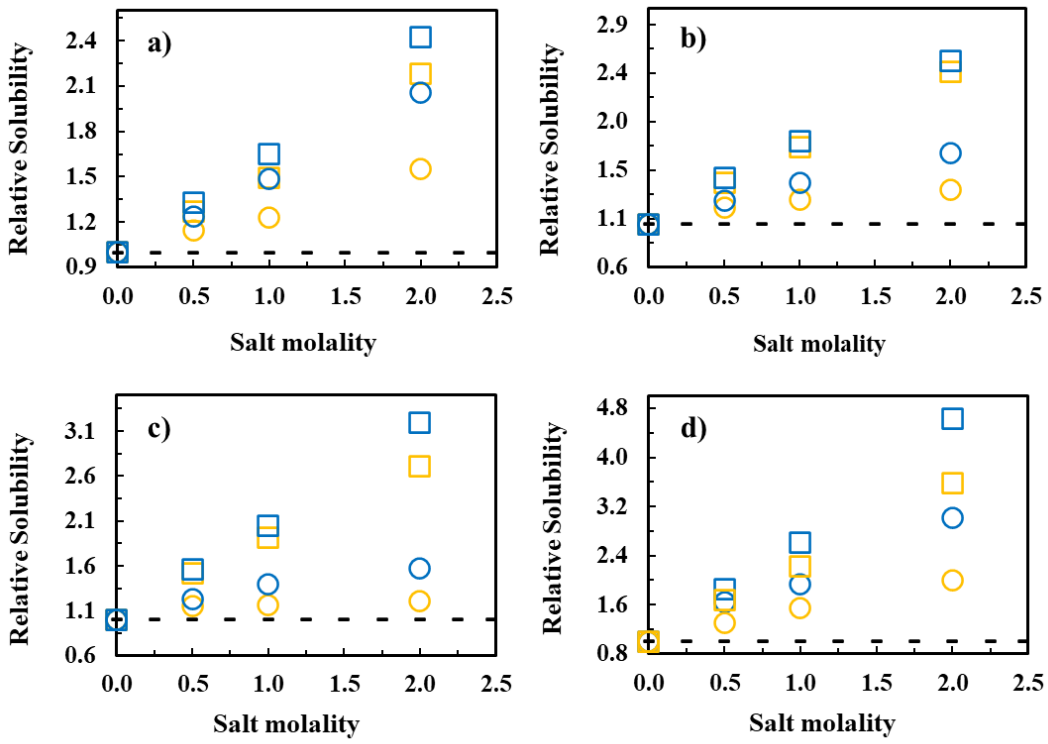

Figure 4. 2. Relative solubility of a) glycine; b) L-leucine; c) L-phenylalanine, and d) L-aspartic acid in aqueous ○, $MgCl_2$; □, $Mg(NO_3)_2$; ○, $CaCl_2$; □, $Ca(NO_3)_2$ solutions with different salt molalities at 298.2 K.

For L-leucine, L-phenylalanine, and L-aspartic acid, no data in the studied aqueous salt solutions were found in the literature, and no comparisons can be presented. The solubility of Gly was studied in aqueous $MgCl_2$ solutions,[47,80] but not at 298.2 K. However, in the temperature range between 293.2 and 303.2 K, in a two mol/kg $MgCl_2$ solution the relative

solubility is close to 1.6,[47] while in this work at 298.2 K the corresponding value is close to 1.55, showing good coherence between both works. As shown in **Figure 4. 3**, a comparison can be made for the relative solubility of Gly in aqueous $CaCl_2$ solutions. In both works, a salting-in effect is observed, but the magnitude differs significantly at higher concentrations. A similar situation was also reported by Tomé *et al.*[40] for DL-alanine in an aqueous $CaCl_2$ solution. As discussed above, in the case of monovalent cations, and by Tomé *et al.*[40], the data from El-Dossoki[42] need to be carefully checked as multiple significant discrepancies have been found.

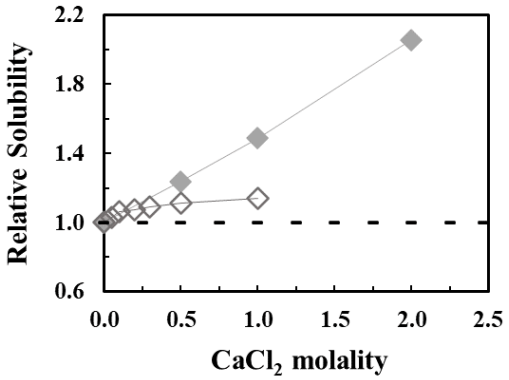

Figure 4. 3. Relative solubility of glycine in aqueous $CaCl_2$ solution at 298.2 K: ◆, (this work); ◇, El-Dossoki[42]. Lines are a guide to the eyes.

Figure 4. 4 shows the relative solubility of glycine, L-leucine, L-phenylalanine, and L-aspartic acid in 2 mol/kg aqueous solutions of the salts combining one of the Na^+, K^+, NH_4^+, Mg^{2+}, Mg^{2+} cations with the Cl^- or NO_3^- anion, at 298.2 K. Comparing the effect of the salts with the same anion, the divalent cations induce a much higher salting-in effect than the salts with the monovalent cations for all AA. In the case of Gly, both salts with the divalent cations and the chloride anion show higher relative solubility than those with the monovalent cations and the nitrate anions. The apolar moiety in Gly is very small, and the balance is much favorable for the interaction of the divalent cation with the carboxylate if compared to the interaction between the nitrate and Gly. For the rest of the AAs, the order differs. Accordingly, the results in the solution with L-leucine and NH_4NO_3 show a salting-in effect of the same magnitude as $MgCl_2$, while in L-phenylalanine, with an apolar aromatic side chain, the salting-in effect of NH_4NO_3 is comparable to that observed in aqueous $CaCl_2$ solutions (with the strongest salting-in cation). This is a consequence of the nitrate anion interaction[27] with the apolar AA moieties leading to a more relevant salting-in effect. Comparing divalent

cations, the salting-in effect enhancement when moving from chloride to nitrate is highest in L-phe, similar for L-leu and L-asp, and the lowest in Gly.

Additionally, that enhancement is more significant in magnesium than calcium salts. When fixing the anion, the salting-in change, moving from magnesium to calcium salts, is more evident in chlorides, while monovalent to divalent cations are more significant in nitrates. Despite its small apolar region, L-asp is the only AA showing a similar salting-in effect in aqueous $MgCl_2$ as in all nitrate solutions of monovalent cations.

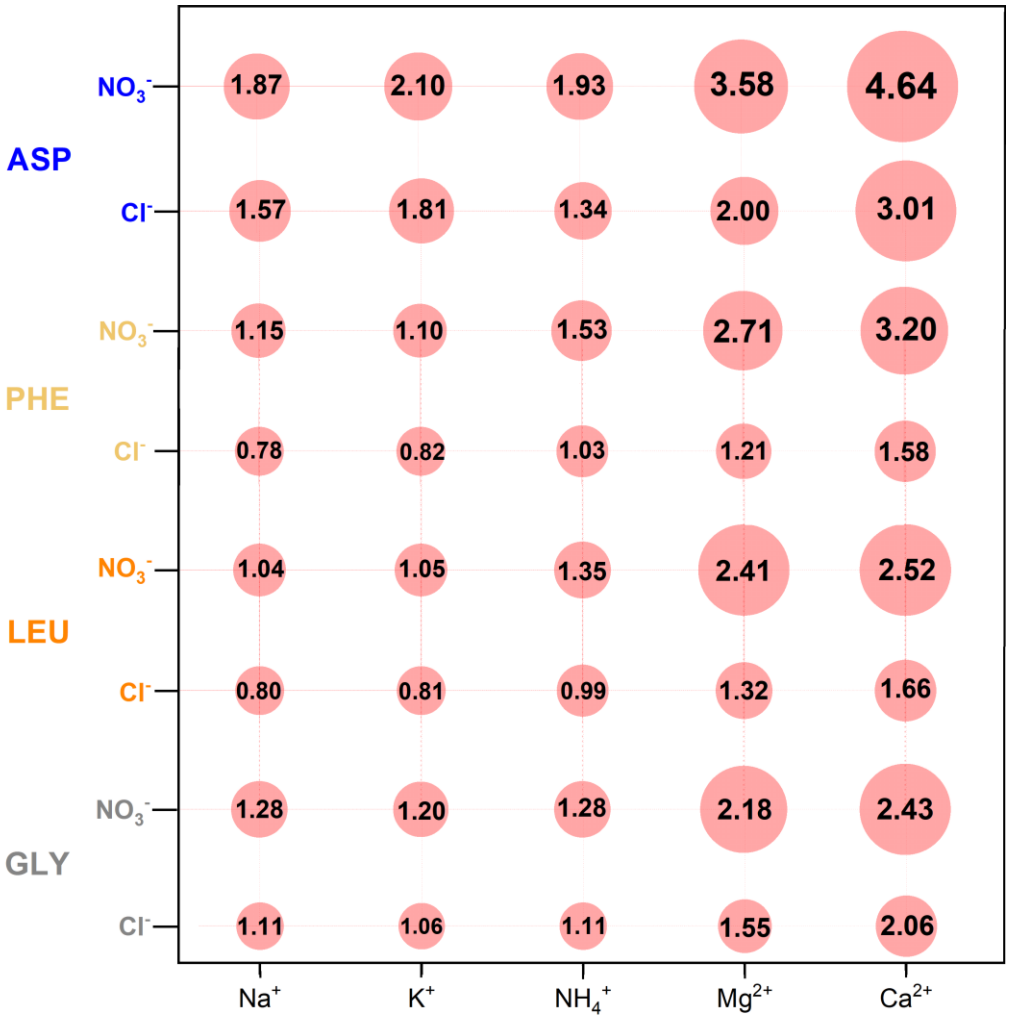

Figure 4. 4. Relative solubility of glycine, L-leucine, L-phenylalanine, and L-aspartic acid in 2 mol/kg aqueous solutions of $MgCl_2$, $Mg(NO_3)_2$, $CaCl_2$, $Ca(NO_3)_2$, NaCl, $NaNO_3$, KCl, KNO_3, NH_4Cl, and, NH_4NO_3 at 298.2 K.

4.2. Effect of the AA structure

The effect of the AA side chain was studied by collecting data from different AA in aqueous solutions of chloride salts, found in the open literature, and analyzed all together with those measured in this work. For aqueous $MgCl_2$ solutions, results were found just for three AA (DL-alanine, L-valine, and L-isoleucine)[79]. **Figure 4. 5** presents the relative solubility change in aqueous $MgCl_2$ solutions, showing a salting-in effect with the seven AA. L-aspartic acid with the polar acidic side chain shows the highest salting-in, followed by glycine with just hydrogen as the side chain, followed by DL – alanine, which has an additional methyl group, compared to glycine. As the AA with larger apolar groups show a very similar salting-in effect, **Figure 4. 5b** is presented to understand the AA ranking more easily. The branched-chain aliphatic AA, L-leucine and L-isoleucine, isomers differing slightly in their chemical structure, are followed by L-valine, which has one methylene group less than leucine or isoleucine but is bulkier. This is consistent with having, among all AA studied, L-phenylalanine as the one presenting the lowest salting-in effect.

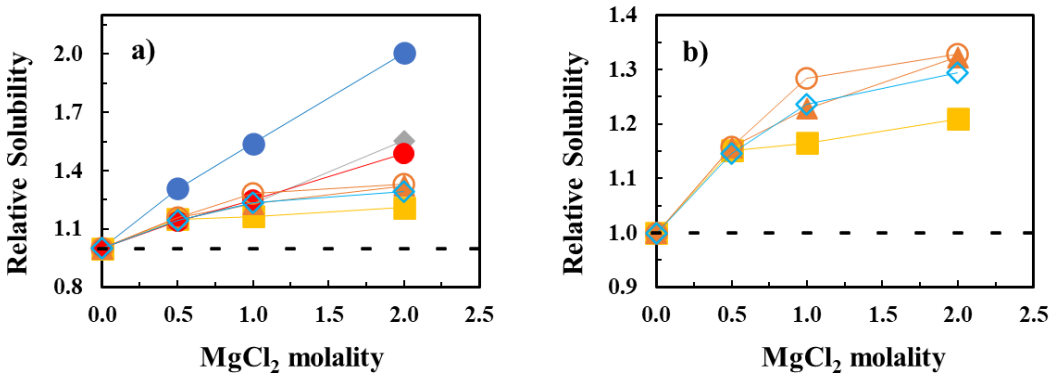

Figure 4. 5. Relative solubility of different AAs in aqueous $MgCl_2$ solution at 298.2 K: ●, L-aspartic acid (this work); ■, L-phenylalanine (this work); ▲, L-leucine (this work); ◆, glycine (this work); ●, DL-alanine[79]; ◇, L-valine[79]; ○, L-isoleucine[79]. Lines are a guide to the eyes.

Figure 4. 6 shows a similar comparison in aqueous $CaCl_2$ solutions. This salt induces a salting-out effect just for L-lysine.[56] Lysine is an alkaline, aliphatic AA whose side chain L-lysine ($(CH_2)_4NH_2$) contains one extra amino group. This demonstrates that besides the weak interaction of chloride anion with the alkyl moieties of the molecule, it also reveals the low interaction of chloride anion with the amine group of the AA. This is surprising as the strong interaction of Ca^{2+} with the carboxylate is somehow lost by the presence of a large hydrophobic group, which did not happen with isoleucine, for instance. However, the data seems

unreliable, also due to the very close salting-out magnitudes caused by NaCl or $CaCl_2$, as reported by El-Dossoki *et al.*[56]. The presence of two -COOH groups in L-aspartic acid and the polar, hydrophilic -OH group in DL-serine (increases the polarity of its hydrocarbon side chain) lead to a more pronounced salting-in effect. The thiol side chain (-SH) of L-cysteine seems to be a reason for the very pronounced salting-in at the salt infinite dilution, bringing some doubts again on data reliability as no changes in the solubility are observed at higher salt molalities. In terms of the AA with a completely apolar side chain, after glycine, salting-in decreases in the order DL-alanine (R = $-CH_3$), L-leucine (R = $-C_4H_9$), L-phenylalanine (R = - $CH_2C_6H_5$), L-valine (R = $-C_3H_7$), and L-isoleucine (R = $-C_4H_9$) showing a salting-in effect with very close magnitudes. In aqueous $MgCl_2$ solution the relative solubility order of AA with apolar side chains follow: DL-ala > L-leu > L-ile > L-val > L-phe while in $CaCl_2$: DL-ala > L-leu > L-phe > L-ile. All the AA studied in this work and DL-alanine, L-isoleucine and L-valine show salting-in effects with higher magnitudes in aqueous $CaCl_2$ solutions.

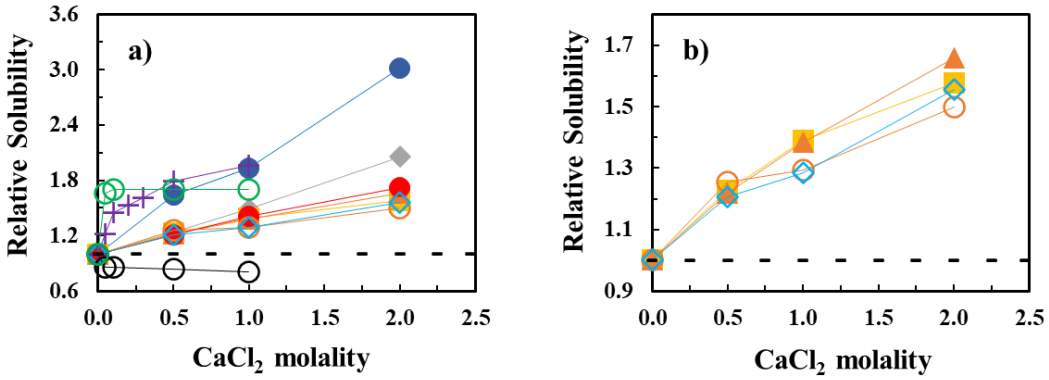

Figure 4. 6. Relative solubility of different AAs in aqueous $CaCl_2$ solution at 298.2 K: ●, L-aspartic acid (this work); ■, L-phenylalanine (this work); ▲, L-leucine (this work); ◆, glycine (this work); ●, DL-alanine[40]; +, DL-serine[42]; ○, L-cysteine[56]; ◇, L-valine[40]; ○, L-isoleucine[40]; ○, L-lysine[56]. Lines are a guide to the eyes.

4.3. The pH of the saturated solutions

The pH of each saturated solution was also measured at 298.2 K and the values listed in **Table 4. 2**. As the pHs of the solutions are very close to the isoelectric point, it can be concluded that AAs in the saturated solutions are in the zwitterionic form (dipolar ions), which was also confirmed by the microspecies distribution as a function of pH presented in **Appendix B**.

Table 4. 2. pH values for the different AA saturated solutions in aqueous electrolyte solutions at 298.2 K.[a]

	pH range in the ternary solution			
Amino acids	$MgCl_2$	$Mg(NO_3)_2$	$CaCl_2$	$Ca(NO_3)_2$
Glycine	5.56-5.97*	5.79-5.97*	5.91-5.97*	5.97*-6.04
Leucine	5.72-5.98*	5.72-5.98*	5.94-5.98*	5.98*-6.04
Phenylalanine	5.25-5.48*	5.39-5.48*	5.47-5.48*	5.48*-5.70
Aspartic acid	2.55-2.77*	2.46-2.77*	2.48-2.77*	2.57-2.77*

[a]Saturated solution in water[1]. u (pH) = 0.05, u (T) = 0.1 K.

4.4. The solid phase studies

Cell parameters for glycine, L-aspartic acid, L-phenylalanine and L-leucine from supplier are already described in **Table 3. 4**. **Table 4. 3** presents the structure, cell parameters, CCDC code, and references relative to the crystal forms found in aqueous electrolyte solutions.

As in aqueous solutions of salts with the monovalent cations, here also in all four aqueous electrolyte solutions glycine solid phase is only in the hexagonal crystal system, the γ-form (**Appendix C: Figure C 8**) and the crystalline form of the other amino acids in equilibrium with the saturated solutions containing the different electrolytes does not change compared to the structure found in the solid from the supplier (**Appendix C: Figure C 9**, **Figure C 10** and **Figure C 11**). The only exception is for phenylalanine (**Appendix C: Figure C 11**) in aqueous solutions $MgNO_3$, containing a second phase. Searching in the ICDD database (version 2022), it was impossible to identify this second phase.

Table 4. 3. Cell parameters for amino acids in aqueous solutions of chloride or nitrate salts of divalent (Mg^{2+} or Ca^{2+}) Cations determined by single crystal X-ray diffraction and comparison with published data in CCDC Cambridge database.

Sample	Crystal form	CCDC code	Reference
Gly-$CaCl_2$ Gly-$CaNO_3$ Gly-$MgNO_3$ Gly-$MgCl_2$	Hexagonal P (γ-glycine) a=b=6.96Å;Å; c=5.43 Å a=b=7.01Å;Å; c=5.49 Å a=b=7.02Å;Å; c=5.50 Å powder	1416374	Jiang *et al.*[157]
aa-$CaCl_2$ aa-$CaNO_3$ aa-$MgNO_3$ aa-$MgCl_2$	Monoclinic P a=5.14 Å; b=6.93 Å; c=7.61 Å; β= 100.57° a=5.15 Å; b=6.97 Å; c=7.63 Å; β= 100.53° powder powder	652520	Bendeif and Jelsch[158]
Phe-$CaNO_3$ Phe-$MgNO_3$	Powder powder	1012155	Ihlefeldt *et al.*[159]
Leu-$CaCl_2$ Leu-$CaNO_3$	Powder powder	1508364	Binns *et al.*[160]

Chapter 5 – Solubilities of amino acids in aqueous solutions of salts with the thiocyanate or tosylate anion.

This chapter includes the measurement of the solubility of glycine, L-leucine, L-phenylalanine, and L-aspartic acid in aqueous NaSCN, KSCN, NH_4SCN and Na-tosylate solutions at 298.2 K. The pH of the saturated solutions and the results of the solid phase studies are also presented.

I declare that I have provided all the solubility measurements of AAs in aqueous salt solutions and monitored the pH of the saturated solution. The solid-phase studies were performed by Paula Brandão from CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal.

5.1. Experimental data and analysis

Table 5. 1 reports the measured solubilities and the standard deviation (in brackets) of L-aspartic acid, L-phenylalanine, L-leucine, and glycine in the aqueous NaSCN, KSCN, NH_4SCN and Na-tosylate solutions with a salt molality of 0, 0.5, 1.0 and 2.0 at 298.2 K. In all the studied systems the absolute solubility follows Gly > Phe > Leu > Asp, which matches the solubility in pure water. The maximum coefficient of variation is 2.9% in the system water/potassium thiocyanate/L-aspartic acid at 0.5 mol·kg^{-1} salt concentration.

Table 5. 1. Solubility of the amino acids (g of AA/1000 g of H_2O), (standard deviation in brackets) in aqueous salt solutions at different molalities, T = 298.2 K and p = 0.1 MPa.[a]

Salts	Electrolyte molality (mol/kg of H_2O)	S_{AA} (g of AA/1000 g of H_2O)			
		Glycine	L-leucine	L-phenylalanine	L-aspartic acid
No salt	0.000	238.332*	21.544*	28.347*	5.140*
		(0.127)	(0.070)	(0.083)	(0.031)
NaSCN	0.500	253.508	23.946	32.221	6.615
		(0.043)	(0.123)	(0.121)	(0.064)
	1.000	266.399	24.806	36.221	7.586
		(0.379)	(0.125)	(0.251)	(0.061)
	2.000	299.763	27.167	41.939	10.324
		(0.177)	(0.219)	(0.600)	(0.166)
KSCN	0.500	250.235	23.928	33.245	6.619
		(0.093)	(0.022)	(0.265)	(0.193)
	1.000	260.997	24.606	36.858	8.170
		(0.770)	(0.020)	(0.052)	(0.082)
	2.000	286.383	26.820	42.874	10.616
		(0.283)	(0.161)	(0.043)	(0.091)
NH_4SCN	0.500	252.357	24.956	34.462	6.853
		(0.621)	(0.167)	(0.214)	(0.059)

	1.000	268.018	28.109	41.790	8.820
		(0.503)	(0.325)	(0.799)	(0.098)
	2.000	297.828	31.442	54.377	11.245
		(0.348)	(0.124)	(0.346)	(0.226)
Na-tosyl-	0.500	236.834	22.095	34.267	6.994
ate		(0.272)	(0.066)	(0.466)	(0.114)
	1.000	230.152	24.453	41.407	8.530
		(0.302)	(0.166)	(0.280)	(0.131)
	1.500	–	–	50.709	–
				(0.104)	
	2.000	255.512	32.540	36.888	11.237
		(1.147)	(0.198)	(0.656)	(0.240)

[a] u(salt molality) = 0.001 mol·kg^{-1}, $u_r(S_{AA})$ = 0.05, u (T) = 0.1 K and $u_r(p)$ =0.05.

Figure 5. 1 shows the relative solubilities of all the studied AAs in different aqueous salt solutions at 298.2 K. All the thiocyanate salts induce a salting-in effect over the whole salt concentration range for all the AA. The magnitude of a salting-in effect of each AA is very similar in all the aqueous salt solutions with the different monovalent cations, except the Leu in aqueous NH_4SCN solution, where the magnitude is higher as the relative solubility of Leu in aqueous NH_4SCN solution gets very close to that of Phe, and not glycine as observed for the other salt solutions. The relative solubility follows the order Asp > Phe > Leu ≅ Gly in the aqueous solutions of salt with the sodium and potassium cations as in the aqueous $Mg(NO_3)_2$ and $Ca(NO_3)_2$ solutions. With the ammonium cation the ranking is Asp > Phe ≅ Leu > Gly. That is, L-aspartic acid with the polar side chain presents the highest salting-in effect. Amino acids with the apolar groups – leucine and phenylalanine show higher salting-in effect than glycine that is the simplest AA, thus demonstrating the interaction of the thiocyanate anion with the apolar moieties of the AA. In line with the conclusions of **Chapter 1** and from the MD simulations studies[27] the results here reported also show that the monovalent cations do not interact with the hydrophobic groups of the AA and the binding with the carboxylate groups of the AA is less favorable.

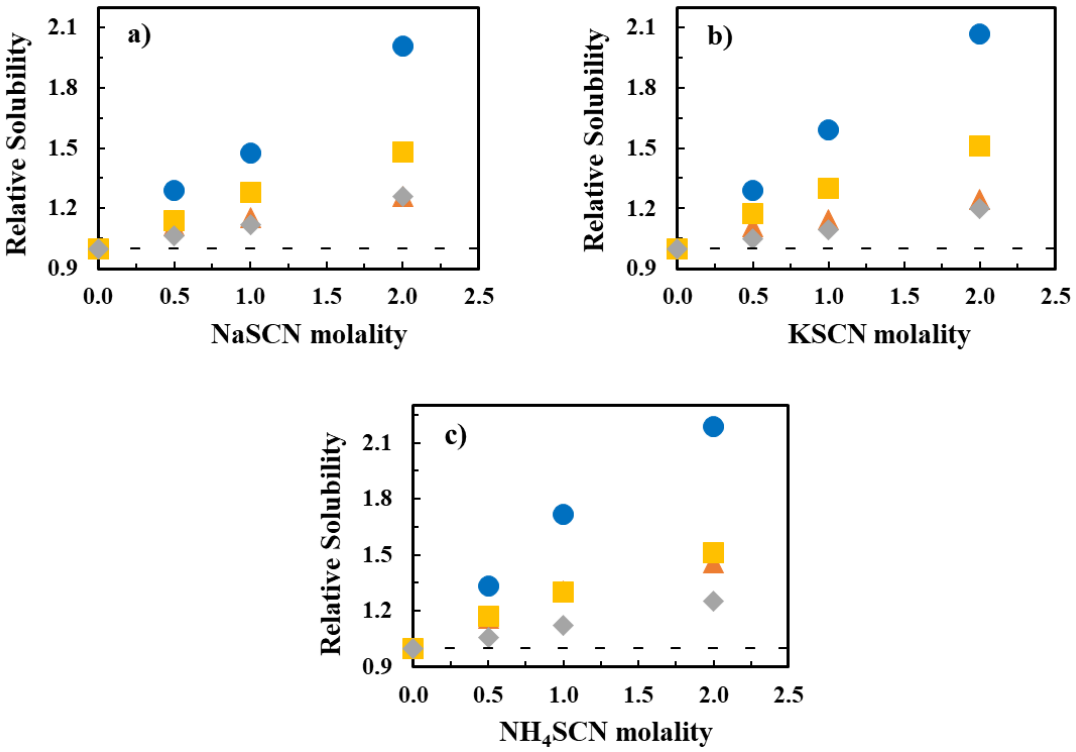

Figure 5. 1. Relative solubility of ◇, glycine; ▲, L-leucine; □, L-phenylalanine, and ●, L-aspartic acid in aqueous a) NaSCN; b) KSCN, and c) NH_4SCN solutions with different molalities at 298.2 K.

During the extensive literature review on the solubility of AA in electrolyte solutions (**Table 1. 1**) no solubility data of AA in aqueous solutions of salts with the thiocyanate anions was found and therefore, no comparison could be provided.

The relative solubilities of all the studied four AA in Na-tosylate solution at 298.2 K is given in **Figure 5. 2**. Globally, Na-tosylate induces a salting-in effect for all the AA, excepting glycine, where the effect is very weak. Until 1 mol/kg of H_2O the relative solubility follows the same order as in aqueous NaSCN and KSCN solutions being L-asp > L-phe > L-leu > Gly. At mol/kg of H_2O the solubility of phenylalanine decreases and the ranking changes to L-asp > L-leu > L-phe > Gly. Due to this change, the solubility of phenylalanine was also studied at 1.5 mol/kg of H_2O to check the magnitude of a salting-in effect. It was observed that the relative solubility decreases after 1.5 mol/kg of H_2O. The solid-phase analysis of the sample taken after the solubility studies in 2 mol/kg of H_2O of Na-tosylate solution showed peaks that do not belong to the phenylalanine structure suggesting that the solid phase was not the expected. As the sodium cation does not show a strong interaction with the amino

acids[27], it can be concluded that toluenesulfonate anion interacts with the apolar parts of the amino acids and increasing their solubilities.

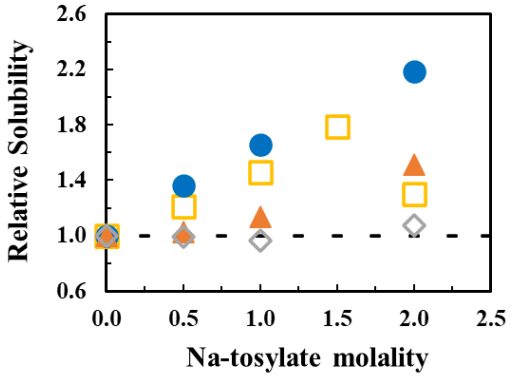

Figure 5. 2. Relative solubility of ◇, glycine; ▲, L-leucine; □, L-phenylalanine, and ●, L-aspartic acid in aqueous Na-tosylate solution with different molalities at 298.2 K.

To evaluate the effect of the inorganic and organic anions on the solubility of the studied amino acids the salts with the sodium cations were chosen. **Figure 5. 3** shows the relative solubility of glycine, L-leucine, L-phenylalanine and L-aspartic acid in aqueous NaCl, $NaNO_3$, NaSCN and Na-tosylate solutions at 298.2 K. Additionally, to evaluate the effect of the anion (SCN^- is placed on the extreme left side of the Hofmeister series) the solubility data in aqueous Na_2SO_4 solution[70] was also added for glycine, selected considering the consistency analysis discussed in **Chapter 3**. As the solubility of phenylalanine in 2 mol/kg of H_2O of aqueous Na-tosylate solution decreased sharply, and the solid-phase analysis showed that the precipitate was not pure phenyalanine, a measurement was also carried out at 1.5 mol/kg of H_2O. Thus, the relative solubilities of L-leucine, L-phenylalanine, and L-aspartic acid followed the same order: tosylate > thiocyanate > nitrate > chloride, what shows that the anion effect on the solubility of these amino acids is in agreement to the Hofmeister series. For glycine the tosylate anion completely reversed the effect, inducing a very weak salting-in, and even a salting-out at 0.5 and 1 mol/kg of H_2O. The reason of this can be the fact that the glycine from the supplier used in the analysis with Na-tosylate was α-form, and not a mixture of α and γ form, as in all other systems. This shows the importance of checking the solid phase present in solubility measurements. The thiocyanate anion, which is the strongest inorganic salting-in anion, for glycine induced a salting-in effect with lower magnitude than the nitrate and even sulphate anions. Although the sulphate anion is a strong salting-out agent in the Hofmeister series, it induces a salting-in effect with very small amino

acids like glycine[27]. The solubility ranking observed is thus nitrate > sulphate > thiocyanate > chloride > tosylate. Even if the increase on the solubility with sodium tosylate is not significant, meaning that it does not act as real hydrotrope for L-aspartic acid due to its small apolar region and its significant polarity, it is the salt with the most relevant effect, showing the importance of the hydrophobic parts of both solute and hydrotrope, but also the much higher effect of the interaction between the apolar part of the AA with the anions.

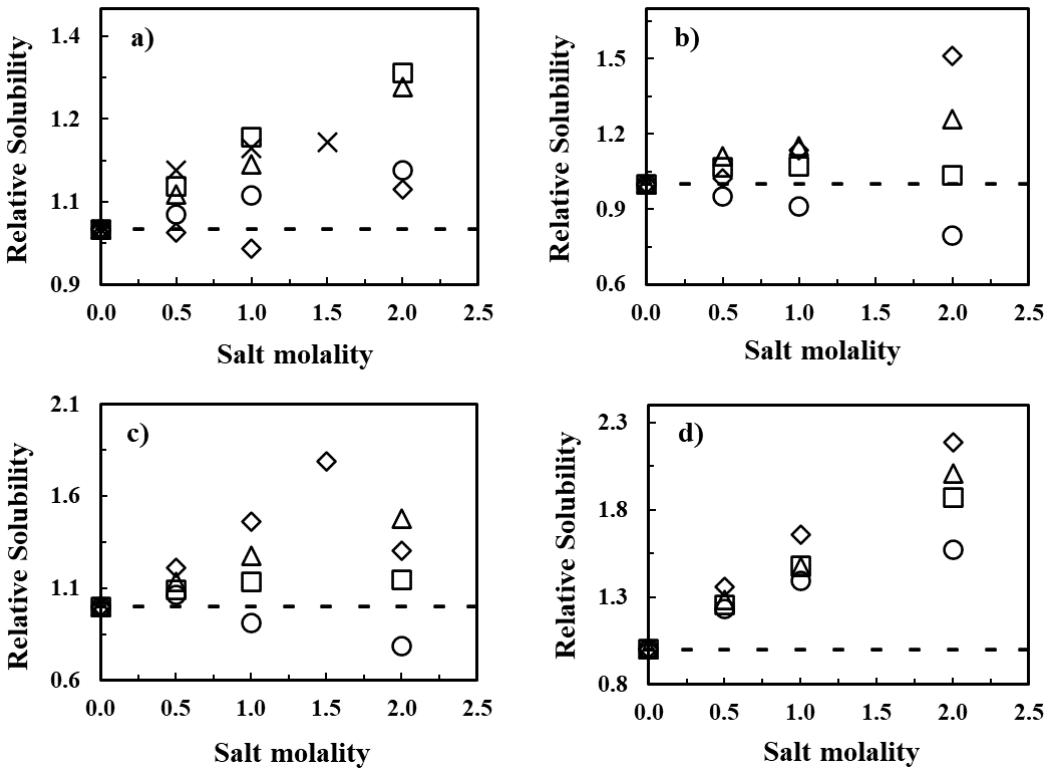

Figure 5. 3. Relative solubility of a) glycine; b) L-leucine; c) L-phenylalanine, and d) L-aspartic acid in aqueous ○, NaCl; □, $NaNO_3$; △, NaSCN; x, Na_2SO_4[70] and ◇, Na-tosylate solutions with different molalities at 298.2 K.

5.2. The pH of the saturated solutions

The pH of the saturated solutions was also measured at 298.2 K and is presented in **Table 5.2.** All amino acids are mainly in the zwitterionic form in the saturated solutions, and this can be confirmed by the microspecies distribution as a function of pH presented in **Appendix B**.

Table 5. 2. pH range of saturated solutions of glycine, L-leucine, L-phenylalanine and L-aspartic acid in aqueous NaSCN, KSCN, NH_4SCN and Na-tosylate solutions at 298.2 K.

	pH range in the ternary solution			
AA	NaSCN	KSCN	NH_4SCN	Na-tosylate
Gly	5.97*-6.11	5.97*-6.24	5.97*-6.03	5.97*-7.22
Leu	5.95-5.98*	5.98*-6.84	5.51-5.98*	5.98*-8.15
Phe	5.48*-5.54	5.48*-6.32	5.48*-5.60	5.48*-8.03
Asp	2.68-2.77*	2.77*-2.79	2.73-2.77*	2.77*-3.80

* Saturated solution in water[1]. u (pH) = 0.05, u (T) = 0.1 K.

5.3. The solid phase studies

The solids from the supplier were analyzed by single crystal X-ray diffraction and the information is already presented in **Table 3. 4**. As in aqueous solutions of salts with the monovalent and divalent cations, here also in all three aqueous tosylate solutions glycine solid phase is a mixture of two phases, a monoclinic corresponding to the α-form and a hexagonal corresponding to γ-form (**Appendix C: Figure C 1**). The other amino acids from the supplier, including the glycine (monoclinic, α-form, **Figure C 21**) used in analysis with Na-tosylate, show a single phase and are well characterized in the literature (**Figure C 3**, **Figure C** and **Figure C 5**). In all four aqueous solutions glycine solid phase **(Figure C 12)** is only in the hexagonal crystal system, the γ-form. The crystalline forms of the other amino acids (**Figure C 13, Figure C 14 and Figure C 16**) in equilibrium with the saturated solutions containing the different salts do not change compared to the structures found in the solids from the suppliers. The exception is for phenylalanine in aqueous Na-tosylate solution at 2 mol/kg of H_2O. The analysis of the crystals obtained after the crystallization showed that the peaks do not belong to phenylalanine (**Figure C 15**) and could be a mixture of compounds from some chemical modification of the the initial reagents. The crystals were also obtained after the solubility studies of phenylalanine in 1.5 mol/kg of H_2O Na-tosylate solution (**Figure C 14**) and it was found that the solid phase did not change compared to the structure found in the original solid from the supplier.

Chapter 6 – Solubilities of aromatic and dicarboxylic amino acids in aqueous solutions of salts.

This chapter includes the solubility measurements of L-aspartic acid, L-glutamic acid, L-tryptophan and L-tyrosine in aqueous KCl and $(NH_4)_2SO_4$ solutions at 298.2 K. The relative solubility data obtained in this work were compared to literature data for other amino acids in the same electrolyte solutions to establish a relative solubility ranking connected to their structure. Finally, all the solubility data were modeled using the electrolyte Perturbed-Chain Statistical Association Theory (ePC-SAFT). The pH results and the results of the solid phase studies are also presented.

I declare that I have provided all the solubility measurements of AAs in aqueous salt solutions, and monitored the pH of the saturated solution, except for the solubility measurements of L-aspartic acid in aqueous $(NH_4)_2SO_4$ solution and L-glutamic acid, and L-tryptophan in aqueous KCl and $(NH_4)_2SO_4$ solutions, which were studied by Carina Silva. I additionaly wrote the manuscript. The solid-phase studies were performed by Paula Brandão from CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal, and ePC-SAFT was modeled by Crystoph Held from the Laboratory of Thermodynamics, Department of Biochemical and Chemical Engineering, TU Dortmund University, Emil-Figge-Str. 70, 44227 Dortmund, Germany.

6.1. Experimental data and analysis

The experimental solubility and the standard deviation (in brackets) of L-aspartic acid, L-glutamic acid, L-tryptophan and L-tyrosine in aqueous KCl and $(NH_4)_2SO_4$ solutions, with salt concentrations up to 2.0 mol/kg, at 298.2 K is given in **Table 6. 1**. The solubility in pure water follows the order: Try > Glu > Asp > Tyr. In aqueous KCl solution, at 2 molal, the solubility of L-glu is higher than the solubility of L-try, and in aqueous $(NH_4)_2SO_4$ solution, except for 0.5 molal, the solubility of L-glutamic acid is higher than the solubility of L-try. The solubility of L-aspartic acid in aqueous $(NH_4)_2SO_4$ solution, at 1.5 and 2 molal is higher than the solubility of L-tryptophan. The maximum coefficient of variation is 3.4% in the system water/ammonium sulfate/L-tyrosine at 0.7 mol·kg^{-1} salt concentration, being lower than 1% in about 67% of cases.

Table 6. 1. Solubility of the amino acids (g of AA/1000 g of H_2O), (standard deviation in brackets) in aqueous KCl and $(NH_4)_2SO_4$ solutions at different molalities, T = 298.2 K and p = 0.1 MPa.[a]

Salt	Salt molality/ (mol/(kg of H_2O)	S_{AA} (g of AA/1000 g of H_2O)			
		L-aspartic acid	L-glutamic acid	L-trypto-phan	L-tyrosine
No salt	0.000	5.140 (0.031)[b]	8.568 (0.029)	12.639 (0.045)	0.427 (0.002)
KCl	0.300		9.760 (0.024)	12.782 (0.072)	0.434 (0.008)
	0.700		10.404 (0.028)	12.952 (0.067)	0.412 (0.004)
	1.000		10.716 (0.060)	12.509 (0.057)	0.394 (0.010)
	1.500		11.264 (0.139)	12.051 (0.032)	0.379 (0.009)
	2.000		11.677 (0.077)	11.134 (0.017)	0.345 (0.017)
$(NH_4)_2SO_4$	0.333	7.228 (0.028)	11.376 (0.038)	13.084 (0.069)	0.457 (0.012)
	0.700	8.899 (0.053)	12.484 (0.050)	12.166 (0.057)	0.437 (0.015)
	1.000	9.999 (0.056)	13.236 (0.004)	11.522 (0.196)	0.407 (0.013)
	1.500	10.998 (0.135)	14.177 (0.114)	9.992 (0.096)	0.353 (0.008)
	2.000	12.116 (0.350)	13.980 (0.332)	8.778 (0.123)	0.300 (0.004)

[a] u(salt molality) = 0.001 mol·kg^{-1}, $u_r(S_{AA})$ = 0.05, u (T) = 0.1 K and u_r (p) =0.05. [b] Solubility data of L-aspartic acid in water and in aqueous KCl solution is given in **Table 3. 1**.

For all amino acids, their solubilities in pure water at 298.2 K were already reported by several authors, as collected in **Table 1. 5**. It should be mentioned that, in many cases, the solid phase structure was not registered. The literature average and standard deviation were calculated for comparison purposes and are also reported in **Table 1. 5**. As can be seen, the values measured in this work generally agree with the literature. For L-tryptophan a difference to the literature average close to 0.9 g per 1000 g of water was found. Nevertheless, the standard deviation of the seven values collected in the literature is also high (1.2 g of AA/1000 g of H_2O).

Figure 6. 1 presents the relative solubility for the studied systems. The solubility of L-Asp in KCl aqueous solutions reported previously is also included for comparison purposes. A salting-in effect is observed in both electrolyte solutions for L-Asp and L-Glu. For the more

hydrophobic amino acids, L-Trp and L-Tyr, a very small salting-in effect at lower concentrations is observed, followed by a decrease in the relative solubility that shows salting-out at higher salt molalities. At higher molalities the ammonium sulfate is a stronger agent both as salting-in (L-Asp and L-Glu) or salting-out (L-Trp and L-Tyr). Considering the four amino acids, the ranking of the relative solubility in KCl is L-Tyr < L-Trp < L- Glu < L-Asp. In the case of the solutions containing $(NH_4)_2SO_4$, the aromatic amino acids swap their position: L-Trp < L-Tyr < L- Glu < L-Asp.

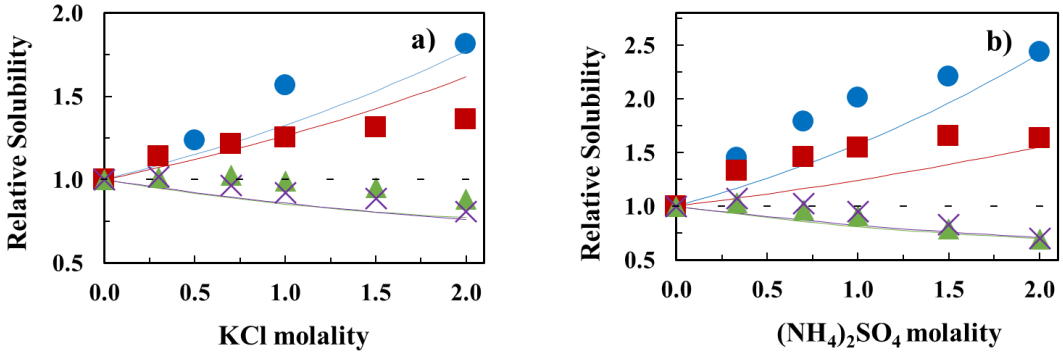

Figure 6. 1. Relative solubility of the amino acids in aqueous solutions of a) KCl and b) $(NH_4)_2SO_4$, at 298.2 K. ●, L-aspartic acid; ■, L-glutamic acid; ▲, L-tryptophan and x, L-tyrosine. Lines were calculated with ePC-SAFT using the parameters from **Table 2. 2**, **Table 2. 3** and **Table 6. 3**.

Regarding the aqueous saline solutions, only the solubility of L-Glu in aqueous KCl solutions was reported by other researchers[58] and are compared with our data in **Figure 6. 2**. As can be seen, the salting-in effect obtained in this work differs considerably from the salting-out published by Abualreish and Noubigh[58]. For the sake of comparison, the impact of NaCl on the solubility of L-Glu measured by Bretti *et al.*[57] is also represented in Figure S5. As can be seen, both NaCl and KCl (our data) induce a similar salting-in effect, which follows the same behavior obtained from the analysis of the results of other amino acids with the same chloride salts[165], thus supporting the consistency of our results.

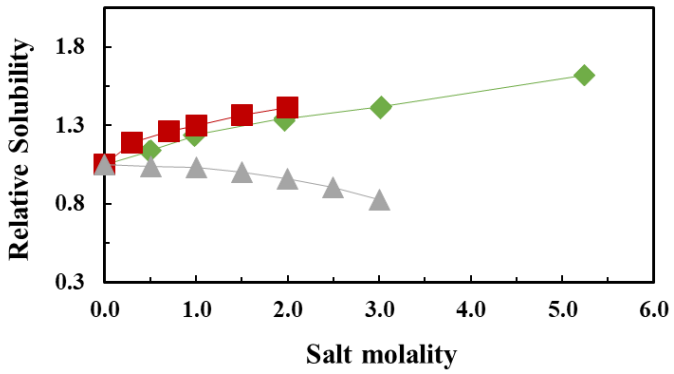

Figure 6. 2. Relative solubility of L-glutamic acid as a function of salt molality at 298.2 K: ◆, NaCl[57]; ■, KCl (this work); ▲, KCl[58]. Lines are a guide to the eyes.

The solubility of L-Trp and L-Tyr in aqueous solutions of NaCl presented in literature could not be compared with our results because of the concentration scale (molarity) or data available only for the DL mixture of isomers. Still, it was possible to notice that they also induce a salting-out effect[44,45,54,60] as observed here for KCl, at larger molalities (0.7 mol·kg^{-1} for L-Tyr and 1.0 mol·kg^{-1} for L-Trp).

The molecular level interpretation of these data can be partly supported by previous results from molecular dynamics studies in which both the anion and cations effects were evaluated. Tome *et al.*[27] proposed a mechanism based on the type and strength of the interactions between the anions and the nonpolar moieties of the amino acids alanine, valine, isoleucine, and 2-aminodecanoic acid. The interactions between the high charge density sulfate anion and the apolar moieties of the amino acids were absent, as this anion showed a stronger interaction with the charged groups. In turn, the chloride anion should have a lower impact on the amino acid's aqueous solubility as it interacts weakly with both polar and apolar segments[27]. Later, Tome *et al.*[79] studied a series of salts that included the monovalent NH_4^+ and K^+, and their influence on the solubility of alanine, valine, and isoleucine. The simulation results showed that no significant interactions occurred between these cations and the apolar parts of the studied amino acids, even if the interactions of the ammonium ion were somewhat stronger. A more significant association of the ammonium cation to the carboxylate group was found, compared to K^+, that may cause the formation of weak single charged positive complexes, which are more soluble in water. Those simulation results[27,79] were obtained for amino acids with apolar side chains such as isoleucine or valine and may assist the interpretation of the results obtained for L-Tyr and L-Trp, which have even larger apolar

side chains. In the case of the anion effect, the sulfate ion should cause a higher salting-out effect than Cl^-, due to its lower interaction with the apolar regions of the amino acids, as confirmed by the higher magnitude of L-Tyr and L-Trp salting-out at higher molality if compared to potassium chloride salt. Therefore, those interactions seem to prevail over the more significant interactions predicted for the NH_4^+ when compared to K^+.

Considering the polar acidic amino acids, both contain an additional polar COOH group with a much higher affinity to interact with the cations. In this case, the attractive interactions of the ions with the oppositely charged groups of the amino acids (NH_3^+ and COO^-) seem to prevail over the interactions with the more hindered and smaller nonpolar moieties available in L-Asp and L-Glu. And, as mentioned above, those interactions with the charged groups are stronger using NH_4^+ than K^+, or SO_4^{2-} compared to Cl^-, supporting the stronger salting-in effect of ammonium sulfate. Comparing the AA, it is also possible to see that the salting-in is more pronounced for aspartic acid, probably due to the additional CH_2 group in the structure of the more hydrophobic glutamic acid.

Figure 6. 3 and **Figure 6. 4** compare the solubility data measured in this work with literature data available for glycine (Gly) and other L-isomers of amino acids (L-alanine, L-Ala; L-isoleucine, L-Ile; L-serine, L-Ser; L-threonine, L-Thr; L-Proline, L-Pro; L-valine, L-Val; L-leucine, L-Leu; L-phenylalanine, L-Phe) in aqueous solutions of KCl and $(NH_4)_2SO_4$, respectively. The chemical structures of the AA are given in **Appendix A: Figure A 1**. The data were selected considering the consistency analysis discussed in **Chapter 3**.

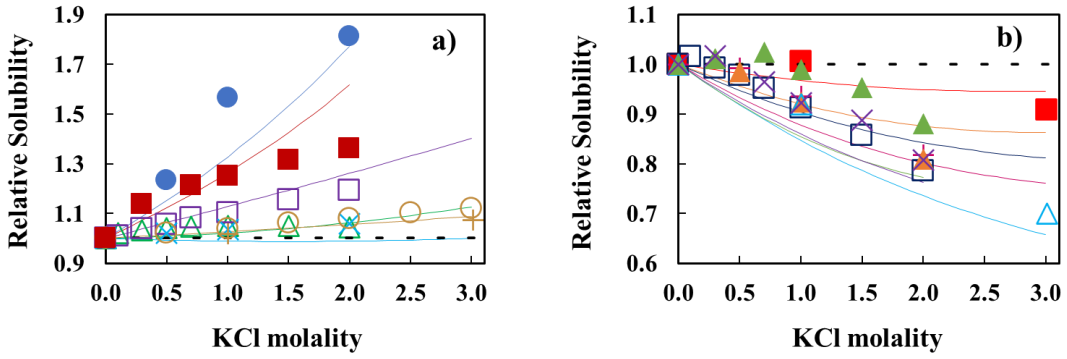

Figure 6. 3. Relative solubility of: a) L-Asp - ●, (this work); L-Glu - ■, (this work); L-Ser - □, [74]; L-Thr - Δ, [74]; L-Pro - ○, [53], +, [49]; Gly - x, (this work) and b) L-Trp - ▲, (this work); L-Tyr - x, (this work); L-Ala - ■, [49]; L-Ile - □, [74]; L-Val - △, [49]; L-Leu - ▲, (this work); L-Phe - +, (this work) in aqueous KCl solution at 298.2 K. Lines were calculated with ePC-SAFT using the parameters from **Table 2. 2**, **Table 2. 3** and **Table 6. 3**.

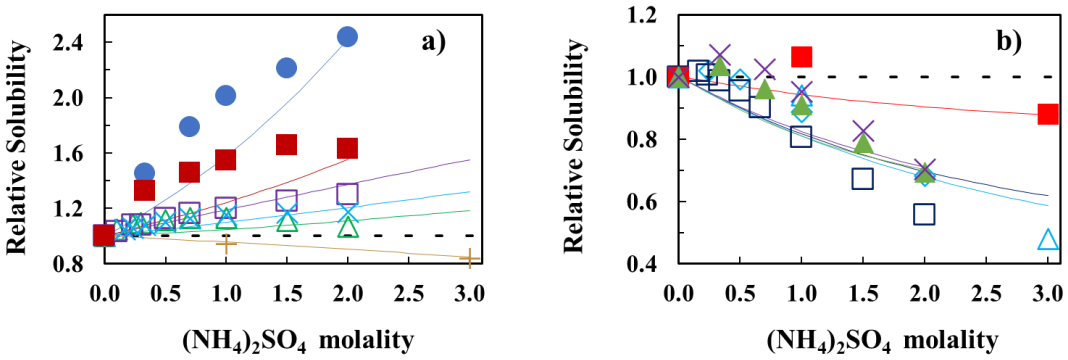

Figure 6. 4. Relative solubility of: a) L-Asp - ●, (this work); L-Glu - ■, (this work); L-Ser - □, [6]; L-Thr - Δ, [6]; L-Pro - +, [49]; Gly - x, [6] and b) L-Trp - ▲, (this work); L-Tyr - x, (this work); L-Ala - ■, [49]; L-Ile - □, [6]; L-Val - △, [49], ◇, [79] in aqueous $(NH_4)_2SO_4$ solution at 298.2 K. Lines were calculated with ePC-SAFT using the parameters from **Table 2. 2**, **Table 2. 3** and **Table 6. 3**.

As can be seen, the ranking of their relative solubility, evaluated at 1.0 mol·kg^{-1} salt concentration, is similar in both salt solutions:

- L-Ile ≈ L-Leu ≈ L-Tyr ≈ L-Val ≈ L-Phe < L-Trp < L-Ala < L-Pro ≈ Gly ≈ L-Thr < L-Ser < L- Glu < L-Asp, in KCl solutions.
- L-Ile < L-Trp ≈ L-Val ≈ L-Pro ≈ L-Tyr < L-Ala < L-Thr ≈ Gly < L-Ser < L-Glu < L-Asp, in $(NH_4)_2SO_4$ solutions.

The pattern identified above may now be generalized for a much larger number of AA. It shows that, in general, $(NH_4)_2SO_4$ induces a more significant effect on their aqueous solubility than KCl, either it is salting-in on the more polar amino acids or salting-out on the less polar. Using as examples the AA in the extremes of the scale, at 2.0 mol·kg^{-1} salt concentration, the relative solubilities of the most polar L-Asp are 2.4 and 1.8 in $(NH_4)_2SO_4$ and KCl solutions, respectively. And, for the apolar L-Ile, the relative solubilities are 0.6 and 0.8 in $(NH_4)_2SO_4$ and KCl solutions, respectively.

Upon adding the salts, a salting-in effect is also observed on the solubility of L-Ser and L-Thr. Both amino acids have a polar hydroxyl group in their side-chain, but L-Thr has an additional -CH_2- group compared to L-Ser which explains their relative solubility ranking. The simplest AA, glycine, also presents a salting-in effect[6]. The remaining amino acids have mostly apolar side-chains (aliphatic and aromatic), and salting-out effects are observed.

6.2. The pH of the saturated solutions

The pH of the saturated solutions was measured at 298.2 K and is presented in **Table 6. 2**. All amino acids are in the zwitterionic form in the saturated solutions, and this can be confirmed by the microspecies distribution as a function of pH presented in **Appendix B**.

Table 6. 2. pH range of saturated solutions of AAs in aqueous $(NH_4)_2SO_4$ or KCl solutions at 298.2 K.

	pH range in the electrolyte solution	
AA	$(NH_4)_2SO_4$	KCl
Asp	2.77*-3.27	2.77*-2.97
Glu	3.22*-3.49	3.19-3.22*
Try	5.37-5.89*	5.89*-5.98
Tyr	5.19-5.66*	5.66*-5.92

* Saturated solution in water[1]. u (pH) = 0.05, u (T) = 0.1 K.

6.3. The solid phase studies

The solid phases of the amino acids, as received from suppliers and filtrated from the saturated ternary solutions containing a 2.0 mol·kg^{-1} salt concentration, were analyzed by powder and single crystal X-ray diffraction. For all solutes, the solid phase recovered from the ternary mixtures kept the structure of the initial sample from the supplier (**Appendix C: Figure C 17**, **Figure C 18**, **Figure C 19** and **Figure C 20**).

Crystals of L-glutamic acid, as received from suppliers, were indexed by single crystal X-ray diffraction with cell parameters a = 5.183 Å (u = 0.013 Å), b = 6.960 Å (u = 0.019 Å), c = 17.35 Å (u = 0.04 Å), orthorhombic P, at 150 K (u = 2 K) and density= 1.561 g/cm^3 (u = 0.012 g/cm^3), where u in the parentheses are standard uncertainties. This result is comparable to that of the sample with CCDC deposition number 1587065 with Z = 4 and density = 1.603 g/cm^3, with data collected at 90 K[165] (**Figure C 17**). Other comparable samples (CCDC deposition numbers 1206528, 1206531, 1515167 and 1877128), with data collected at room temperature, resulted in density values between (1.576-1.590) g /cm^{-3}, which are closer to the value obtained in this work. In the case of L-aspartic acid, the cell parameters of the crystals are given in **Table 3. 4**. The crystal size of L-tryptophan (**Figure C 19**) and L-tyrosine (**Figure C 20**) samples was very small, not allowing the cell parameters determination by single crystal X-ray diffraction. Thus, these samples were analyzed by powder X-ray diffraction showing all the same powder patterns as the samples with CCDC deposition

numbers 986569[166] and 1208550[167], respectively (Figures S3 and S4). These structures are comparable to those reported by Do *et al.* [99], for which the melting properties are available.

6.4. Thermodynamic modeling

The ePC-SAFT model allows the calculation of the solubility of amino acids in aqueous electrolyte solutions and might be applied in a predictive way, i.e., without using binary interaction parameters (k_{ij}) between the inorganic ions and the amino acid. k_{ij} are used to correct the geometric mixing rule of the dispersive energy u_{ij} of a mixture of two components *i* and *j*, obtained from the Lorentz–Berthelot combining rules:

$$u_{ij} = \sqrt{u_i u_j} \cdot (1 - k_{ij}) \tag{6.1}$$

Predicting the amino acid solubility upon salt addition yields results that are quite satisfying for a few systems only (e.g., nonpolar AA in water + $(NH_4)_2SO_4$), while higher deviations for most of the systems were observed (e.g., amino acids with polar substituents in water + $(NH_4)_2SO_4$). Consequently, it was decided to estimate the k_{ij} parameters between ions and amino acids; as ePC-SAFT is an ion-based modeling approach, two k_{ij} parameters (anion - amino acid and cation - amino acid) might be adjusted to experimental solubility data.

To reduce the number of k_{ij} parameters, only one k_{ij} parameter was estimated for each ternary amino acid + water + salt system, using the experimental solubility data from this work and the literature. Knowledge from previous work indicates that ePC-SAFT rather overestimates the solubility of amino acids in salt solutions[141]. Thus, the k_{ij} parameters between any combination of inorganic cation - amino acid was set to $k_{ij} = 0.08$, slightly reducing the interaction energy between inorganic cations and the AA. Therefore, only the k_{ij} parameters between Cl^- - amino acid and SO_4^{2-} - amino acid were adjusted to experimental solubility of amino acid in the respective aqueous electrolyte solution at 298.2 K and 1 bar, and are listed in **Table 6. 3**.

Table 6. 3. ePC-SAFT binary k_{ij} parameters between inorganic anions and amino acid estimated in this work[a]. Valid only with the parameters in **Table 2. 2** and **Table 2. 3**.

Amino acid	k_{ij} Anion-AA	
	Cl^-	SO_4^{2-}
Glycine	-0.1	-0.2
L-Alanine	-0.1	0.2
L-Valine	0	0.3
L-Leucine	-0.1	0.3
L-Isoleucine	-0.1	0.3
L-Phenylalanine	-0.1	0.3
L-Serine	-0.4	-0.5
L-Threonine	-0.15	0
L-Aspartic acid	-0.4	-0.5
L-Glutamic acid	-0.4	0
L-Proline	-0.2	1.0
L-Tyrosine	0.1	0.6
L-Tryptophan	0	0.6

[a] For K^+ and NH_4^+, $k_{cation,AA} = 0.08$.

It becomes obvious from Table 4 that k_{ij} parameters between several ions and amino acids of the same chemical class are very similar. In this manner, it can be inferred that k_{ij} parameters follow some trends:

- # SO_4^{2-} and amino acids with apolar side chains (Ala, Val, Leu, Ile, and Phe): positive k_{ij} values were found, which are 0.2 or 0.3.
- # Cl^- and amino acids with apolar side chains: slightly negative k_{ij} values equal to -0.1.
- # Cl^- / SO_4^{2-} and amino acids with polar/acidic side chains: negative k_{ij} values close to -0.4.

Exceptions were found for the amino acids L-Pro, L-Tyr and L-Trp. Nevertheless, apart from these exceptions, the results suggest that these k_{ij} parameters might be transferable to other systems as well, which will be investigated in future work.

The final correlations were already shown in **Figure 6. 1**, **Figure 6. 3** and **Figure 6. 4**. As can be seen, the model is able to capture the general salting-in and salting-out trends for the

amino acids, though it only predicts the monotonic increase or decrease of the relative solubility, not being able to describe the maximum values observed for some systems.

Finally, to quantitatively evaluate the quality of the ePC-SAFT correlations, the average relative deviations (ARD) between calculated ("calc") and experimental ("exp") solubility values were obtained for each ternary system (water/electrolyte/amino acid), using the following expression:

$$ARD\,(\%) = 100 \cdot \frac{1}{NP} \sum_{k=1}^{NP} \left| \frac{S_k^{exp} - S_k^{calc}}{S_k^{exp}} \right| \tag{6.2}$$

In this equation, NP is the total number of data points for each ternary mixture, and *S* represents the solubility of the amino acid (g amino acid/1000 g of H_2O). The results are presented in **Table 6. 4**. The global ARD for the solubility over all the systems studied in this work is 8% for KCl aqueous solutions and 10% for $(NH_4)_2SO_4$. Higher deviations were obtained for the systems experimentally studied in this work, all higher than 14% in ammonium sulfate aqueous solutions, with the highest ARD of 22% and 17% being obtained for L-tryptophan in KCl and $(NH_4)_2SO_4$ solutions, respectively. The ARD values for all the other systems, obtained from the literature review, is less than 10.6%.

The results can be considered satisfactory as these systems are very difficult to represent not only due to the presence of the electrolyte, but also to the zwitterionic character of the AA. These difficulties have been shown in detail before[74], when correlating the solubility data of AA in aqueous potassium chloride solutions at different temperatures using a complete correlation model such as Pitzer–Simonson–Clegg[168].

Table 6. 4. The average ARD between ePC-SAFT and experimental solubility for N data points.

		ARD Solubility												
		Asp	Glu	Trp	Tyr	Gly	Ala	Ile	Ser	Thr	Val	Pro	Leu	Phe
KCl	ARD (%)	9.6	7.8	21.5	11.8	2.7	4.0	3.2	8.2	2.3	7.4	9.3	10.6	2.9
	N	3	5	5	5	3	2	7	7	7	2	2+6	3	3
	Ref.	a	a	a	a	a	49	74	74	74	49	49,53	a	a
$(NH_4)_2SO_4$	ARD (%)	14.2	14.6	17.2	14.2	1.8	5.8	8.1	10.2	6.1	10.4	7.1	-	-
	N	5	5	5	5	8	2	8	8	8	4+2	2		
	Ref.	a	a	a	a	6	49	6	6	6	49,79	49		

[a] Measured in this work.

Chapter 7 – The effect of pH on the solubility of amino acids.

This chapter includes the effect of pH on the solubility of glycine, diglycine and N-acetylglycine in water and in 1 molal Na_2SO_4 solution at 298.2 K. The obtained solubility results of glycine in water at different pHs were compared with the results found in the literature. Additionally, to check the effect of pH on the solubilities of amino acids with different polarities, the data published in the literature was collected.

I declare that I have provided all the solubility measurements of AAs in aqueous salt solutions and monitored the pH of the saturated solutions.

7.1. Experimental data and analysis

The solubilities of glycine, diglycine and N-acetylglycine with the standard deviation (in brackets) at different pH of the saturated solutions in water and in 1 molal Na_2SO_4 solution at 298.2 K, are given in **Table 7. 1**, together with the correspondent standard deviation. The solubilities of glycine and diglycine were studied in the pH range from 3 to 9/10, and the solubility of N-acetylglycine at pH from -0.25 to 4. In pure water and in 1 molal Na_2SO_4 aqueous solution, without any addition of acid/base, the absolute solubility follows the order: glycine > diglycine > N-acetylglycine, while when pH is higher or lower to the isoelectric confition, the solubility of diglycine becomes larger than of glycine. The maximum coefficient of variation was 2.3% for N-acetylglycine in sodium sulfate solution, when pH is 0.18, being lower than 1% in about 95% of cases.

Table 7. 1. Solubilities (g of solute/1000 g of H_2O) of glycine, diglycine and N-acetylglycine at different pHs in water and 1 molal Na_2SO_4 solution at 298.2 K and p = 0.1 MPa (standard deviation between brackets).[a]

Solvents	Glycine		Diglycine		N-acetylglycine	
	pH	S_{AA} (g of AA/1000 g of H_2O)	pH	S_{AA} (g of AA/1000 g of H_2O)	pH	S_{AA} (g of AA/1000 g of H_2O)
H_2O	3.24	395.814 (0.894)	3.26	643.786 (0.253)	-0.25	35.434 (0.086)
	3.27	394.654 (0.498)	3.62	513.127 (0.218)	-0.06	37.385 (0.160)
	3.60	314.475 (0.278)	4.09	307.709 (0.253)	0.21	38.139 (0.090)
	4.02	260.027 (0.078)	4.31	267.957 (0.583)	1.82*	40.919 (0.098)
	4.19	253.251 (0.784)	5.66*	227.794 (0.781)	2.75	47.520 (0.050)

	4.88	238.771 (0.116)	5.78*	228.860 (0.412)	3.37	64.115 (0.063)
	5.98*	238.332 (0.127)	5.81*	227.695 (0.090)	3.67	85.405 (0.289)
	8.07	240.988 (0.292)	7.73	257.325 (0.166)	3.71	102.523 (0.073)
	8.94	271.909 (0.305)	8.07	301.691 (0.659)	3.92	120.043 (0.126)
			8.17	296.038 (0.058)		
			8.53	385.650 (0.560)		
1 molal Na_2SO_4 solution	3.47	340.342 (0.478)	3.37	652.423 (0.858)	-0.20	24.412 (0.327)
	3.65	320.798 (0.077)	3.66	500.779 (1.529)	0.18	24.118 (0.548)
	3.96	296.633 (0.241)	3.94	411.985 (0.664)	2.53*	30.607 (0.211)
	4.41	281.810 (0.399)	4.18	341.143 (0.488)	3.24	40.347 (0.402)
	6.57*	272.198 (0.534)	4.38	308.651 (0.802)	3.79	69.327 (0.366)
	9.06	283.305 (0.297)	6.14*	271.661 (0.300)	3.94	95.935 (1.309)
	9.22	287.545 (0.068)	7.58	282.451 (0.596)		
	9.60	305.783 (0.168)	7.87	290.412 (0.717)		
	9.78	320.603 (0.384)	8.11	305.554 (1.592)		
			8.62	349.949 (0.406)		
			8.62	352.103 (0.595)		

*Solubility in solvents without acid/base addition. [a]$u(pH) = 0.05$, u(salt molality) = 0.001 mol·kg^{-1}, $u_r(S_{AA})$= 0.05, $u(T) = 0.1$ K and $u_r(p)$ =0.05.

The solubility values of diglycine and N-acetylglycine in pure water measured in this work at 298.2 K agree with the literature (**Table 1. 7**).

Figure 7. 1 shows the solubilities of glycine, diglycine, and N-acetylglycine in pure water as a function of the pH at 298.2 K. As can be seen, adding either acid or base produced relevant changes to the solubility in pure water. In pure water, glycine and diglycine are in zwitterion form, and N-acetylglycine is in its neutral one. The addition of H_2SO_4 to water and glycine/diglycine produced cationic species (positively charged amino group) and the

addition of NaOH produced anionic species (negatively charged carboxyl group), as shown in **Appendix B: Figure B 1** and **Figure B 9,** respectively. As N-acetylglycine does not have an amine group, at low pH presents just in neutral form, and the addition of acid does not change solubility significantly. However, adding a base leads to the formation of anionic species, and solubility increases considerably (**Appendix B: Figure B 10**). This effect is well related to the chemical equilibrium shift. The total solubility considers the zwitterion or neutral form plus the anion or cation forms. The pH change converts a zwitterion to an anion/cation, reducing its concentration in solution and enhancing the solubilization of more solute to compensate for the decrease of the zwitterion concentration, ending by increasing the solubility. Outside the isoelectric zones, the solubility of glycine increases around 1.7 times when pH is 3.24. At this pH, the maximum solubility was found for diglycine too, and the solubility increased approximately 2.8 times. The maximum increase of solubility was observed in the case of N-acetylglycine at pH 3.92, where it increased around 6 times, all when compared to the isoelectric point solubility.

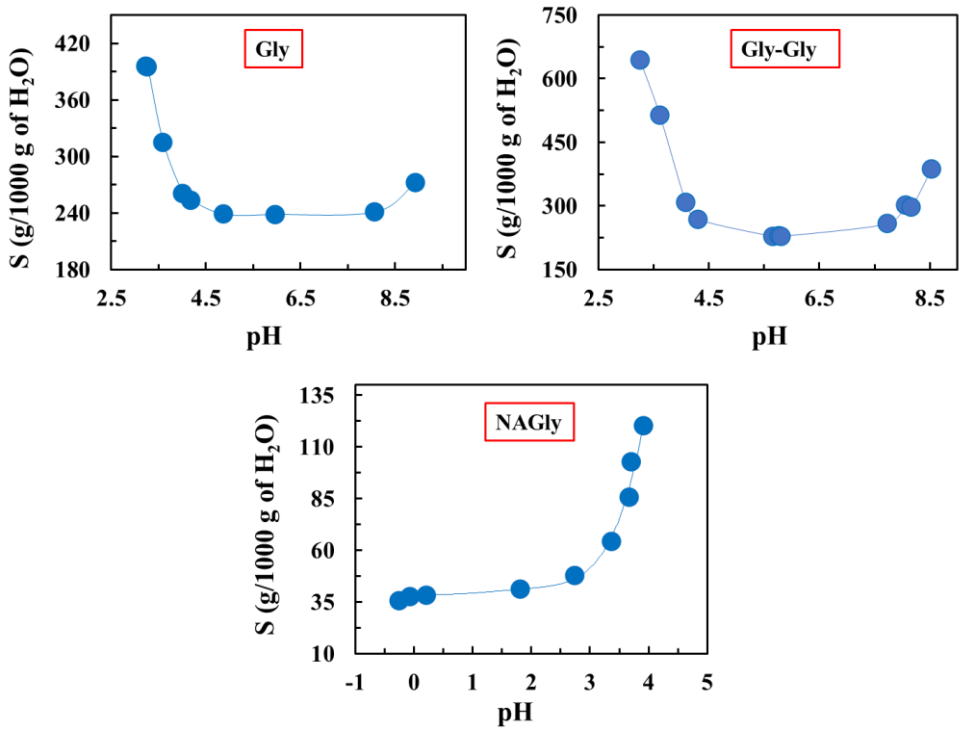

Figure 7. 1. Solubility of glycine (Gly), diglycine (Gly-Gly) and N-acetylglycine (NAGly) in water at 298.2 K as a function of pH.

Figure 7. 2 presents the solubility of glycine, diglycine, and N-acetylglycine in 1 molal Na_2SO_4 solution as a function of pH at 298.2 K. The addition of acid/base to 1 molal Na_2SO_4 solution produced a similar effect as in water.

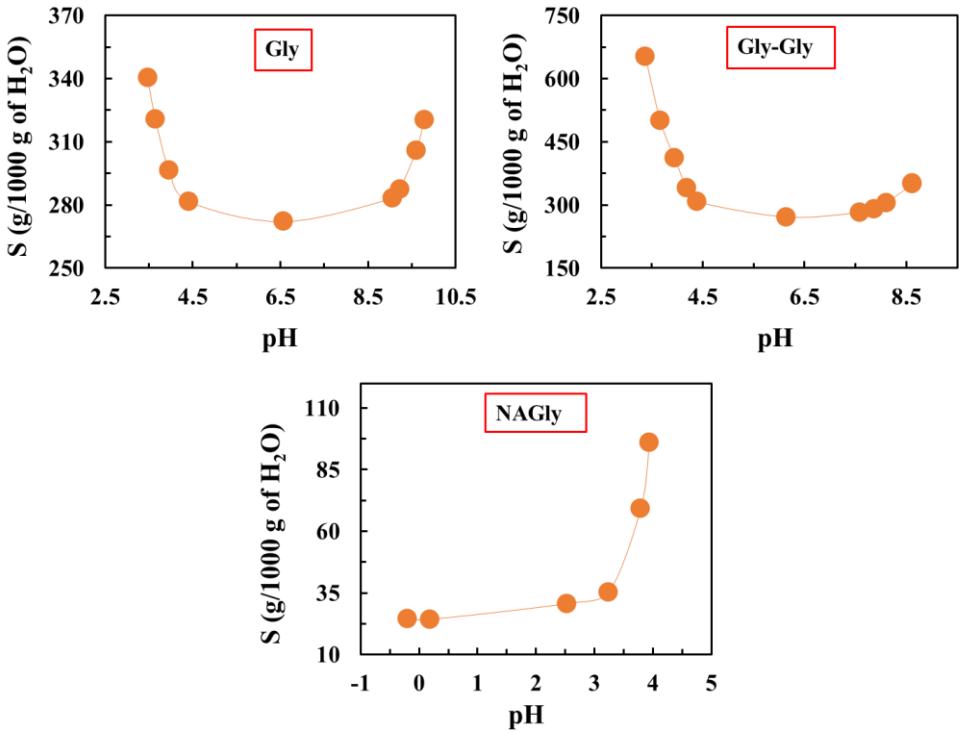

Figure 7. 2. Solubility of glycine (Gly), diglycine (Gly-Gly) and N-acetylglycine (NAGly) at 298.2 K in 1 molal Na_2SO_4 solution as a function of pH.

To check the effect of Na_2SO_4 on the solubility of glycine, dipeptide, and N-acetylglycine at different pHs, **Figure 7. 1** and **Figure 7. 2** were combined in **Figure 7. 3**. While the solubilities of glycine and diglycine in aqueous salt solution at each pH were found to be higher than the solubilities in pure water, the solubility of N-acetylglycine was higher in water. In the case of glycine and diglycine, the increase of solubility induced by Na_2SO_4 is more detectable at the isoelectric point, and pHs where the solubility is close to the solubility in pure water. When the solubility starts to increase and differ significantly from the solubility at the isoelectric point, the effect of Na_2SO_4 diminishes significantly, showing the dominance of the chemical equilibrium changes over the interactions of the salt with the biomolecules. The solubility of N-acetylglycine at each pH point is higher in water than in an aqueous salt solution, but this difference also decreases at higher pHs.

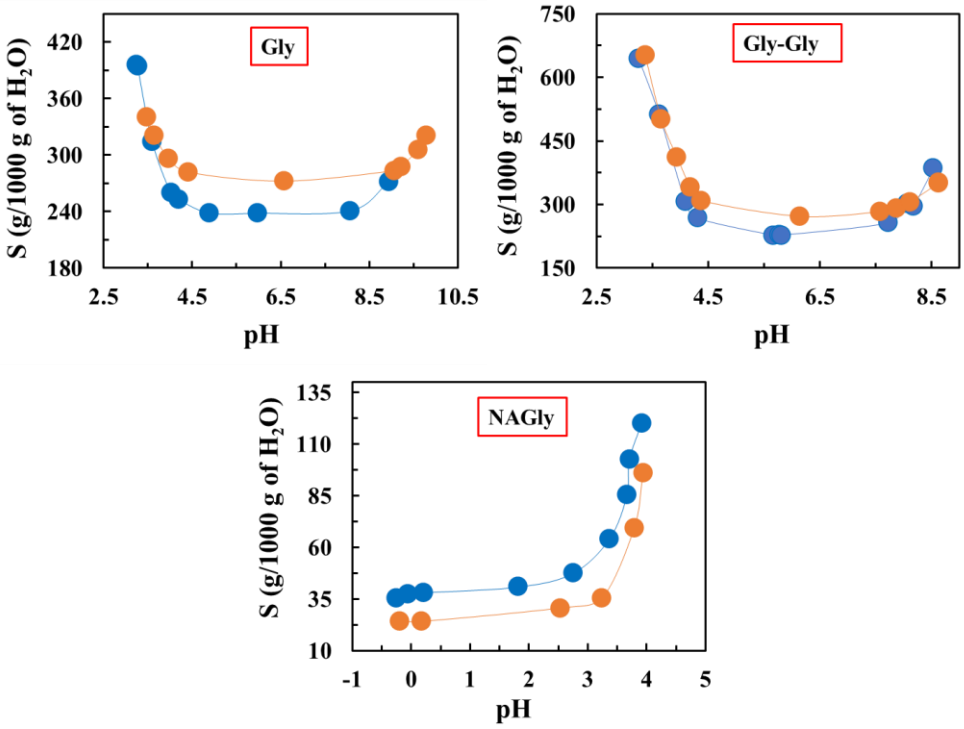

Figure 7. 3. Solubility of glycine (Gly), diglycine (Gly-Gly) and N-acetylglycine (NAGly) at 298.2 K in ●, water and in ●,1 molal Na_2SO_4 solution as a function of pH.

Figure 7. 4 shows the relative solubility of glycine, diglycine and N-acetylglycine in 1 molal Na_2SO_4 solution at different pHs at 298.2 K. The fact that the salt induces a salting-in effect for glycine and diglycine, and a salting-out for N-acetylglycne can be seen. As the Na^+ cation does not affect much the solubility, a salting-in effect in the case of glycine and diglycine can be explained by the interaction of the SO_4^{2-} anion with small these two small molecules, where the amino positively charged group is more accessible[27]. As N-acetylglycine is neutral and has just an apolar methyl group, the solubility of that molecule decreases with the presence of the sulfate anion in the solution. Like observed before, the magnitude of a salting-in effect over glycine and diglycine is higher close to the pH of the isoelectric point and decreases with adding acid or base. In the case of N-acetylglycine the magnitude of the salting-out effect decreases at higher pHs.

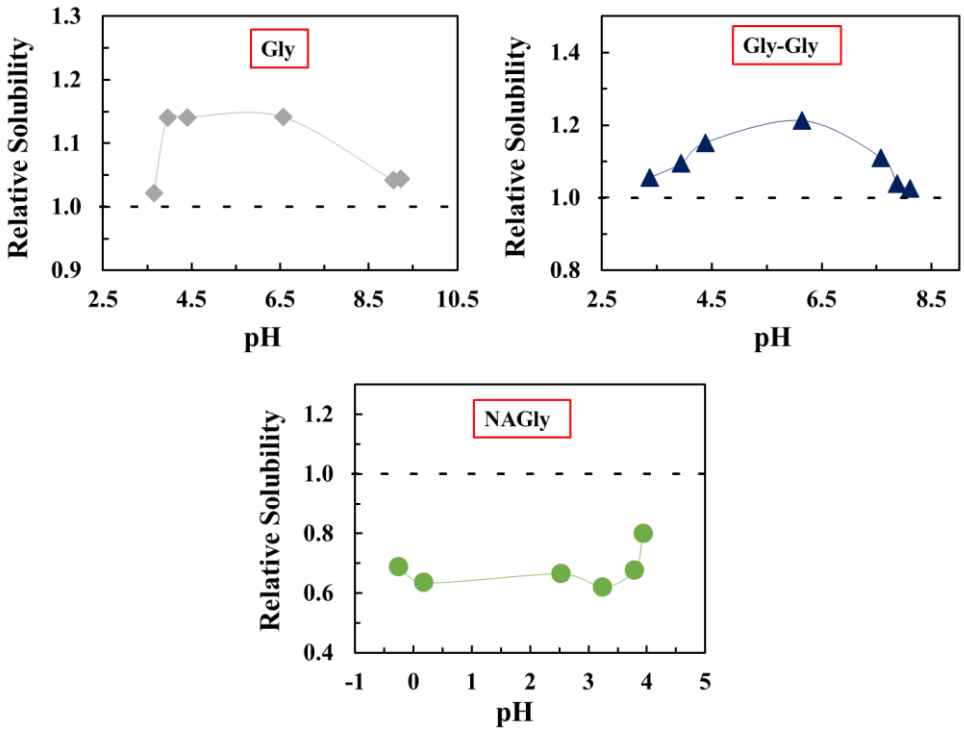

Figure 7. 4. Relative solubility of ◆, glycine (Gly), ▲, diglycine (Gly-Gly) and ●, N-acetylglycine (NAGly) at 298.2 K in water and in 1 molal Na_2SO_4 solution as a function of pH.

The solubility data of glycine and diglycine at various pHs is given in the work Lu *et al.*[43], but as the data are presented in different units, they were not included. Anyway, the comparison of solubility data of glycine and diglycine found in this work with the data found in Lu *et al.*[43] (assuming the density of the solution as the density of water) was performed in **Appendix E: Figure E 1**. No more data have been reported for glycine, diglycine, and N-acetylglycine in an aqueous Na_2SO_4 solution, or diglycine and N-acetylglycine in water at different pH, so no comparison could be provided. Only the solubility of diglycine and N-acetylglycine were found in 1 molal Na_2SO_4. The compiled results were 273.6 g/1000 g of H_2O[109] and 270.86 g/1000 g of H_2O[108] for diglycine, and 29.238 g/1000 g of H_2O[109] for N-acetylglycine and are close to the results of this work.

The solubility data of glycine in pure water at different pH could be compared with the results of Needham *et al.*[122] in **Figure 7. 5**, where HCl and NaOH were used to modify the pH of the solution. The results found in this work are higher than the results found in the literature. Even at the isoelectric point the solubility of glycine in water is different. Therefore, it is expected the observed shift between the two curves. The reason for this can be the

different crystal forms of the studied glycine. In the work of Needham *et al.*[122] the solid phase of glycine was not identified.

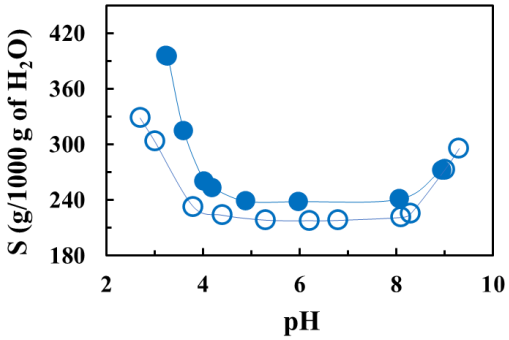

Figure 7. 5. Solubility of glycine in water as a function of pH at 298.2 K: ●, (this work); ○, Needham *et al.*[122].

The solubility dependence of different AAs on pH is given in **Figure 7. 6**, as a) small AAs: glycine, alanine; b) with the hydrophobic side chains: valine, leucine, isoleucine; c) with the aromatic side chains: phenylalanine and tyrosine; d) with the acidic side chain: aspartic and glutamic acid, and e) with amide group in the side chain: asparagine and glutamine. The solubility of AAs in water follows the order: Gly > L-/DL-alanine > L-val > L-gln > L-ile > L-phe > L-asn > L-leu > DL-phe > L-glu > L-asp > L-tyr, as is given in the literature[169]. L-serine[119] is not included in this figure because the molal solubility in water of this AA is reported to be 4.013 mol/kg, which is very high compared to the literature[169]. Excepting L-asp and L-glu, which are acidic AAs and therefore the isoelectric point is 2.77 and 3.22, respectively, the isoelectric points of all other AAs are between pH 5.4 and 7.25. In total, the solubility of all AAs increases outside the isoelectric zones. For clarity, the solubility of AAs is not shown after 500 g/1000 g of H_2O in **Figure 7. 6b** and **e**. The size of the constant solubility band around the isoelectric point is very similar for all the AAs, except the L-aspartic acid and L-glutamic acid.

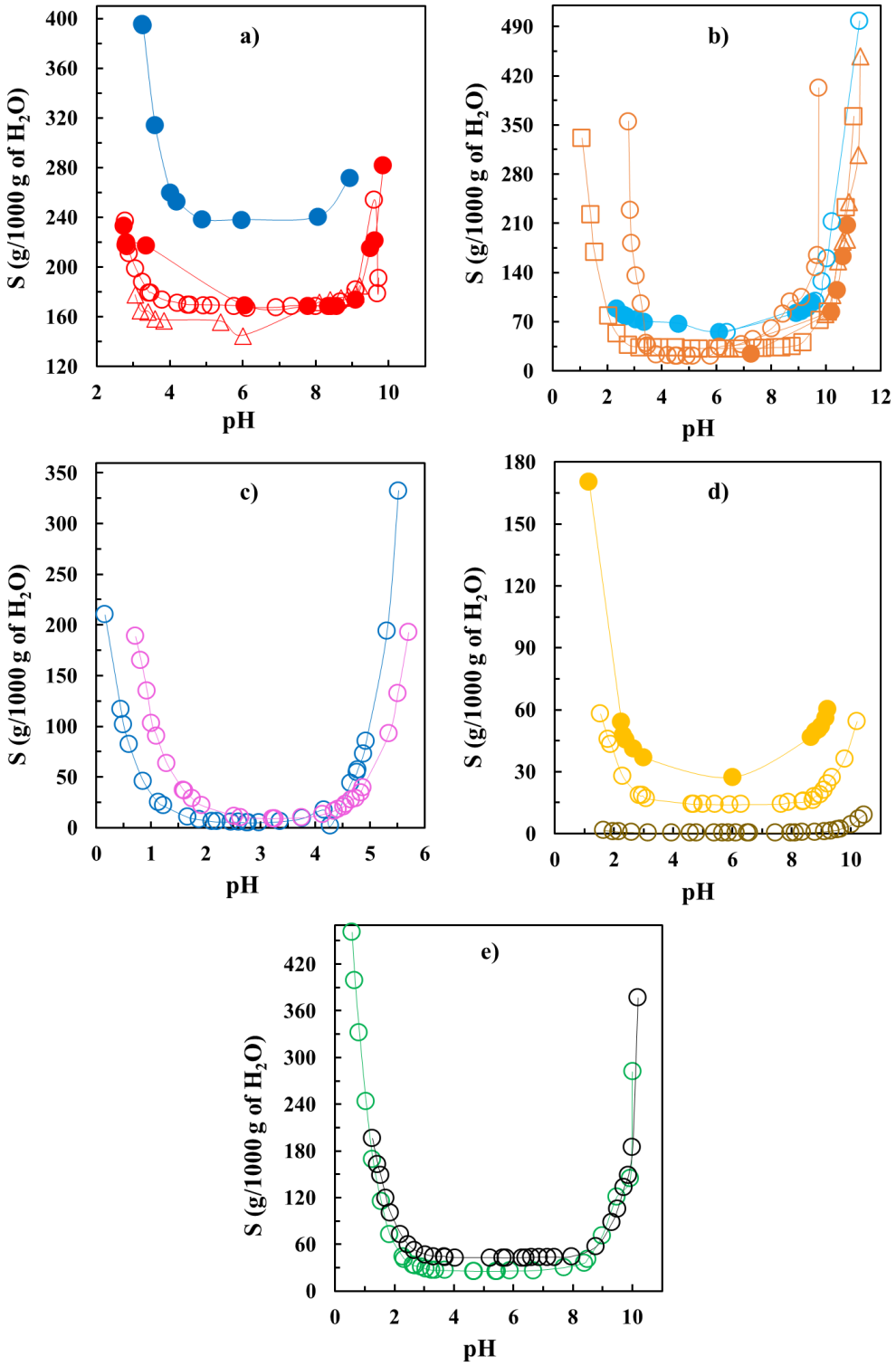

Figure 7. 6. Solubility of a) ●, glycine (this work); ●, DL-alanine[125]; ○, DL-alanine[119]; △, L-alanine[122]; b) ○, L-valine[124]; ●, L-valine[122]; □, L-isoleucine[119]; △, L-isoleucine[124]; ○, L-leucine[119]; ●, L-leucine[124]; c) ○, L-aspartic acid[103]; ○, L-glutamic acid[103]; d) ●, L-phenylalanine[122]; ○, DL-phenylalanine[119]; ○, L-tyrosine[103]; e) ○, L-asparagine[103], and ○, L-glutamine[103]; in water as a function of pH at 298.2 K.

Chapter 8 – Conclusions and future work

The solubility of glycine, L and DL-valine, and L-leucine (nonpolar, aliphatic hydrophobic group); L-phenylalanine, L-tryptophan, and L-tyrosine (nonpolar, hydrophobic aromatic side chain), and L-aspartic or L-glutamic acids (second negatively charged hydrophilic carboxyl group) was studied in aqueous solutions of salts, with concentrations ranging from 0 to 2 mol/kg at 298.2 K. Regarding the electrolytes, cations such as sodium, potassium, ammonium, magnesium, and calcium and anions such as chloride, nitrate, thiocyanate, and sulfate were selected, covering a wide range of the ions in the Hofmeister series. Different quantitative methods of analysis were applied, depending on the solubility magnitude and hydration form of the salts: gravimetry, refraction index, or UV-Vis spectrophotometry. The pH of the saturated solution was measured, and in the observed pH range, all the amino acids were mainly present as zwitterions. An extensive literature review on the solubility of AAs and peptides in electrolyte solutions, the effect of pH on the solubility of AAs, and molecular dynamics was carried out.

The study of the solubility of glycine, L-leucine, L-phenylalanine, and L-aspartic acid was carried out in aqueous solutions of salts with the monovalent and divalent cations: Na^+, K^+, NH_4^+, Mg^{2+}, Ca^{2+} and Cl^-, NO_3^- anions. In all systems, the absolute solubility follows the order Gly > Phe > Leu > Asp, as in pure water. The relative solubilities in aqueous solutions of all the salts with the chloride anions and aqueous $NaNO_3$ and KNO_3, evaluated at 2 molal, follow the order: Asp > Gly > Phe ≅ Leu. In aqueous NH_4NO_3, $Mg(NO_3)_2$, and $Ca(NO_3)_2$ solutions the ranking is; Asp > Phe > Leu ≅ Gly. Excepting NaCl and KCl, which induce a salting-out effect in the solutions with Phe and Leu, in all other systems a salting-in was observed.

Comparing the effect of the salts with the same anion the divalent cations are much stronger salting-in agents than the monovalent cations for all the AAs. Consistently to the Hofmeister series, the Ca^{2+} induces a much stronger salting-in effect than Mg^{2+}. The monovalent cations, and especially the alkali cations, do not significantly affect the AA solubility in water. On the other hand, the nitrate anion induces a higher solubility enhancement than the chloride anions, also in agreement with the Hofmeister series. It was noticed a complex interplay between the significative divalent cation/carboxylate interaction and that between the nitrate with the apolar moieties of the AA, turning, for instance, ammonium nitrate as effective as calcium chloride in the increase of L-phe solubility.

To establish patterns on the effect of the anion, and specific structural features of the AA, an extensive database on the solubility of amino acids in aqueous electrolyte solutions has been constituted. The collected data, some very recent, show significative differences and inconsistencies.

Considering the effect of anions on the solubility of glycine and alanine, when combined with the monovalent cations Na^+, K^+, and NH_4^+, generally, the Hoffmeister series is verified. Acetate is a strong salting-out agent, while nitrate anion presents the highest salting-in effect, followed by iodide, bromide, chloride, and fluoride anion. A major difference is observed with the sulfate anion, which is a salting-in agent to glycine, and a salting-out agent for AA when the alkyl-chain length increases, as also explained by molecular dynamics.

Analyzing the effects of NaCl and KCl in a wide variety of AAs, their maximum salting-in effect is observed over acidic AA, followed by polar, neutral AAs such as serine and cysteine. Concerning salting-out, it is maximum over basic AA such as lysine and in apolar aliphatic AA with large alkyl-chains. In both salts, glycine, cyclic proline, and alanine present the least significative salting-in/out effect. Generally, $NaNO_3$ and KNO_3 salts induce salting-in in all AAs following a ranking quite similar to the chloride salts, with the smallest effects being observed for AAs such as glycine, proline, and phenylalanine.

To perform data assessment and analyze the impact of optical isomerism the solubility of L and DL-valine was studied in aqueous $NaNO_3$ and KNO_3 solutions. Unlike some of the publications, the results obtained in this work showed that the $NaNO_3$ and KNO_3 salts induced a slight salting-in effect, except for the L-valine in both salt solutions at 2 molal, and also that isomerism does not play a role in the impact of salts on the solubility of AA.

To contribute to the fill the gap concerning the impact of thiocyanate anion, which is located at the right extreme of the Hofmeister series, the solubilities of glycine, L-leucine, L-phenylalanine, and L-aspartic acid were studied in aqueous NaSCN, KSCN, and NH_4SCN solutions. The salts with the thiocyanate anions induced a salting-in effect with all the AAs the ranking for the salting-in effect follows Asp > Phe > Leu > Gly. The relative solubility of Gly was very close to Leu in aqueous solutions of salts with the sodium and potassium cation, while in aqueous NH_4SCN solution this similarity was found between Leu and Phe.
The study was then extended, with the same AA as above, for salts containing an organic anion (tosylate). Sodium cation was selected as its effect on the solubility of AA is well

studied with different inorganic anions, and it is quite common in an organic salt family usually applied as hydrotropes. Sodium tosylate induced a salting-in effect with all the AAs, except for glycine in aqueous Na-tosylate solution at 0.5 and 1 molal. Until 1 molal, the order of the salting magnitude in aqueous sodium tosylate solution is the same as in aqueous NaSCN and KSCN solutions, but at 2 molal the solubility of phenylalanine decreases, and the ranking changes to L-asp > L-leu > L-phe > Gly, which can be understood taking into account the structure of the solid phase.

Summarizing the studies in this work over the AA glycine, L-leucine, L-phenylalanine, and L-aspartic acid, and sodium-containing salts, the relative solubility followed tosylate > thiocyanate > nitrate > chloride, being the order of inorganic anions as in Hofmeister series. For glycine, however, the order was nitrate > thiocyanate > chloride > tosylate, showing once more the specificity of the simplest AA, which usually fails to obey trends.

In our review, we observed a large body of reliable information on the solubility of AA in aqueous KCl and $(NH_4)_2SO_4$ solutions. Therefore, the available information was extended to AA containing two carboxylic acids or an aromatic group. At 2.0 $mol \cdot kg^{-1}$ salt concentration, both electrolytes induced salting-in in the polar acidic L-Asp and L-Glu and salting-out in the more hydrophobic aromatic L-Tyr and L-Trp. Those effects are stronger in ammonium sulfate solutions than in potassium chloride. These are amino acids for which both experimental and modeling results are scarce in the literature, reinforcing the importance of this study by considering AA with aromatic or two carboxylic groups. The obtained relative solubility data were compared to literature data for other amino acids in the same electrolyte solutions and all the data were described using ePC-SAFT, an ion-specific modeling approach. The results showed that the model captures the general salting-in and salting-out trends for the amino acids, though it only predicts the monotonic increase or decrease of the relative solubility, not describing the maximum values observed for some systems.

Finally, the effect of the pH on the solubility of glycine, diglycine, and N-acetylglycine in water, and one molal Na_2SO_4 solution, at 298.2 K, was checked. The solubilities of glycine and diglycine were carried out in the pH range from 3 to 9/10, and the solubility of N-acetylglycine at pH from -0.25 to 4. To change the pH, H_2SO_4 or NaOH was used. In pure water and one molal Na_2SO_4 aqueous solution, the absolute solubility follows the order: glycine > diglycine > N-acetylglycine, but with the addition of acid/base, the solubility of diglycine

becomes larger than of glycine. The solubility of N-acetylglycine only changes at high pHs as the addition of base leads to the formation of anionic species.

The addition of acid/base to 1 molal Na_2SO_4 solution produces a similar effect as in water. The salt induces a salting-in effect for glycine and diglycine, and a salting-out effect for N-acetylglycine, as the sulfate anion induces a salting-in effect only for small molecules, where the positively charged group is more accessible. The solubility of N-acetylglycine decreases due to the presence of an apolar methyl group. The effect of the salt is more pronounced at the isoelectric point, and the magnitude of a salting-in/salting-out effect decreases with adding acid or base. In addition, the solubility dependence of different AAs on pH was collected. The isoelectric point of all AAs was found between pH 5.4 and 7.25, except the acidic AAs L-asp and L-glu, for which the isoelectric point is 2.77 and 3.22. respectively.

To check the reliability, the obtained results were compared with the solubility data found in the literature, whenever possible. Solubility data were not found for L-aspartic acid, L-leucine, and L-phenylalanine in aqueous solutions of KCl, $NaNO_3$, KNO_3, NH_4Cl, NH_4NO_3, $MgCl_2$, $CaCl_2$; L-glutamic acid in aqueous $(NH_4)_2SO_4$ solution; L-tryptophan and L-tyrosine in aqueous KCl and $(NH_4)_2SO_4$ solutions; for all the AAs in aqueous solutions of $Mg(NO_3)_2$, $Ca(NO_3)_2$, NaSCN, KSCN, and NH_4SCN; and for glycine in aqueous Na_2SO_4 solution, diglycine and N-acetylglycine in water and in aqueous Na_2SO_4 solution, which demonstrates clearly the relevant contribution of the new data acquired in this work.

The solids from the supplier and solids settled in equilibrium with the saturated solution, after vacuum filtration and drying, were very often analyzed by single crystal and powder X-ray diffraction. Glycine from the supplier used in all the studies, except the solubility study in Na-tosylate solution (only α-form), presents a mixture of two phases, a monoclinic corresponding to the α-form and a hexagonal corresponding to γ-form. All the other amino acids from the supplier show a single phase. In all aqueous solutions of the salts with the inorganic and organic cation, the glycine samples crystallize only in the hexagonal crystal system, the γ-form. Generally, the crystalline form of the other amino acids in equilibrium with the saturated solutions does not change compared to the structures found in the solids from the suppliers. The exception is phenylalanine in aqueous $MgNO_3$ or Na-tosylate solutions. In the case of an aqueous $Mg(NO_3)_2$ solution a second phase of phenylalanine was found, which could not be identified. The analysis of the crystals obtained after the crystallization of the sample showed that the peaks do not belong to phenylalanine and could be a

mixture of compounds from some chemical modification of the initial compounds. The crystals were also obtained after the solubility studies of phenylalanine in 1.5 molal Na-tosylate solution. In this case, the solid phase did not change compared to the structure of the original material from the supplier.

Future research should be focused on the solubility of amino acids containing more than one amino or carboxylic acid group, aromatic and heteroatomic amino acids, in aqueous electrolyte solutions. For most of the AAs, these studies should be extended to aqueous solutions containing polyvalent cations. As very few experimental data are available for peptides and proteins, another vector needing a lot of attention is the application of different methodologies to analyze and compare the importance of the peptide bond in terms of the interactions with the ions. A relevant question still under great attention is the relative importance of the C=O and N-H interactions with the ions.

The interactions of the AAs and peptides in aqueous saline solutions must also be studied by applying techniques such as density measurements (for partial molar volumes), NMR spectroscopy, and molecular dynamics simulations. In this respect, another approach that can give important insights is the predictive quantum-chemistry based model COSMO-RS to describe the activity coefficients of the AA and the impact of the presence of the different ions in solution. All these can support future insights into the behavior of proteins in relevant aqueous electrolyte solutions.

It is also of great importance to extend the studies on the impact of organic salts, namely hydrotropes based on the anions salicylate, tosylate, and caprylate, on the solubility of different amino acids. In this case, special care must be taken into account concerning changes in the solid phase.

References

(1) Nelson, D. L.; Cox, M. M. *Lehninger Principles of Biochemistry*, 4th ed.; W. H. Freeman and Company: New York, 2005.

(2) Chi, E. Y.; Krishnan, S.; Randolph, T. W.; Carpenter, J. F. Physical Stability of Proteins in Aqueous Solution: Mechanism and Driving Forces in Nonnative Protein Aggregation. *Pharm Res* **2003**, *20* (9), 1325–1336. https://doi.org/10.1023/a:1025771421906.

(3) Selkoe, D. J. Folding Proteins in Fatal Ways. *Nature* **2003**, *426*, 900–904. https://doi.org/10.1038/nature02264.

(4) Kakizuka, A. Protein Precipitation: A Common Etiology in Neurodegenerative Disorders? *Trends in Genetics* **1998**, *14* (10), 396–402. https://doi.org/10.1016/s0168-9525(98)01559-5.

(5) Chiti, F.; Dobson, C. M. Protein Misfolding, Amyloid Formation, and Human Disease: A Summary of Progress over the Last Decade. *Annu Rev Biochem* **2017**, *86* (1), 27–68. https://doi.org/10.1146/annurev-biochem-061516-045115.

(6) Ferreira, L. A.; Macedo, E. A.; Pinho, S. P. The Effect of Ammonium Sulfate on the Solubility of Amino Acids in Water at (298.15 and 323.15) K. *Journal of Chemical Thermodynamics* **2009**, *41* (2), 193–196. https://doi.org/10.1016/j.jct.2008.09.019.

(7) Kramer, R. M.; Shende, V. R.; Motl, N.; Pace, C. N.; Scholtz, J. M. Toward a Molecular Understanding of Protein Solubility: Increased Negative Surface Charge Correlates with Increased Solubility. *Biophys J* **2012**, *102* (8), 1907–1915. https://doi.org/10.1016/j.bpj.2012.01.060.

(8) Wu, L.; Wu, T.; Wu, J.; Chang, R.; Lan, X.; Wei, K.; Jia, X. Effects of Cations on the "Salt in" of Myofibrillar Proteins. *Food Hydrocoll* **2016**, *58*, 179–183. https://doi.org/10.1016/j.foodhyd.2016.02.028.

(9) Nahar, M. K.; Zakaria, Z.; Hashim, U.; Bari, M. F. Effect of PH and Salt Concentration on Protein Solubility of Slaughtered and Non-Slaughtered Broiler Chicken Meat. *Sains Malays* **2017**, *46* (5), 719–724. https://doi.org/10.17576/jsm-2017-4605-06.

(10) Sathe, S. K.; Zaffran, V. D.; Gupta, S.; Li, T. Protein Solubilization. *Journal of the American Oil Chemists' Society* **2018**, *95* (8), 883–901. https://doi.org/10.1002/aocs.12058.

(11) Melander, W.; Horvath, C. Salt Effects on Hydrophobic Interactions in Precipitation and Chromatography of Proteins: An Interpretation of the Lyotropic Series'. *Arch Biochem Biophys* **1977**, *183* (1), 200–215. https://doi.org/10.1016/0003-9861(77)90434-9.

(12) Andreetta-Gorelkina, I. V.; Greiff, K.; Rustad, T.; Aursand, I. G. Reduction of Salt in Haddock Mince: Effect of Different Salts on the Solubility of Proteins. *Journal of Aquatic Food Product Technology* **2016**, *25* (4), 518–530. https://doi.org/10.1080/10498850.2013.879241.

(13) Kalyuzhnyi, Y. v.; Vlachy, V. Explicit-Water Theory for the Salt-Specific Effects and Hofmeister Series in Protein Solutions. *J Chem Phys* **2016**, *144* (21). https://doi.org/10.1063/1.4953067.

(14) Murakami, S.; Hayashi, T.; Kinoshita, M. Effects of Salt or Cosolvent Addition on Solubility of a Hydrophobic Solute in Water: Relevance to Those on Thermal Stability of a Protein. *J Chem Phys* **2017**, *146* (5). https://doi.org/10.1063/1.4975165.

(15) Zhou, H. X. Interactions of Macromolecules with Salt Ions: An Electrostatic Theory for the Hofmeister Effect. *Proteins: Structure, Function and Genetics* **2005**, *61* (1), 69–78. https://doi.org/10.1002/prot.20500.

(16) Roberts, D.; Keeling, R.; Tracka, M.; van der Walle, C. F.; Uddin, S.; Warwicker, J.; Curtis, R. Specific Ion and Buffer Effects on Protein-Protein Interactions of a Monoclonal Antibody. *Mol Pharm* **2015**, *12* (1), 179–193. https://doi.org/10.1021/mp500533c.

(17) Nandi, P. K.; Robinson, D. R. The Effects of Salts on the Free Energy of the Peptide Group. *Journal of the American Chemical Society* **1972**, *94* (4), 1299–1307. https://doi.org/10.1021/ja00759a042.

(18) Wu, G. Amino Acids: Metabolism, Functions, and Nutrition. *Amino acids*. May 2009, pp 1–17. https://doi.org/10.1007/s00726-009-0269-0.

(19) Drauz, K.; Gayson, I.; Kleemann, A.; Krimmer, H.-P.; Leuchtenberger, W.; Weckbecker, C. Amino Acids. *Ullmann's Encyclopedia of Industrial Chemistry*; 2012; Vol. 3, pp 1–58. https://doi.org/10.1002/14356007.a02_057.pub2.

(20) Wu, G. Functional Amino Acids in Growth, Reproduction, and Health. *Advances in Nutrition*. November 2010, pp 31–37. https://doi.org/10.3945/an.110.1008.

(21) Mohanty, A. K.; Parija, S.; Mlsra, M. Ce(IV)-N-Acetylglycine Initiated Graft Copolymerization of Acrylonitrile onto Chemically Modified Pineapple Leaf Fibers. *J Appl Polym Sci* **1996**, *60*, 931–937. https://doi.org/10.1080/03602559608000928.

(22) Harburn, J. J.; Rath, N. P.; Spilling, C. D. Efficient Synthesis of Tyrosine-Derived Marine Sponge Metabolites via Acylation of Amines with a Coumarin. *Journal of Organic Chemistry* **2005**, *70* (16), 6398–6403. https://doi.org/10.1021/jo050846r.

(23) Das, D. K.; Sarkar, S.; Khan, M.; Belal, M.; Khan, A. T. A Mild and Efficient Method for Large Scale Synthesis of 3-Aminocoumarins and Its Further Application for the Preparation of 4-Bromo-3-Aminocoumarins. *Tetrahedron Lett* **2014**, *55* (35), 4869–4874. https://doi.org/10.1016/j.tetlet.2014.07.035.

(24) Si-Kwan, K.; Ubukata, M.; Isono, K. N-Acetylglycine Side Chain Is Critical for the Antimicrobial Activity of Xanthostatin. *J. Microbiol. Biotechnol* **2003**, *13* (6), 998–1000.

(25) Cheng, X. C.; Kihara, T.; Kusakabe, H.; Fang, R. P.; Ni, Z. F.; Shen, Y. C.; Ko, K.; Yamaguchi, I.; Isono, K.; Isono, K. Xanthostatin, a New Antibiotic. *Agric Biol Chem* **2014**, *51* (1), 279–281. https://doi.org/10.1080/00021369.1987.10867987.

(26) Wang, J.; Wang, J.; Liu, J.; Wang, S.; Pei, J. Solubility of D-Aspartic Acid and L-Aspartic Acid in Aqueous Salt Solutions from (293 to 343) K. *J Chem Eng Data* **2010**, *55* (4), 1735–1738. https://doi.org/10.1021/je9007102.

(27) Tomé, L. I. N.; Jorge, M.; Gomes, J. R. B.; Coutinho, J. A. P. Toward an Understanding of the Aqueous Solubility of Amino Acids in the Presence of Salts: A Molecular Dynamics Simulation Study. *Journal of Physical Chemistry B* **2010**, *114* (49), 16450–16459. https://doi.org/10.1021/jp104626w.

(28) Kunz, W. Specific Ion Effects in Colloidal and Biological Systems. *Curr Opin Colloid Interface Sci* **2010**, *15* (1–2), 34–39. https://doi.org/10.1016/j.cocis.2009.11.008.

(29) Holm, R. H.; Kennepohl, P.; Solomon, E. I. Structural and Functional Aspects of Metal Sites in Biology. *Chem Rev* **1996**, *96*, 2239–2314. https://doi.org/10.1021/cr9500390.

(30) Celander, D. W.; Cech, T. R. Visualizing the Higher Order Folding of a Catalyic RNA Molecule. *Science (1979)* **1991**, *251*, 401–407. https://doi.org/10.1126/science.1989074.

(31) Pyle, A. M. Metal Ions in the Structure and Function of RNA. *Journal of Biological Inorganic Chemistry*. 2002, pp 679–690. https://doi.org/10.1007/s00775-002-0387-6.

(32) Zot, H. G.; Potter, J. D. A Structural Role for the Ca2+-Mg2+ Sites on Troponin C in the Regulation of Muscle Contraction. Preparation and Properties of Troponin C Depleted Myofibrils. *Journal of Biological Chemistry* **1982**, *257* (13), 7678–7683. https://doi.org/10.1016/S0021-9258(18)34434-X.

(33) Serra, M. J.; Baird, J. D.; Dale, T.; Fey, B. L.; Retatagos, K.; Westhof, E. Effects of Magnesium Ions on the Stabilization of RNA Oligomers of Defined Structures. *RNA* **2002**, *8* (3), 307–323. https://doi.org/10.1017/s1355838202024226.

(34) Santosh, M. S.; Lyubartsev, A. P.; Mirzoev, A. A.; Bhat, D. K. Molecular Dynamics Investigation of Dipeptide - Transition Metal Salts in Aqueous Solutions. *Journal of Physical Chemistry B* **2010**, *114* (49), 16632–16640. https://doi.org/10.1021/jp108376j.

(35) Wang, Y.; Zhou, Y.; Li, P. jun; Wang, X. xi; Cai, K. zhou; Chen, C. gui. Combined Effect of CaCl2 and High Pressure Processing on the Solubility of Chicken Breast Myofibrillar Proteins under Sodium-Reduced Conditions. *Food Chem* **2018**, *269*, 236–243. https://doi.org/10.1016/j.foodchem.2018.06.107.

(36) Dillip, G. R.; Bhagavannarayana, G.; Raghavaiah, P.; Deva Prasad Raju, B. Effect of Magnesium Chloride on Growth, Crystalline Perfection, Structural, Optical, Thermal and NLO Behavior of γ-Glycine Crystals. *Mater Chem Phys* **2012**, *134* (1), 371–376. https://doi.org/10.1016/j.matchemphys.2012.03.004.

(37) Dudev, T.; Lim, C. Principles Governing Mg, Ca, and Zn Binding and Selectivity in Proteins. *Chem Rev* **2003**, *103* (3), 773–787. https://doi.org/10.1021/cr020467n.

(38) Nidhi, K.; Indrajeet, S.; Khushboo, M.; Gauri, K.; Jyoti Sen, D. Hydrotropy: A Promising Tool for Solubility Enhancement: A Review. *International Journal of Drug Development & Research* **2011**, *3* (2), 26–33. https://doi.org/10.52711/2231-5713.2022.00025.

(39) Kumar, V. S.; Raja, C.; Jayakumar, C. A Review on Solubility Enhancement Using Hydrotropic Phenomena. *Int J Pharm Pharm Sci* **2014**, *6* (6), 1–7.

(40) Tomé, L. I. N.; Sousa, C. S. R.; Gomes, J. R. B.; Ferreira, O.; Coutinho, J. A. P.; Pinho, S. P. Understanding the Cation Specific Effects on the Aqueous Solubility of Amino Acids: From Mono to Polyvalent Cations. *The Royal Society of Chemistry Advances* **2015**, *5* (20), 15024–15034. https://doi.org/10.1039/C5RA00501A.

(41) Roy, S.; Guin, P. S.; Mahali, K.; Dolui, B. K. Solubility and Transfer Gibbs Free Energetics of Glycine, DL-Alanine, DL-nor-Valine and DL-Serine in Aqueous Sodium Fluoride and Potassium Fluoride Solutions at 298.15 K. *Indian J Chem* **2017**, *56A*, 399–406.

(42) El-Dossoki, F. I. Effect of the Charge and the Nature of Both Cations and Anions on the Solubility of Zwitterionic Amino Acids, Measurements and Modeling. *J Solution Chem* **2010**, *39* (9), 1311–1326. https://doi.org/10.1007/s10953-010-9580-3.

(43) Lu, J.; Wang, X. J.; Yang, X.; Ching, C. B. Solubilities of Glycine and Its Oligopeptides in Aqueous Solutions. *J Chem Eng Data* **2006**, *51* (5), 1593–1596. https://doi.org/10.1021/je0600754.

(44) Carta, R.; Tola, G. Solubilities of L-Cystine, L-Tyrosine, L-Leucine, and Glycine in Aqueous Solutions at Various PHs and NaCl Concentrations. *J Chem Eng Data* **1996**, *41* (3), 414–417. https://doi.org/10.1021/JE9501853.

(45) Carta, R. Solubilities of L-Cystine, L-Tyrosine, L-Leucine, and Glycine in Sodium Chloride Solutions at Various PH Values. *Journal of Chemical Thermodynamics* **1998**, *30*, 379–387. https://doi.org/10.1006/JCHT.1997.0313.

(46) Khoshkbarchi, M. K.; Vera, J. H. Effect of NaCl and KCl on the Solubility of Amino Acids in Aqueous Solutions at 298.2 K: Measurements and Modeling. *Ind Eng Chem Res* **1997**, *36* (6), 2445–2451. https://doi.org/10.1021/IE9606395.

(47) Ansari, Z. H.; Li, Z. Solubilities and Modeling of Glycine in Mixed NaCl-MgCl2 Solutions in a Highly Concentrated Region. *J Chem Eng Data* **2016**, *61* (10), 3488–3497. https://doi.org/10.1021/acs.jced.6b00403.

(48) Ansari, Z. H.; Li, Z. Solid-Liquid Equilibria for the Glycine-Alcohol-NaCl-H2O System. *J Chem Eng Data* **2017**, *62* (10), 3551–3560. https://doi.org/10.1021/acs.jced.7b00549.

(49) Held, C.; Reschke, T.; Müller, R.; Kunz, W.; Sadowski, G. Measuring and Modeling Aqueous Electrolyte/Amino-Acid Solutions with EPC-SAFT. *Journal of Chemical Thermodynamics* **2014**, *68*, 1–12. https://doi.org/10.1016/j.jct.2013.08.018.

(50) Roy, S.; Hossain, A.; Mahali, K.; Dolui, B. K. Thermodynamics and Mechanisms of Glycine Solvation in Aqueous NaCl and KCl Solutions at 298.15 K. *Russian Journal of Physical Chemistry A* **2015**, *89* (11), 2111–2119. https://doi.org/10.1134/S0036024415110151.

(51) Gao, W.; Li, Z. Determination and Chemical Modeling of Phase Equilibria for the Glycine-KCl-NaCl-H 2O System and Its Application to Produce Crystals with Anti-caking Characteristics. *Ind Eng Chem Res* **2012**, *51* (24), 8315–8325. https://doi.org/10.1021/ie300787k.

(52) Roy, S.; Hossain, A.; Dolui, B. K. Solubility and Chemical Thermodynamics of D,L-Alanine and D,L-Serine in Aqueous NaCl and KCl Solutions. *J Chem Eng Data* **2016**, *61* (1), 132–141. https://doi.org/10.1021/acs.jced.5b00351.

(53) Roy, S.; Guin, P. S.; Mahali, K.; Dolui, B. K. Role of Electrolytes in the Solubility of L-Proline and Its Transfer Free Energetics. *J Mol Liq* **2016**, *223* (11), 927–933. https://doi.org/10.1016/j.molliq.2016.09.018.

(54) Roy, S.; Guin, P. S.; Mahali, K.; Dolui, B. K. Amino Acid Solubility under the Influence of NaCl at 298.15 K. *J Mol Liq* **2016**, *218*, 316–318. https://doi.org/10.1016/j.molliq.2016.02.054.

(55) Bretti, C.; Giuffrè, O.; Lando, G.; Sammartano, S. Modeling Solubility and Acid–Base Properties of Some Amino Acids in Aqueous NaCl and (CH3)4NCl Aqueous Solutions at Different Ionic Strengths and Temperatures. *Springerplus* **2016**, *5* (1), 928. https://doi.org/10.1186/s40064-016-2568-8.

(56) El-Dossoki, F. I.; El-Damarany, M. M. Solvation of Basic and Neutral Amino Acids in Aqueous Electrolytic Solutions: Measurements and Modeling. *J Chem Eng Data* **2015**, *60* (10), 2989–2999. https://doi.org/10.1021/acs.jced.5b00393.

(57) Bretti, C.; Cigala, R. M.; Giuffrè, O.; Lando, G.; Sammartano, S. Modeling Solubility and Acid-Base Properties of Some Polar Side Chain Amino Acids in NaCl and (CH3)4NCl Aqueous Solutions at Different Ionic Strengths and Temperatures. *Fluid Phase Equilib* **2018**, *459*, 51–64. https://doi.org/10.1016/j.fluid.2017.11.030.

(58) Abualreish, M. J.; Noubigh, A. Evaluation of Thermodynamic Properties and Correlation of L-Glutamic Acid Solubility in Some Aqueous Chloride Solutions from 298.15 to 323.15 K. *Can J Chem* **2019**, *97* (8), 615–620. https://doi.org/10.1139/cjc-2019-0018.

(59) Mondal, S.; Dolui, B. K.; Roy, S.; Ghosh, S.; Mahali, K. Study of the Solubility and Transfer Thermodynamics of D,L-Phenylalanine in Aqueous Sodium Chloride and D,L-Serine in Aqueous Sodium Nitrate Solutions. *J Solution Chem* **2016**, *45* (12), 1755–1772. https://doi.org/10.1007/s10953-016-0527-1.

(60) Bretti, C.; Crea, F.; Stefano, C. de; Sammartano, S.; Dipartimento, G. V. Some Thermodynamic Properties of DL-Tyrosine and DL-Tryptophan. Effect of the Ionic Medium, Ionic Strength and Temperature on the Solubility and Acid-Base Properties. *Fluid Phase Equilib* **2012**, *314*, 185–197. https://doi.org/10.1016/j.fluid.2011.10.007.

(61) Guin, P. S.; Mahali, K.; Dolui, B. K.; Roy, S. Solubility and Thermodynamics of Solute-Solvent Interactions of Some Amino Acids in Aqueous Sodium Bromide and Potassium Bromide Solutions. *J Chem Eng Data* **2018**, *63* (3), 534–541. https://doi.org/10.1021/acs.jced.7b00647.

(62) Datta, A.; Roy, S. Thermodynamics of Solute–Solvent Interactions and Solubility of Some Amino Acids in Aqueous Sodium Iodide Solutions at T = 298.15 K. *Russian Journal of Physical Chemistry A* **2021**, *95*, S62–S70. https://doi.org/10.1134/S0036024421140041.

(63) Pradhan, A. A.; Vera, J. H. Effect of Anions on the Solubility of Zwitterionic Amino Acids. *J Chem Eng Data* **2000**, *45* (1), 140–143. https://doi.org/10.1021/je9902342.

(64) Roy, S.; Guin, P. S.; Mondal, S.; Ghosh, S.; Dolui, B. K. Solubility of Glycine and DL-nor-Valine in Aqueous Solutions of NaNO3 and KNO3 and Measurements of Transfer Thermodynamics. *J Mol Liq* **2016**, *222*, 313–319. https://doi.org/10.1016/j.molliq.2016.07.050.

(65) Talukdar, H.; Rudra, S.; Kundu, K. K. Thermodynamics of Transfer of Glycine, Diglycine, and Triglycine from Water to Aqueous Solutions of Urea, Glycerol, and Sodium Nitrate. *Can J Chem* **1988**, *66* (3), 461–468. https://doi.org/10.1139/v88-080.

(66) Sun, H.; Wang, L.; Liu, B. Solubility of α-Glycine in Water with Additives at a Temperature Range of (293.15–343.15) K: Experimental Data and Results of Thermodynamic Modeling. *Fluid Phase Equilib* **2017**, *434*, 167–175. https://doi.org/10.1016/j.fluid.2016.12.003.

(67) Roy, S.; Mahali, K.; Mondal, S.; Mondal, R. P.; Dolui, B. K. Physico-Chemical Studies of DL-Alanine in Aqueous Sodium Nitrate Solution. *Russ J Gen Chem* **2015**, *85* (1), 162–167. https://doi.org/10.1134/S1070363215010284.

(68) Chowdhury, S.; Mandal, P.; Hossain, A.; Guin, P. S.; Roy, S.; Mahali, K. Electrolytic Effect on the Solubility and Solvation Thermodynamics of L -Serine and L -Isoleucine in Aqueous Media. *J Chem Eng Data* **2019**, *64* (10), 4286–4297. https://doi.org/10.1021/acs.jced.9b00363.

(69) Roy, S.; Mondal, S.; Dolui, B. K. Solvation Thermodynamics of DL-Phenylalanine in Aqueous NaNO3 Solution at 298.15 K. *Russian Journal of Physical Chemistry A* **2018**, *92* (4), 734–738. https://doi.org/10.1134/S003602441804026X.

(70) Ferreira, L. A.; Macedo, E. A.; Pinho, S. P. Effect of KCl and Na2SO4 on the Solubility of Glycine and DL-Alanine in Water at 298.15 K. *Ind Eng Chem Res* **2005**, *44* (23), 8892–8898. https://doi.org/10.1021/ie050613q.

(71) Roy, S.; Guin, P. S.; Mahali, K.; Hossain, A.; Dolui, B. K. Evaluation and Correlation of Solubility and Solvation Thermodynamics of Glycine, DL-Alanine and DL-Valine in Aqueous Sodium Sulphate Solutions at Two Different Temperatures. *J Mol Liq* **2017**, *234*, 124–128. https://doi.org/10.1016/j.molliq.2017.03.068.

(72) Ramasami, P. Solubilities of Amino Acids in Water and Aqueous Sodium Sulfate and Related Apparent Transfer Properties. *J Chem Eng Data* **2002**, *47* (5), 1164–1166. https://doi.org/10.1021/JE025503U.

(73) Chowdhury, S.; Mandal, P.; Islam Sarikul, M.; Hossain, A.; Guin, P. S.; Roy, S.; Mahali, K. Solubility and Transfer Solvation Thermodynamics of L-Isoleucine and L-Serine in Water to Aqueous Solution of Na2SO4 and K2SO4 from 288.15 K to 303.15 K. *Chem Phys Lett* **2018**, *706*, 432–439. https://doi.org/10.1016/j.cplett.2018.06.032.

(74) Ferreira, L. A.; Macedo, E. A.; Pinho, S. P. KCl Effect on the Solubility of Five Different Amino Acids in Water. *Fluid Phase Equilib* **2007**, *255* (2), 131–137. https://doi.org/10.1016/j.fluid.2007.04.004.

(75) Venkatesu, P.; Lee, M.-J.; Lin, H.-M. Transfer Free Energies of Peptide Backbone Unit from Water to Aqueous Electrolyte Solutions at 298.15 K. *Biochem Eng J* **2006**, *32* (3), 157–170. https://doi.org/10.1016/j.bej.2006.09.015.

(76) Hossain, A.; Roy, S. Solubility and Solute-Solvent Interactions of DL-Alanine and DL-Serine in Aqueous Potassium Nitrate Solutions. *J Mol Liq* **2018**, *249*, 1133–1137. https://doi.org/10.1016/j.molliq.2017.11.104.

(77) Hossain, A.; Mahali, K.; Dolui, B. K.; Guin, P. S.; Roy, S. Solubility Analysis of Homologous Series of Amino Acids and Solvation Energetics in Aqueous Potassium Sulfate Solution. *Heliyon* **2019**, *5* (8), e02304. https://doi.org/10.1016/j.heliyon.2019.e02304.

(78) Zeng, Y.; Li, Z. Phase Equilibria for the Glycine−Methanol−NH4Cl−H2O System. *Ind Eng Chem Res* **2014**, *53* (43), 16864–16872. https://doi.org/10.1021/IE502846M.

(79) Tome, L. I. N.; Pinho, S. P.; Jorge, M.; Gomes, J. R. B.; Coutinho, J. A. P. Salting-in with a Salting-out Agent: Explaining the Cation Specific Effects on the Aqueous Solubility of Amino Acids. *J Phys Chem B* **2013**, *117*, 6116–6128. https://doi.org/10.1021/jp4021307.

(80) Ansari, Z. H.; Zeng, Y.; Zhang, Y.; Demopoulos, G. P.; Li, Z. Modeling of Glycine Solubility in Aqueous HCl–MgCl2 System and Its Application in Phase Transition of Glycine by Changing Media and Supersaturation. *J Cryst Growth* **2017**, *467*, 116–125. https://doi.org/10.1016/j.jcrysgro.2017.03.037.

(81) Shi, G.; Dang, Y.; Pan, T.; Liu, X.; Liu, H.; Li, S.; Zhang, L.; Zhao, H.; Li, S.; Han, J.; Tai, R.; Zhu, Y.; Li, J.; Ji, Q.; Mole, R. A.; Yu, D.; Fang, H. Unexpectedly Enhanced Solubility of Aromatic Amino Acids and Peptides in an Aqueous Solution of Divalent Transition-Metal Cations. *Phys Rev Lett* **2016**, *117* (23), 1–6. https://doi.org/10.1103/PhysRevLett.117.238102.

(82) Mondal, S.; Ghosh, S.; Hossain, A.; Mahali, K.; Roy, S.; Dolui, B. K. Thermodynamics of DL-α-Aminobutyric Acid Induced Solvation Mechanism in Aqueous KCl Solutions at 288.15-308.15 K. *Russian Journal of Physical Chemistry A* **2016**, *90* (9), 1798–1805. https://doi.org/10.1134/S003602441609020X.

(83) Roy, S.; Guin, P. S.; Dolui, B. K. Solubility and Solvation Thermodynamics of DL-nor-Valine in Aqueous Solutions of NaCl and KCl. *J Mol Liq* **2015**, *211*, 294–300. https://doi.org/10.1016/j.molliq.2015.07.030.

(84) Roy, S.; Mahali, K.; Pal, S.; Mondal, S.; Dolui, B. K. Solubility of α-Amino Butyric Acid in Water-NaNO3 Mixture and Analysis of Related Thermodynamic Parameters. *Analytical Chemistry: An Indian Journal* **2015**, *15* (2), 65–73.

(85) Rowland, D. Thermodynamic Properties of the Glycine + H2O System. *J Phys Chem Ref Data* **2018**, *47* (2). https://doi.org/10.1063/1.5016677.

(86) Dunn, M. S.; Ross, F. J.; Read, L. S. The Solubility of Amino Acids. *J Biol Chem* **1933**, *103*, 579–595. https://doi.org/10.1016/s0021-9258(18)75836-5.

(87) Nozaki, Y.; Tanford, C. The Solubility of Aminoacids, Diglycine, and Triglycine in Aqueous Guanidine Hydrochloride Solutions. *J Biol Chem* **1970**, *245* (7), 1648–1652. https://doi.org/10.1016/S0021-9258(19)77141-5.

(88) Dalton, J. B.; Schmidt, C. L. A. The Solubilities of Certain Amino Acids and Related Compounds in Water, the Densities of Their Solutions at Twenty-Five Degrees, and the Calculated Heats of Solution and Partial Molal Volumes. *J Biol Chem* **1933**, *103*, 549–578. https://doi.org/10.1016/s0021-9258(18)75234-4.

(89) Soto, A.; Arce, A.; Khoshkbarchi, M. K.; Vera, J. H. Measurements and Modelling of the Solubility of a Mixture of Two Amino Acids in Aqueous Solutions. *Fluid Phase Equilib* **1999**, *158–160*, 893–901. https://doi.org/10.1016/S0378-3812(99)00087-4.

(90) Matsuo, H.; Suzuki, Y.; Sawamura, S. Solubility of α-Amino Acids in Water under High Pressure: Glycine, L-Alanine, L-Valine, L-Leucine, and L-Isoleucine. *Fluid Phase Equilib* **2022**, *200*, 227–237. https://doi.org/10.1016/S0378-3812(02)00029-8.

(91) Gekko, K.; Ohmae, E.; Kameyama, K.; Takagi, T. Acetonitrile-Protein Interactions: Amino Acid Solubility and Preferential Solvation. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **1998**, *1387*, 195. https://doi.org/10.1016/s0167-4838(98)00121-6.

(92) Romero, C. M.; Oviedo, C. D. Effect of Temperature on the Solubility of α-Amino Acids and α,ω-Amino Acids in Water. *J Solution Chem* **2013**, *42* (6), 1355–1362. https://doi.org/10.1007/s10953-013-0031-9.

(93) Vasantha, T.; Kavitha, T.; Kumar, A.; Venkatesu, P.; Rama Devi, R. S. Evaluating the Transfer Free Energies of Amino Acids from Water to Ammonium-Based Ionic Liquids at 298.15 K. *J Mol Liq* **2015**, *208*, 130–136. https://doi.org/10.1016/j.molliq.2015.04.007.

(94) Thomas, D. W. Studies on the Purification and Properties of L-Leucine. Studies on the Mode of Action of Trypsin and Chymotrypsin, California Institute of Technology, Pasadena, California, 1951. https://doi.org/10.7907/DKCB-ER97.

(95) Nozaki, Y.; Tanford, C. The Solubility of Amino Acids and Related Compounds in Aqueous Urea Solutions. *J Biol Chem* **1963**, *238*, 4074–4081. https://doi.org/10.1016/S0021-9258(18)51830-5.

(96) Sasahara, K.; Uedaira, H. Solubility of Amino Acids in Aqueous Poly (Ethylene Glycol) Solutions. *Colloid Polym Sci* **1993**, *271*, 1035–1041. https://doi.org/10.1007/BF00659292.

(97) Bowden, N. A.; Sanders, J. P. M.; Bruins, M. E. Solubility of the Proteinogenic α-Amino Acids in Water, Ethanol, and Ethanol–Water Mixtures. *J Chem Eng Data* **2018**, *63* (3), 488–497. https://doi.org/10.1021/acs.jced.7b00486.

(98) Zhu, W.; Fan, Y.; Xu, Q.; Liu, X.; Heng, B.; Yang, W.; Hu, Y. Saturated Solubility and Thermodynamic Evaluation of L-Tryptophan in Eight Pure Solvents and Three Groups of Binary Mixed Solvents by the Gravimetric Method at T = 278.15-333.15 K. *J Chem Eng Data* **2019**, *64* (9), 4154–4168. https://doi.org/10.1021/acs.jced.9b00562.

(99) Do, H. T.; Chua, Y. Z.; Kumar, A.; Pabsch, D.; Hallermann, M.; Zaitsau, D.; Schick, C.; Held, C. Melting Properties of Amino Acids and Their Solubility in Water. *RSC Adv* **2020**, *10*, 44205–44215. https://doi.org/10.1039/D0RA08947H.

(100) He, Q.; Cong, Y.; Zheng, M.; Farajtabar, A.; Zhao, H. Solubility of L-Tyrosine in Aqueous Solutions of Methanol, Ethanol, n-Propanol and Dimethyl Sulfoxide: Experimental Determination and Preferential Solvation Analysis. *J Chem Thermodyn* **2018**, *124*, 123–132. https://doi.org/10.1016/j.jct.2018.05.011.

(101) Jin, X. Z.; Chao, K.-C. Solubility of Four Amino Acids in Water and of Four Pairs of Amino Acids in Their Water Solutions. *J Chem Eng Data* **1992**, *37*, 199–203. https://doi.org/10.1021/je00006a016.

(102) Apelblat, A.; Manzurola, E. Solubilities of L-Aspartic, DL-Aspartic, DL-Glutamic, p-Hydroxybenzoic, o-Anisic, p-Anisic, and Itaconic Acids in Water from T=278 K to T=345 K. *J Chem Thermodyn* **1997**, *29* (12), 1527–1533. https://doi.org/10.1006/jcht.1997.0267.

(103) Lee, C.-Y.; Chen, J.-T.; Chang, W.-T.; Shiah, I.-M. Effect of PH on the Solubilities of Divalent and Trivalent Amino Acids in Water at 298.15K. *Fluid Phase Equilib* **2013**, *343*, 30–35. https://doi.org/10.1016/j.fluid.2013.01.010.

(104) Mo, Y.; Dang, L.; Wei, H. Solubility of α-Form and ß-Form of L-Glutamic Acid in Different Aqueous Solvent Mixtures. *Fluid Phase Equilib* **2011**, *300*, 105–109. https://doi.org/10.1016/j.fluid.2010.10.020.

(105) Pabba, S.; Kumari, A.; Narra, T.; Thella, P. K.; Satyavathi, B.; Shah, K.; Kundu, S.; Bhargava, S. K. Measurement and Modeling of Solid–Liquid Equilibria of L-

Glutamic Acid in Pure Solvents and Aqueous Binary Mixtures. *J Chem Eng Data* **2019**, *64* (3), 1155–1165. https://doi.org/10.1021/acs.jced.8b01084.

(106) Hermanto, M. W.; Kee, N. C.; Tan, R. B. H.; Chiu, M.-S.; Braatz, R. D. Robust Bayesian Estimation of Kinetics for the Polymorphic Transformation of L-Glutamic Acid Crystals. *AIChE Journal* **2008**, *54* (12), 3248–3259. https://doi.org/10.1002/aic.11623.

(107) Manzurola, E.; Apelblat, A. Solubilities of L-Glutamic Acid, 3-Nitrobenzoic Acid, p-Toluic Acid, Calcium-L-Lactate, Calcium Gluconate, Magnesium-DL-Aspartate, and Magnesium-L-Lactate in Water. *J Chem Thermodyn* **2002**, *34* (7), 1127–1136. https://doi.org/10.1006/jcht.2002.0975.

(108) Breil, M. P.; Mollerup, J. M.; Rudolph, E. S. J.; Ottens, M.; van der Wielen, L. A. M. Densities and Solubilities of Glycylglycine and Glycyl-L-Alanine in Aqueous Electrolyte Solutions. *Fluid Phase Equilib* **2004**, *215* (2), 221–225. https://doi.org/10.1016/j.fluid.2003.08.010.

(109) Maria da Silva dos Santos, Y. Efeitos de Sais Na Solubilidade de Diglicina e N-Acetilglycina Em Água. Projeto de Mestre em Tecnologia Biomédica, Instituto Politécnico de Bragança, 2016.

(110) Lampreia, I. M. S.; Magalhães, S. R. J.; Rodrigues, S. I. M.; Mendonça, Â. F. S. S. Solubility of Proline-Leucine Dipeptide in Water and in Aqueous Sodium Chloride Solutions from T = (288.15 to 313.15) K. *Journal of Chemical Thermodynamics* **2006**, *38*, 240–244. https://doi.org/10.1016/j.jct.2005.05.009.

(111) Pérez-Sánchez, G.; Santos, Y. S.; Ferreira, O.; Coutinho, J. A. P.; Gomes, J. R. B.; Pinho, S. P. The Cation Effect on the Solubility of Glycylglycine and N-Acetylglycine in Aqueous Solution: Experimental and Molecular Dynamics Studies. *J Mol Liq* **2020**, *310* (113044). https://doi.org/10.1016/j.molliq.2020.113044.

(112) Nozaki, Y.; Tanford, C. The Solubility of Amino Acids and Related Compounds in Aqueous Ethylene Glycol Solutions. *J Biol Chem* **1965**, *240* (9), 3568–3573. https://doi.org/10.1016/s0021-9258(18)97181-4.

(113) Nozaki, Y.; Tanford, C. The Solubility of Amino Acids and Two Glycine Peptides in Aqueous Ethanol and Dioxane Solutions: Establishment of a Hydrophobicity Scale. *J Biol Chem* **1971**, *246* (7), 2211–2217. https://doi.org/10.1016/S0021-9258(19)77210-X.

(114) Gekko, K. Mechanism of Polyol-Induced Protein Stabilization: Solubility of Amino Acids and Diglycine in Aqueous Polyol Solutions. *J. Biochem* **1981**, *90*, 1633–1641. https://doi.org/10.1093/oxfordjournals.jbchem.a133638.

(115) Smith, E. R. B.; Smith, P. K. Thermodynamic Properties of Solutions of Amino Acids and Related Substances. *Journal of Biological Chemistry* **1940**, *135*, 273–279. https://doi.org/10.1016/s0021-9258(18)73184-0.

(116) Mcmeekin, T. L.; Cohn, E. J.; Weare, J. H. Studies in the Physical Chemistry of Amino Acids, Peptides and Related Substances. III. The Solubility of Derivatives of the Amino Acids in Alcohol-Water Mixtures. *J Am Chem Soc* **1935**, *57*, 626–633. https://doi.org/10.1021/Ja01307A010.

(117) Guo, Y.; He, H.; Huang, H.; Qiu, J.; Han, J.; Hu, S.; Liu, H.; Zhao, Y.; Wang, P. Solubility Determination and Thermodynamic Modeling of N-Acetylglycine in Different Solvent Systems. *J Chem Eng Data* **2021**, *66* (3), 1344–1355. https://doi.org/10.1021/acs.jced.0c00983.

(118) Breil, M. P.; Mollerup, J. M.; Rudolph, E. S. J.; Ottens, M.; van der Wielen, L. A. M. Determination of the Activity Coefficients of Glycylglycine and Glycyl-L-Alanine in Sodium Chloride Solutions by an Electrochemical Cell with Ion-Selective Electrodes: Experimental Measurements and Thermodynamic Theory. *Fluid Phase Equilib* **2001**, *191* (1–2), 127–140. https://doi.org/10.1016/S0378-3812(01)00622-7.

(119) Tseng, H. C.; Lee, C. Y.; Weng, W. L.; Shiah, I. M. Solubilities of Amino Acids in Water at Various PH Values under 298.15 K. *Fluid Phase Equilib* **2009**, *285* (1–2), 90–95. https://doi.org/10.1016/j.fluid.2009.07.017.

(120) Prausnitz, J. M. *Molecular Thermodynamics of Fluid-Phase Equilibria*; Prentice-Hall: Englewood Cliffs, 1969.

(121) Hitchcock, D. The Solubility of Tyrosine in Acid and in Alkali. *J Gen Physiol* **1924**, 747–757. https://doi.org/10.1085/jgp.6.6.747.

(122) Needham, T. E.; Paruta, A. N.; Gerraughty, R. J. Solubility of Amino Acids in Pure Solvent Systems. *J Pharm Sci* **1971**, *60* (4), 565–567. https://doi.org/10.1002/jps.2600600410.

(123) Zumstein, R. C.; Rousseau, R. W. Solubility of L-Isoleucine in and Recovery of L-Isoleucine from Neutral and Acidic Aqueous Solutions. *Industrial & Engineering Chemistry Research* **1989**, *28* (8), 1226–1231. https://doi.org/10.1021/ie00092a016.

(124) Gatewood Brown, M.; Rousseau, R. W. Effect of Sodium Hydroxide on the Solubilities of L-Isoleucine, L-Leucine, and L-Valine. *Biotechnol Prog* **1994**, *10* (3), 253–257. https://doi.org/10.1021/bp00027a003.

(125) Pradhan, A. A.; Vera, J. H. Effect of Acids and Bases on the Solubility of Amino Acids. *Fluid Phase Equilib* **1998**, *152*, 121–132. https://doi.org/10.1016/S0378-3812(98)00387-2.

(126) Fuchs, D.; Fischer, J.; Tumakaka, F.; Sadowski, G. Solubility of Amino Acids: Influence of the PH Value and the Addition of Alcoholic Cosolvents on Aqueous Solubility. *Ind Eng Chem Res* **2006**, *45* (19), 6578–6584. https://doi.org/10.1021/IE0602097.

(127) Franco, L. F. M.; Mattedi, S.; Pessôa Filho, P. de A. A New Approach for the Thermodynamic Modeling of the Solubility of Amino Acids and β-Lactam Compounds as a Function of PH. *Fluid Phase Equilib* **2013**, *354*, 38–46. https://doi.org/10.1016/j.fluid.2013.06.009.

(128) Voges, M.; Prikhodko, I. v.; Prill, S.; Hübner, M.; Sadowski, G.; Held, C. Influence of PH Value and Ionic Liquids on the Solubility of L-Alanine and L-Glutamic Acid in Aqueous Solutions at 30 °C. *J Chem Eng Data* **2017**, *62* (1), 52–61. https://doi.org/10.1021/acs.jced.6b00367.

(129) Paterová, J.; Rembert, K. B.; Heyda, J.; Kurra, Y.; Okur, H. I.; Liu, W. R.; Hilty, C.; Cremer, P. S.; Jungwirth, P. Reversal of the Hofmeister Series: Specific Ion Effects on Peptides. *Journal of Physical Chemistry B* **2013**, *117* (27), 8150–8158. https://doi.org/10.1021/jp405683s.

(130) Bröhl, A.; Albrecht, B.; Zhang, Y.; Maginn, E.; Giernoth, R. Influence of Hofmeister Ions on the Structure of Proline-Based Peptide Models: A Combined Experimental and Molecular Modeling Study. *J Phys Chem B* **2017**, *121* (9), 2062–2072. https://doi.org/10.1021/acs.jpcb.6b12465.

(131) Jungwirth, P.; Cremer, P. S. Beyond Hofmeister. *Nat Chem* **2014**, *6* (4), 261–263. https://doi.org/10.1038/nchem.1899.

(132) Balos, V.; Kim, H.; Bonn, M.; Hunger, J. Dissecting Hofmeister Effects: Direct Anion–Amide Interactions Are Weaker than Cation–Amide Binding. *Angewandte Chemie - International Edition* **2016**, *128* (28), 8257–8261. https://doi.org/10.1002/anie.201602769.

(133) Balos, V.; Bonn, M.; Hunger, J. Quantifying Transient Interactions between Amide Groups and the Guanidinium Cation. *Physical Chemistry Chemical Physics* **2015**, *17* (43), 28539–28543. https://doi.org/10.1039/C5CP04619J.

(134) Balos, V.; Bonn, M.; Hunger, J. Correction: Quantifying Transient Interactions between Amide Groups and the Guanidinium Cation. *Physical Chemistry Chemical Physics*. Royal Society of Chemistry 2016, pp 1346–1347. https://doi.org/10.1039/C5CP90226F.

(135) Hladílková, J.; Heyda, J.; Rembert, K. B.; Okur, H. I.; Kurra, Y.; Liu, W. R.; Hilty, C.; Cremer, P. S.; Jungwirth, P. Effects of End-Group Termination on Salting-out Constants for Triglycine. *J Phys Chem Lett* **2013**, *4* (23), 4069–4073. https://doi.org/10.1021/jz4022238.

(136) Heyda, J.; Vincent, J. C.; Tobias, D. J.; Dzubiella, J.; Jungwirth, P. Ion Specificity at the Peptide Bond: Molecular Dynamics Simulations of N-Methylacetamide in Aqueous Salt Solutions. *Journal of Physical Chemistry B* **2010**, *114* (2), 1213–1220. https://doi.org/10.1021/jp910953w.

(137) Du, H.; Wickramasinghe, R.; Qian, X. Effects of Salt on the Lower Critical Solution Temperature of Poly (N-Isopropylacrylamide). *Journal of Physical Chemistry B* **2010**, *114* (49), 16594–16604. https://doi.org/10.1021/jp105652c.

(138) Rembert, K. B.; Paterova, J.; Heyda, J.; Hilty, C.; Jungwirth, P.; Cremer, P. S. Molecular Mechanisms of Ion-Specific Effects on Proteins. *J Am Chem Soc* **2012**, *134* (24), 10039–10046. https://doi.org/10.1021/ja301297g.

(139) Gross, J.; Sadowski, G. Perturbed-Chain SAFT: An Equation of State Based on a Perturbation Theory for Chain Molecules. *Ind Eng Chem Res* **2001**, *40*, 1244–1260. https://doi.org/10.1021/ie0003887.

(140) Cameretti, L. F.; Sadowski, G.; Mollerup, J. M. Modeling of Aqueous Electrolyte Solutions with Perturbed-Chain Statistical Associated Fluid Theory. *Ind Eng Chem Res* **2005**, *44*, 3355–3362. https://doi.org/10.1021/ie0488142.

(141) Held, C.; Reschke, T.; Mohammad, S.; Luza, A.; Sadowski, G. EPC-SAFT Revised. *Chemical Engineering Research and Design* **2014**, *92*, 2884–2897. https://doi.org/10.1016/j.cherd.2014.05.017.

(142) Do, H. T.; Franke, P.; Volpert, S.; Klinksiek, M.; Thome, M.; Held, C. Measurement and Modelling Solubility of Amino Acids and Peptides in Aqueous 2-Propanol Solutions. *Physical Chemistry Chemical Physics* **2021**, *23*, 10852–10863. https://doi.org/10.1039/D1CP00005E.

(143) Chua, Y. Z.; Do, H. T.; Schick, C.; Zaitsau, D.; Held, C. New Experimental Melting Properties as Access for Predicting Amino-Acid Solubility. *RSC Adv* **2018**, *8* (12), 6365–6372. https://doi.org/10.1039/C8RA00334C.

(144) Wolbach, J. P.; Sandler, S. I. Using Molecular Orbital Calculations to Describe the Phase Behavior of Cross-Associating Mixtures. *Ind Eng Chem Res* **1998**, *37* (8), 2917–2928. https://doi.org/10.1021/ie9607255.

(145) Bülow, M.; Ascani, M.; Held, C. EPC-SAFT Advanced - Part I: Physical Meaning of Including a Concentration-Dependent Dielectric Constant in the Born Term and in the Debye-Hückel Theory. *Fluid Phase Equilib* **2021**, *535*. https://doi.org/10.1016/j.fluid.2021.112967.

(146) Cameretti, L. F.; Sadowski, G. Modeling of Aqueous Amino Acid and Polypeptide Solutions with PC-SAFT. *Chemical Engineering and Processing: Process Intensification* **2008**, *47* (6), 1018–1025. https://doi.org/10.1016/j.cep.2007.02.034.

(147) Held, C.; Cameretti, L. F.; Sadowski, G. Measuring and Modeling Activity Coefficients in Aqueous Amino-Acid Solutions. *Ind Eng Chem Res* **2011**, *50* (1), 131–141. https://doi.org/10.1021/ie100088c.

(148) Iitaka, Y. Crystal Structure of β-Glycine. *Nature* **1959**, *183*, 390–391. https://doi.org/10.1107/S0365110X60000066.

(149) Iitaka, Y. The Crystal Structure of γ-Glycine. *Acta Crystallogr* **1961**, *14* (1), 1–10. https://doi.org/10.1107/S0365110X61000012.

(150) Han, G.; Chow, P. S.; Tan, R. B. H. Salt-Dependent Growth Kinetics in Glycine Polymorphic Crystallization. *CrystEngComm* **2016**, *18* (3), 462–470. https://doi.org/10.1039/C5CE01974E.

(151) Ferrari, E. S.; Davey, R. J.; Cross, W. I.; Gillon, A. L.; Towler, C. S. Crystallization in Polymorphic Systems: The Solution-Mediated Transformation of β to α Glycine. *Cryst Growth Des* **2003**, *3* (1), 53–60. https://doi.org/10.1021/cg025561b.

(152) Han, G.; Chow, P. S.; Tan, R. B. H. Effects of Common Inorganic Salts on Glycine Polymorphic Transformation: An Insight into Salt-Dependent Polymorphic Selectivity. *Cryst Growth Des* **2016**, *16* (11), 6499–6505. https://doi.org/10.1021/acs.cgd.6b01177.

(153) Yang, X.; Wang, X.; Ching, C. B. Solubility of Form α and Form γ of Glycine in Aqueous Solutions. *J Chem Eng Data* **2008**, *53* (5), 1133–1137. https://doi.org/10.1021/je7006988.

(154) Sakai, H.; Hosogai, H.; Kawakita, T.; Onuma, K.; Tsukamoto, K. Transformation of α-Glycine to γ-Glycine. *J Cryst Growth* **1992**, *116* (3–4), 421–426. https://doi.org/10.1016/0022-0248(92)90651-X.

(155) Kitamura, M. Strategy for Control of Crystallization of Polymorphs. *CrystEngComm* **2009**, *11* (6), 949–964. https://doi.org/10.1039/B809332F.

(156) Hossain, A.; Roy, S.; Dolui, B. K. Effects of Thermodynamics on the Solvation of Amino Acids in the Pure and Binary Mixtures of Solutions: A Review. *J Mol Liq* **2017**, *232*, 332–350. https://doi.org/10.1016/j.molliq.2017.02.080.

(157) Jiang, Q.; Shtukenberg, A. G.; Ward, M. D.; Hu, C. Non-Topotactic Phase Transformations in Single Crystals of β-Glycine. *Cryst Growth Des* **2015**, *15* (6), 2568–2573. https://doi.org/10.1021/acs.cgd.5b00187.

(158) Bendeif, E. E.; Jelsch, C. The Experimental Library Multipolar Atom Model Refinement of L-Aspartic Acid. *Acta Crystallogr C* **2007**, *63* (6), o361–o364. https://doi.org/10.1107/S0108270107021671.

(159) Ihlefeldt, F. S.; Pettersen, F. B.; von Bonin, A.; Zawadzka, M.; Görbitz, C. H. The Polymorphs of L-Phenylalanine. *Angewandte Chemie International Edition* **2014**, *53* (49), 13600–13604. https://doi.org/10.1002/anie.201406886.

(160) Binns, J.; Parsons, S.; McIntyre, G. J. Accurate Hydrogen Parameters for the Amino Acid L-Leucine. *Acta Crystallogr B Struct Sci Cryst Eng Mater* **2016**, *72* (6), 885–892. https://doi.org/10.1107/S2052520616015699.

(161) Dalhus, B.; Görbitz, C. H. Crystal Structures of Hydrophobic Amino Acids I. Redeterminations of L-Methionine and L-Valine at 120 K. *Acta Chem Scand* **1996**, *50* (6), 544–548. https://doi.org/10.3891/acta.chem.scand.50-0544.

(162) Flaig, R.; Koritsanszky, T.; Dittrich, B.; Wagner, A.; Luger, P. Intra- and Intermolecular Topological Properties of Amino Acids: A Comparative Study of Experimental and Theoretical Results. *J Am Chem Soc* **2002**, *124* (13), 3407–3417. https://doi.org/10.1021/ja011492y.

(163) Dudev, T.; Lim, C. Effect of Carboxylate-Binding Mode on Metal Binding/Selectivity and Function in Proteins. *Acc Chem Res* **2007**, *40* (1), 85–93. https://doi.org/10.1021/ar068181i.

(164) Tian, J.; Yin, Y.; Sun, H.; Luo, X. Magnesium Chloride: An Efficient 13C NMR Relaxation Agent for Amino Acids and Some Carboxylic Acids. *Journal of Magnetic Resonance* **2002**, *159*, 137–144. https://doi.org/10.1016/S1090-7807(02)00016-2.

(165) Ruggiero, M. T.; Sibik, J.; Zeitler, J. A.; Korter, T. M. Examination of L-Glutamic Acid Polymorphs by Solid-State Density Functional Theory and Terahertz Spectroscopy. *J Phys Chem A* **2016**, *120* (38), 7490–7495.

(166) Görbitz, C. H.; Törnroos, K. W.; Day, G. M. Single-Crystal Investigation of L-Tryptophan with $Z' = 16$. *Acta Crystallogr B* **2012**, *68* (5), 549–557. https://doi.org/10.1107/S0108768112033484.

(167) Frey, M. N.; Koetzle, T. F.; Lehmann, M. S.; Hamilton, W. C. Precision Neutron Diffraction Structure Determination of Protein and Nucleic Acid Components. X. A Comparison between the Crystal and Molecular Structures of L-Tyrosine and L-Tyrosine Hydrochloride. *J Chem Phys* **1973**, *58* (6), 2547–2556.

(168) Clegg, S. L.; Pitzer, K. S. Thermodynamics of Multicomponent, Miscible, Ionic Solutions: Generalized Equations for Symmetrical Electrolytes. *J Phys Chem* **1992**, *96*, 3513–3520. https://doi.org/10.1021/j100187a061.

(169) Lide, D. R. *CRC Handbook of Chemistry and Physics*; 2005.

(170) Will G. Energiedispersion Und Synchrotronstrahlung: Eine Neue Methode Und Eine Neue Strahlenquelle Fur Die Rontgenbeugung. *Fortschritte der Mineralogie* **1981**, *59*, 31–94.

(171) Schlemper, E. O.; Hamilton, W. C. Neutron-Diffraction Study of the Structures of Ferroelectric and Paraelectric Ammonium Sulfate. *J Chem Phys* **1966**, *44* (12), 4498–4509. https://doi.org/10.1063/1.1726666.

List of publications

1) Aliyeva, M.; Brandão, P.; Gomes, J. R. B.; Coutinho, J. A. P.; Ferreira, O.; Pinho, S. P.; Ferreira, O. Electrolyte Effects on the Amino Acid Solubility in Water: Solubilities of Glycine, L-Leucine, L-Phenylalanine, and L-Aspartic Acid in Salt Solutions of (Na^+, K^+, NH_4^+)/(Cl^-, NO_3^-). *Industrial & Engineering Chemistry Research*, **2022**, *61* (16), 5620-5631. https://doi.org/ 10.1021/acs.iecr.1c04562

My contribution to this work was the measurement of all the experimental solubility data, monitoring pH, calculations, their interpration and write the manuscript.

2) Aliyeva, M.; Brandão, P.; Gomes, J. R. B.; Coutinho, J. A. P.; Ferreira, O.; Pinho, S. P.; Ferreira, O. Solubilities of Amino Acids in Aqueous Solutions of Chloride or Nitrate Salts of Divalent (Mg^{2+} or Ca^{2+}) Cations. *Journal of Chemical & Engineering Data*, **2022**, *67* (6), 1565-1572. https://doi.org/10.1021/acs.jced.2c00148

My contribution to this work was the measurement of solubility of L-aspartic acid in aqueous KCl solution and L-tyrosine in aqueous KCl and $(NH_4)_2SO_4$ solutions, monitoring pH of these systems, calculations, their interpration and write the manuscript.

3) Aliyeva, M.; Brandão, P.; Gomes, J. R. B.; Coutinho, J. A. P.; Held, C.; Ferreira, O.; Pinho, S. P.; Ferreira, O. Salt Effects on the Solubility of Aromatic and Dicarboxylic Amino Acids in Water. *The Journal of Chemical Thermodynamics*, submitted for publication, **2022**, 177, 106929. https://doi.org/10.1016/j.jct.2022.106929

My contribution to this work was the measurement of all the experimental solubility data, monitoring pH, calculations, their interpration and write the manuscript.

Appendix

Appendix A – Chemical structures of amino acids listed in this work

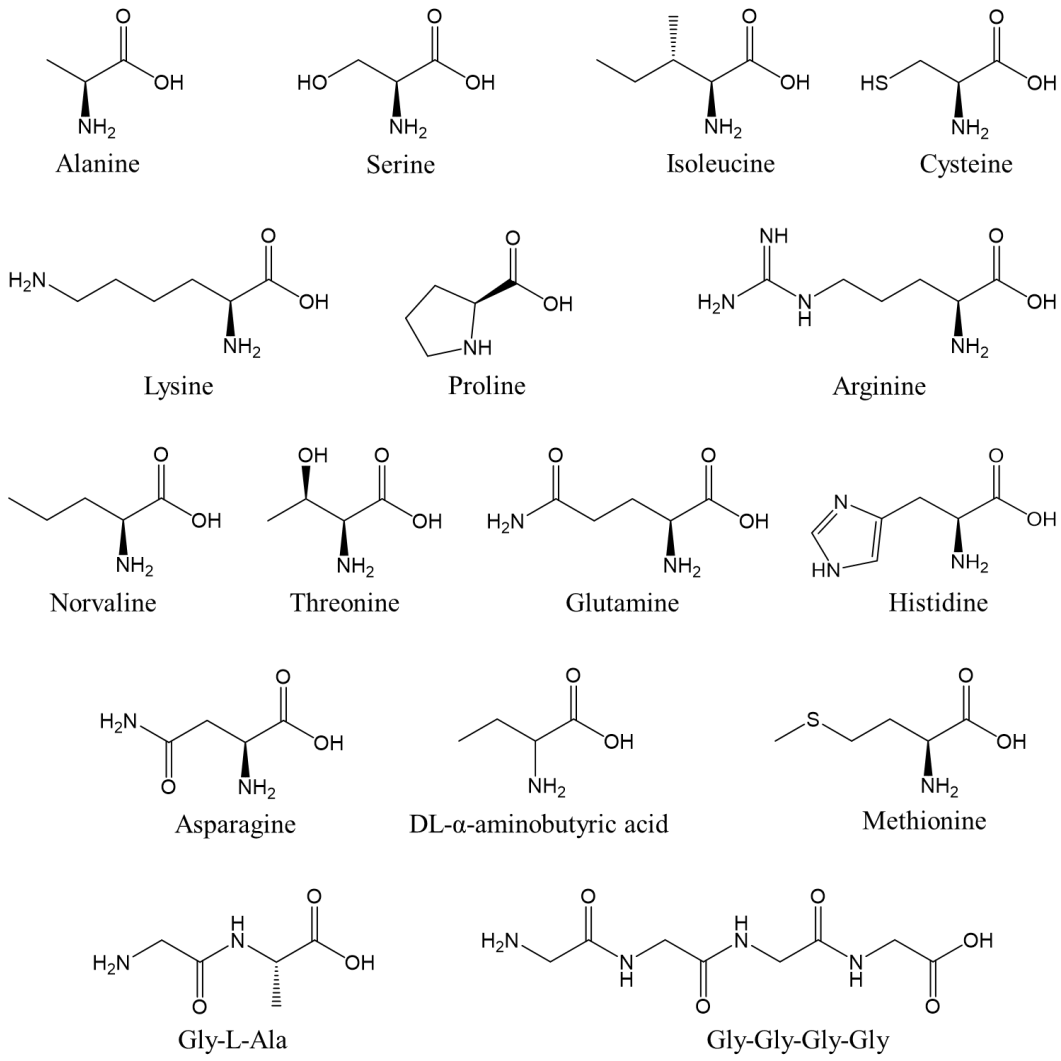

Figure A 1. The chemical structures of all amino acids and peptides listed in Figures in this work.

Appendix B – Chemical species distribution of amino acids and peptides

All the calculations were performed using the Software Chemicalize (ChemAxon Ltd, 1998-2022, https://chemicalize.com/#/).

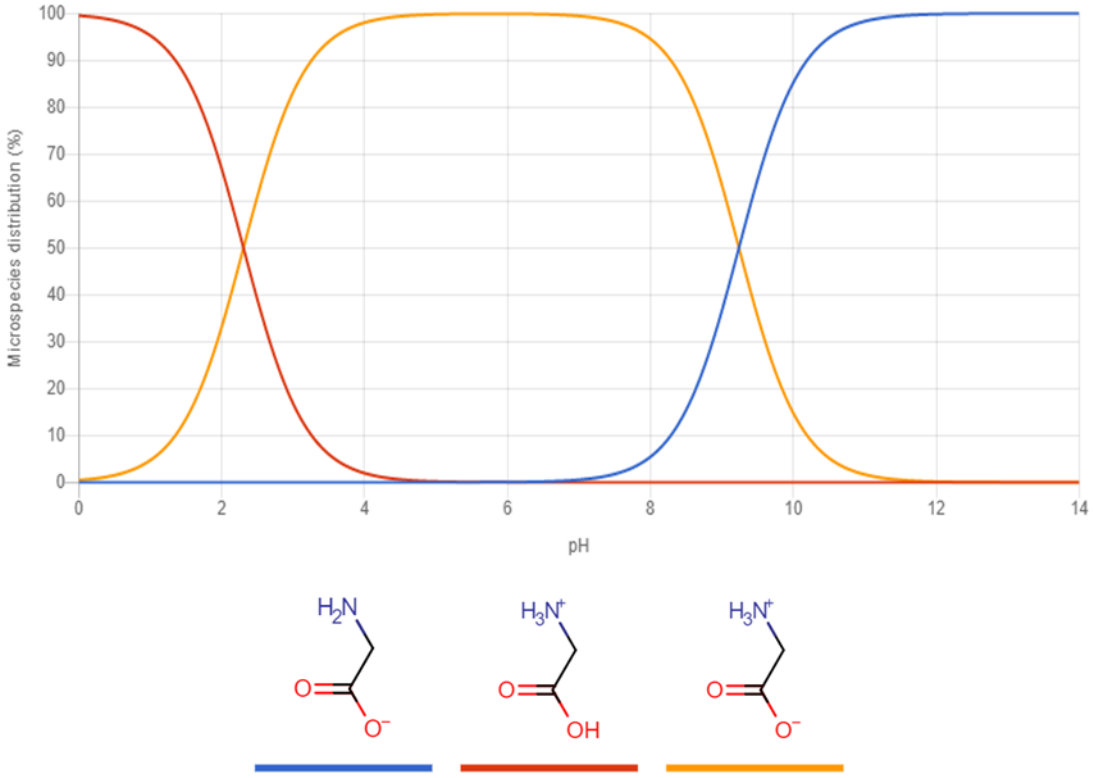

Figure B 1. Chemical species distribution as a function of pH of glycine.

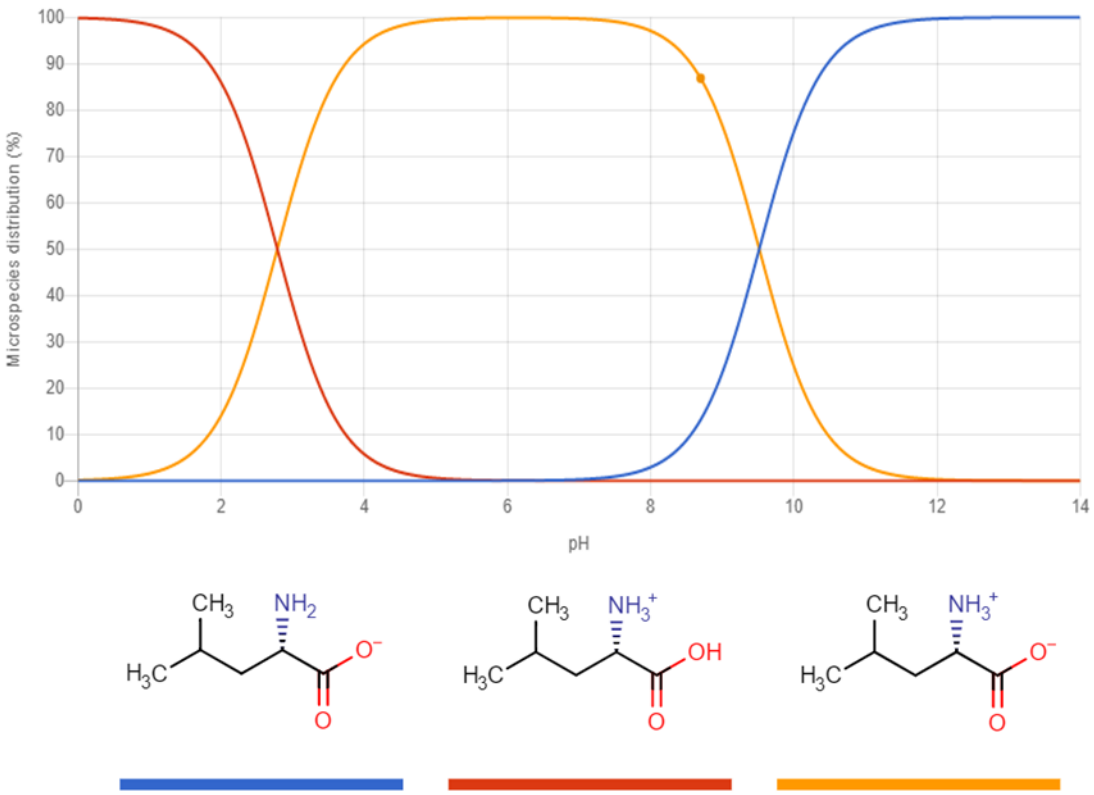

Figure B 2. Chemical species distribution as a function of pH of L-leucine.

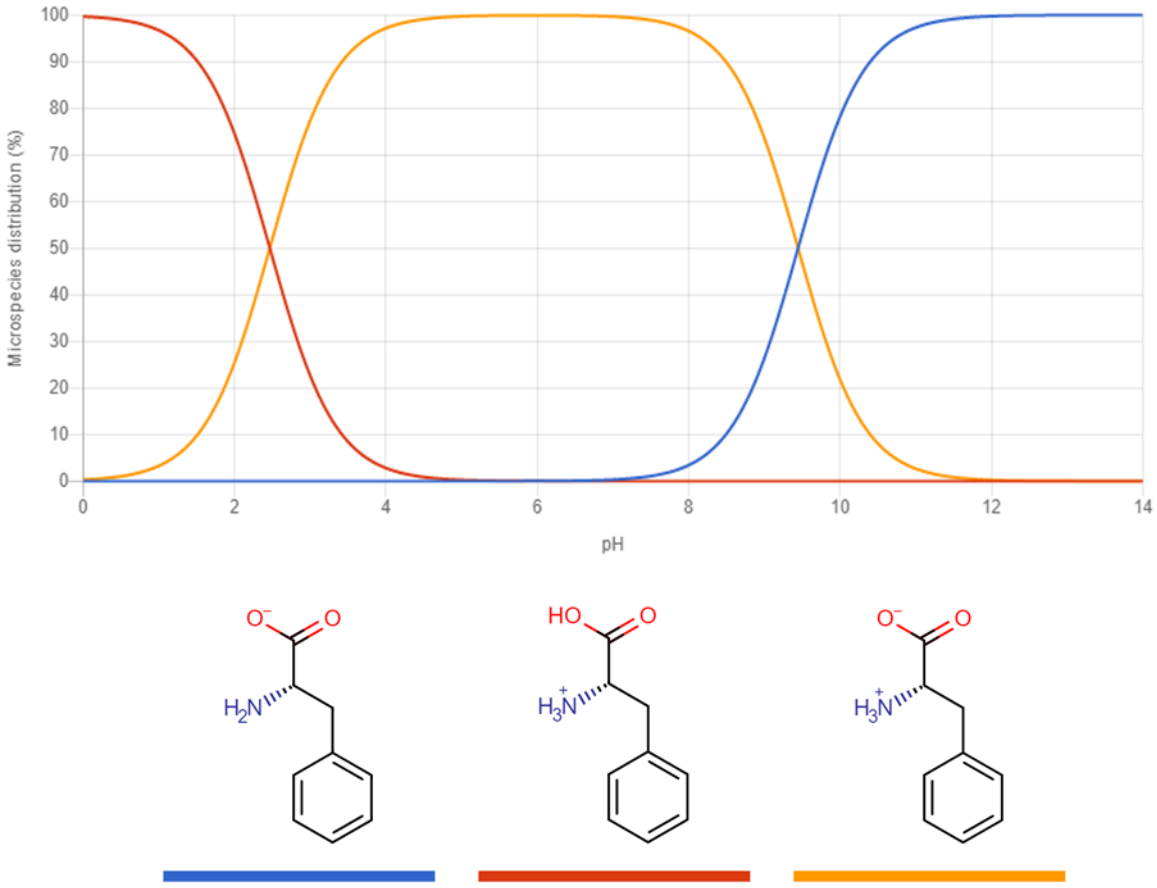

Figure B 3. Chemical species distribution as a function of pH of L-phenylalanine.

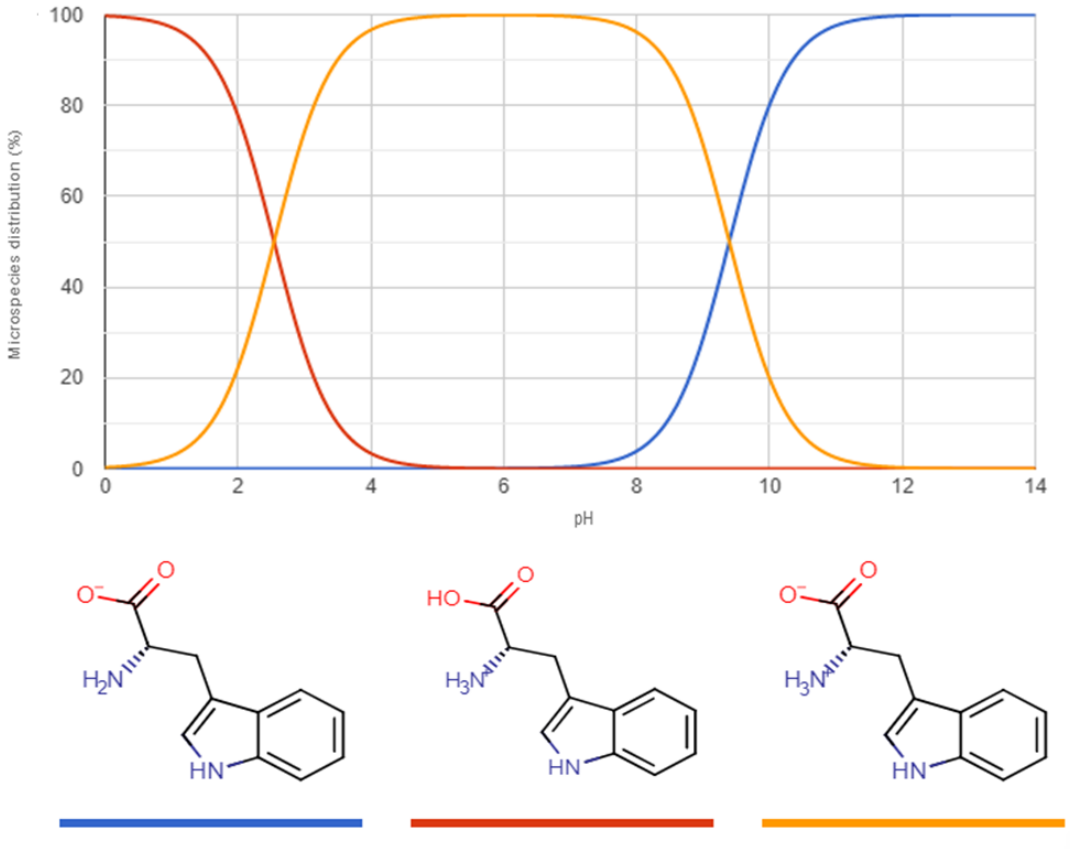

Figure B 4. Chemical species distribution as a function of pH of L-tryptophan.

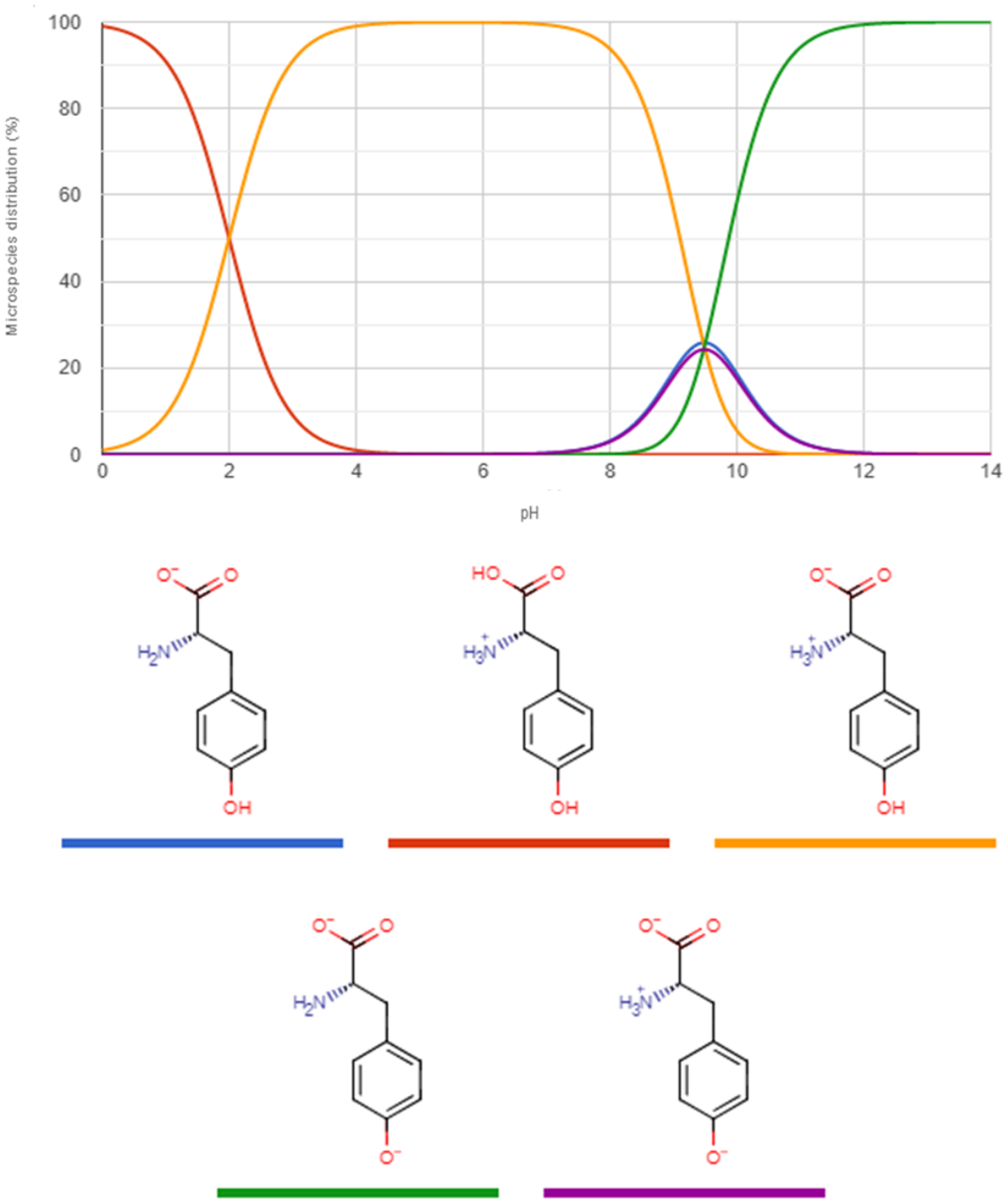

Figure B 5. Chemical species distribution as a function of pH of L-tyrosine.

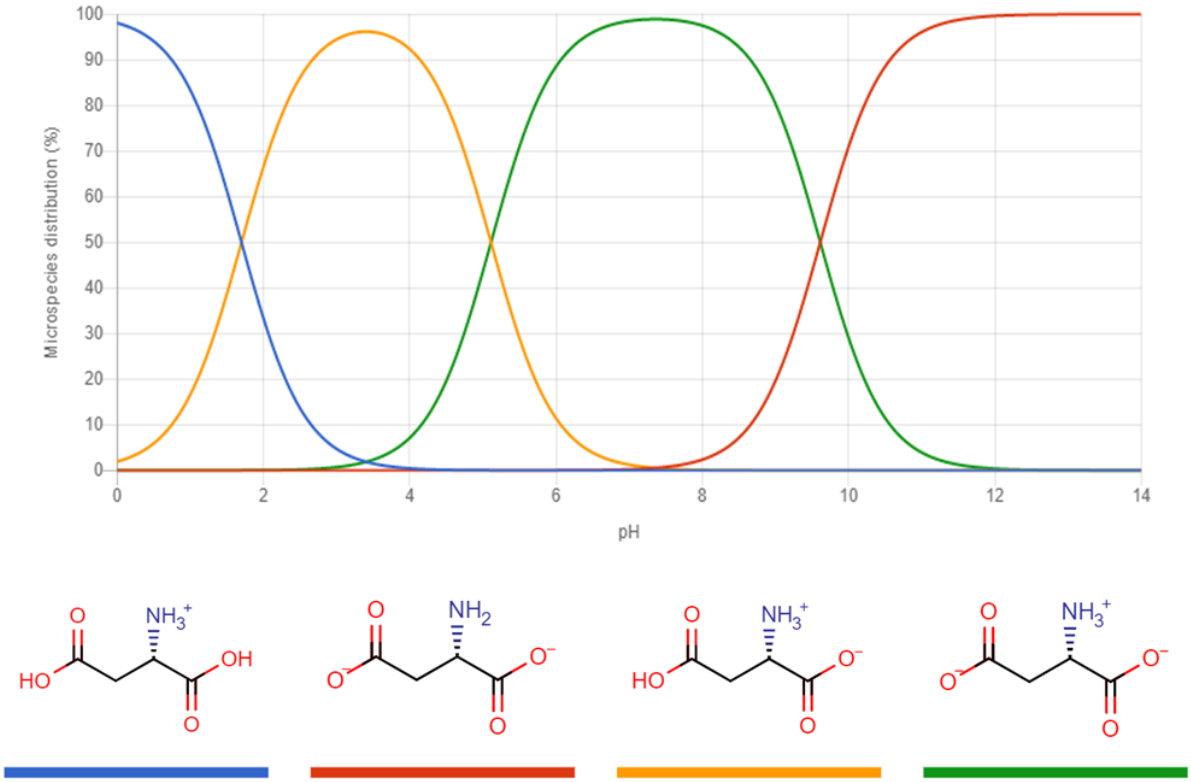

Figure B 6. Chemical species distribution as a function of pH of L-aspartic acid.

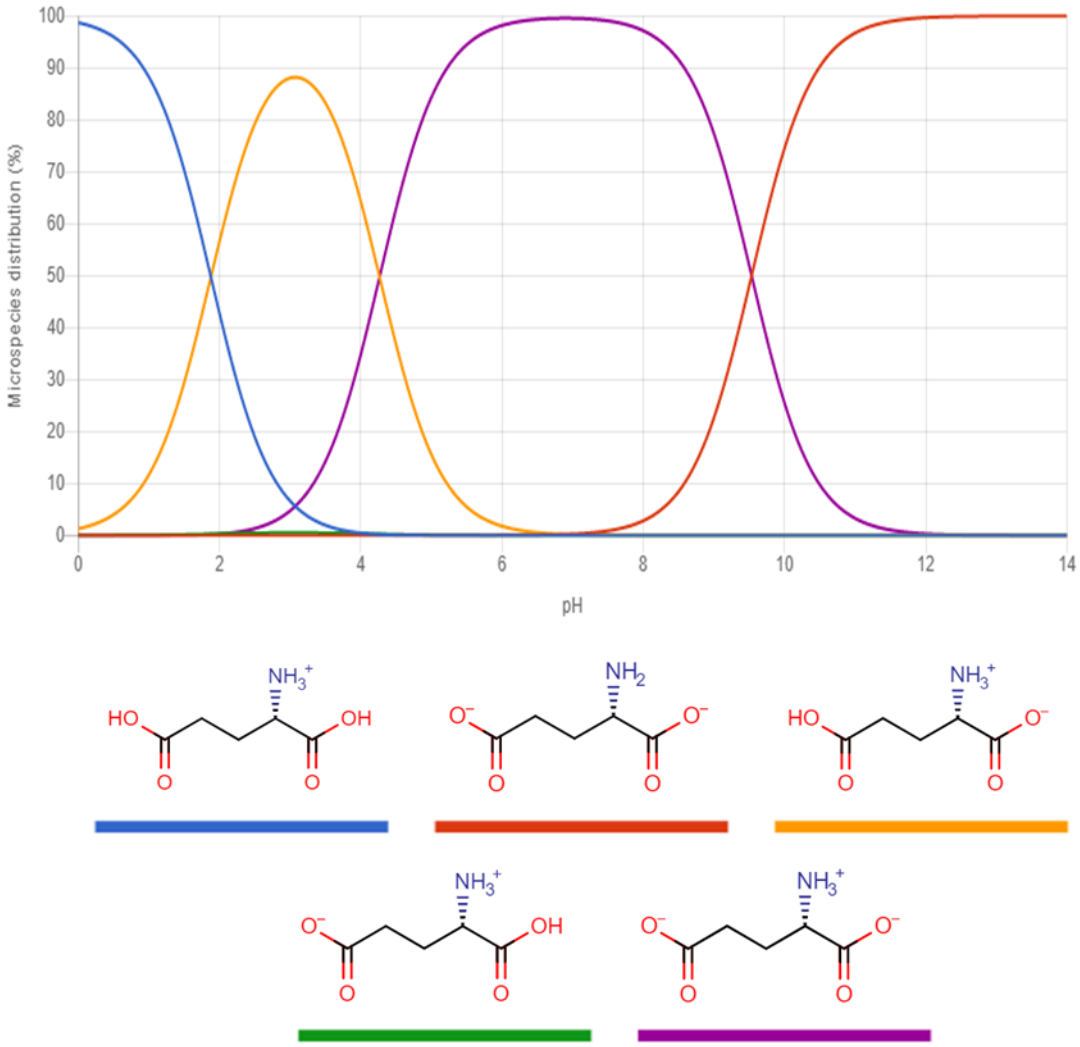

Figure B 7. Chemical species distribution as a function of pH of L-glutamic acid.

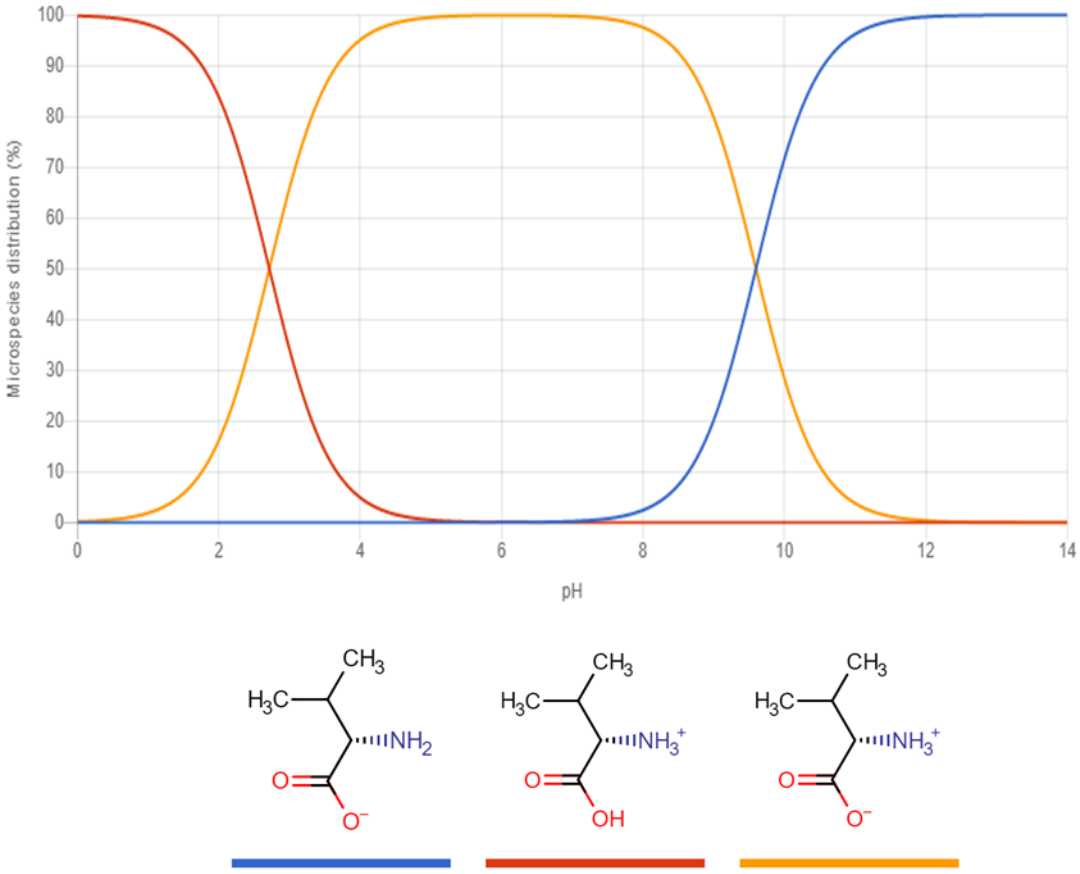

Figure B 8. Chemical species distribution as a function of pH of L-valine (DL-valine).

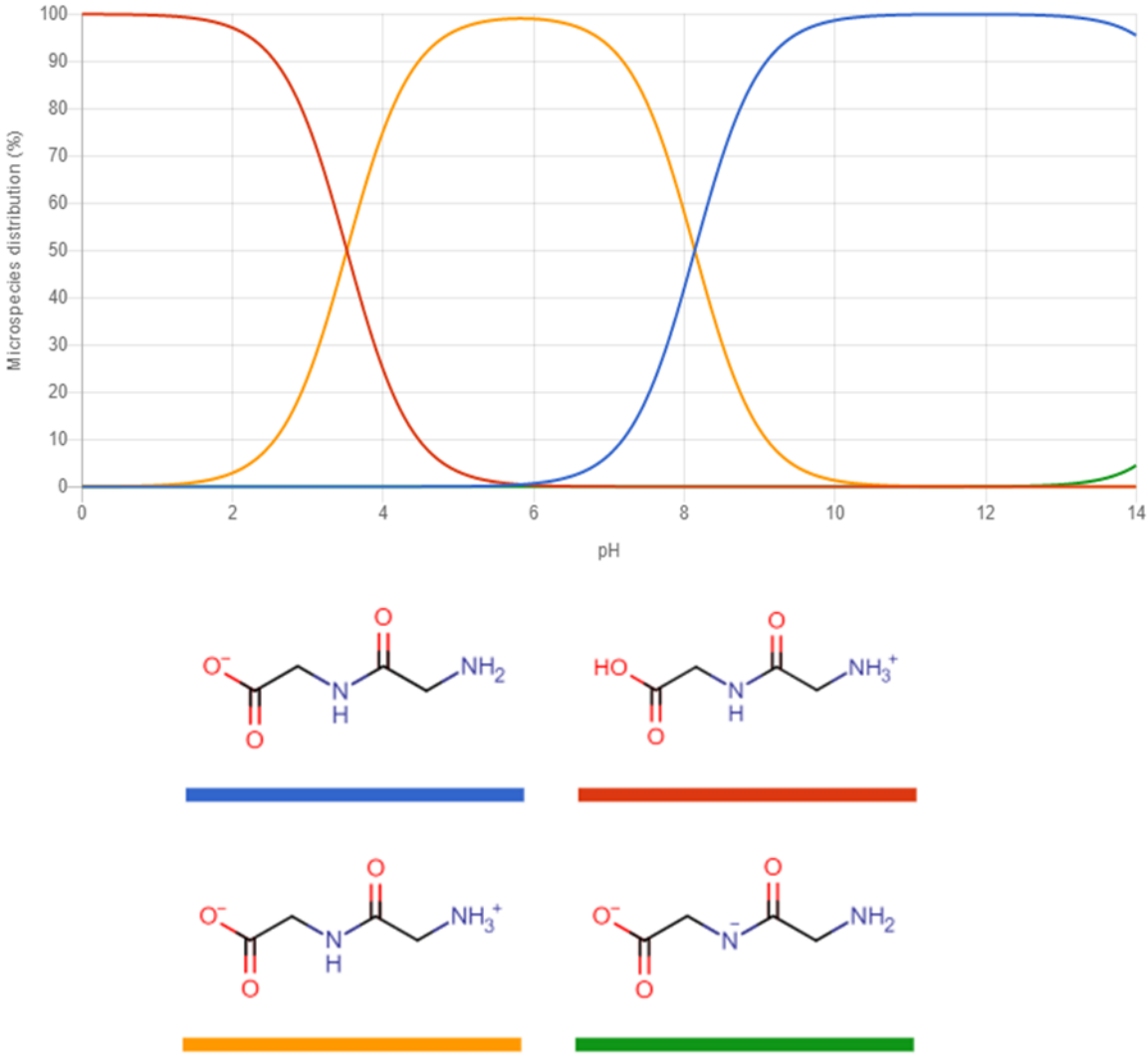

Figure B 9. Chemical species distribution as a function of pH of diglycine.

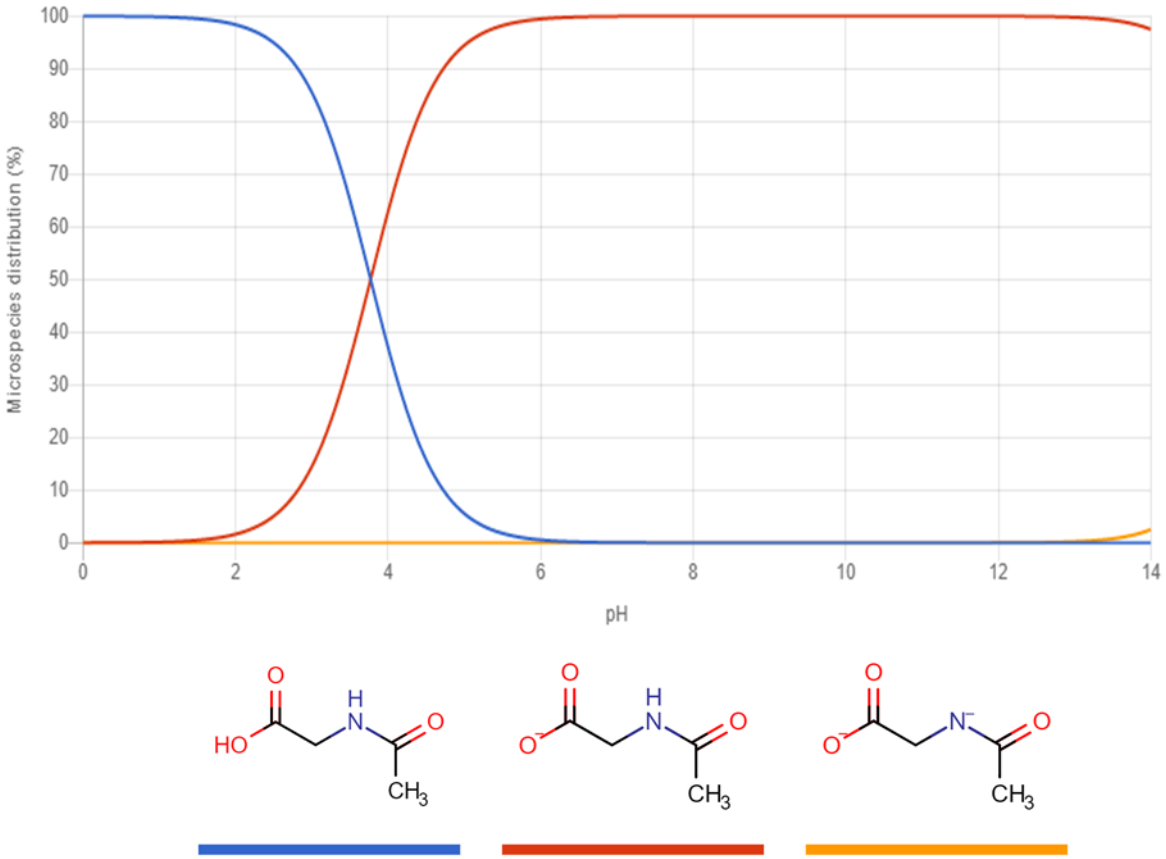

Figure B 10. Chemical species distribution as a function of pH of N-acetylglycine.

Appendix C – Solid phase studies

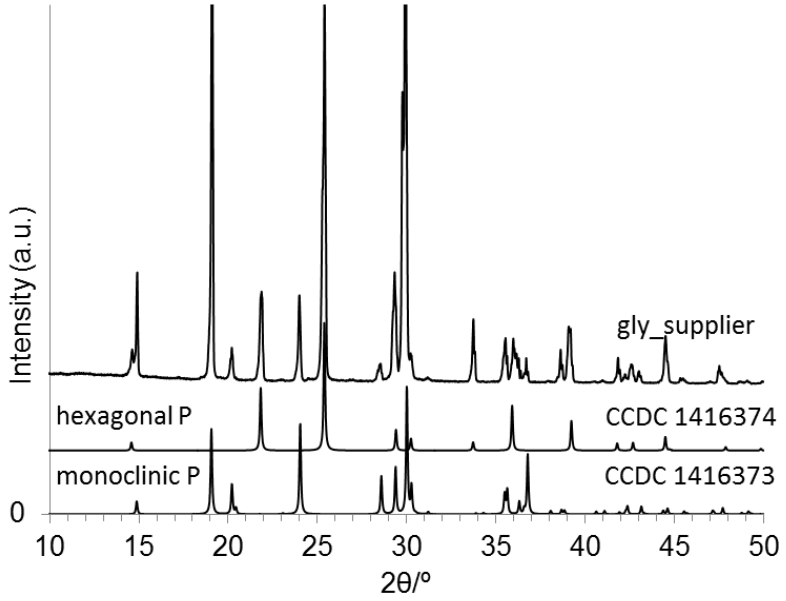

Figure C 1. Comparison of the experimental X–ray powder diffraction pattern of glycine from supplier with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 1416373 (α form) and CCDC 1416374 (γ form).

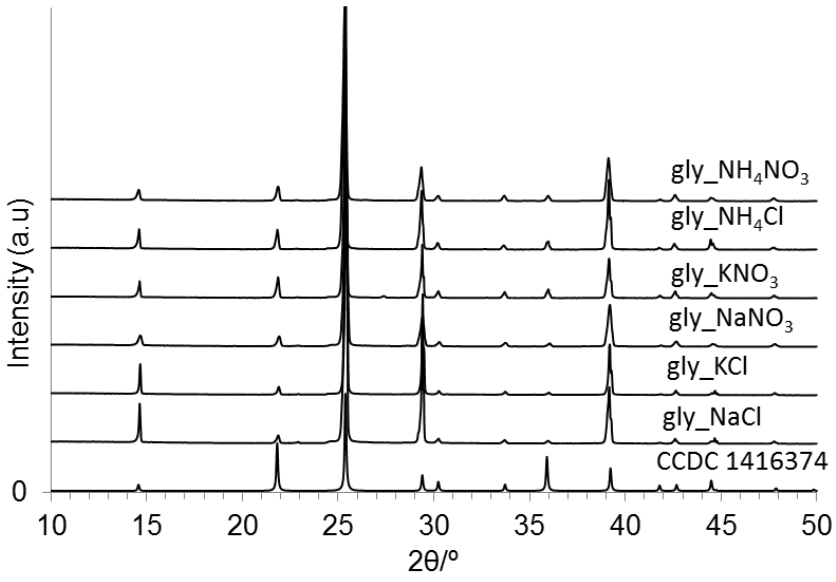

Figure C 2. Comparison of the experimental X–ray powder diffraction pattern of glycine obtained after crystallization with the different salts solutions with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 1416374 (γ-glycine).

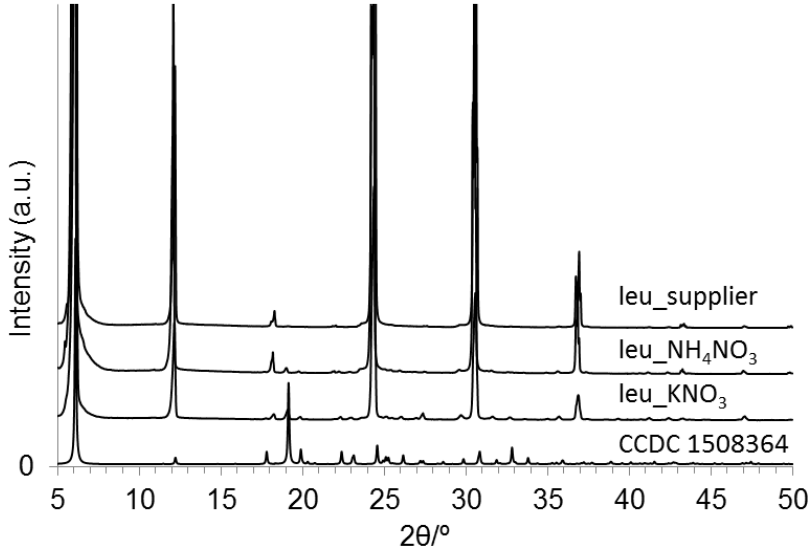

Figure C 3. Comparison of the experimental X–ray powder diffraction pattern of L-leucine from supplier and L-leucine samples obtained after crystallization with the different salts solutions with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 1508364.

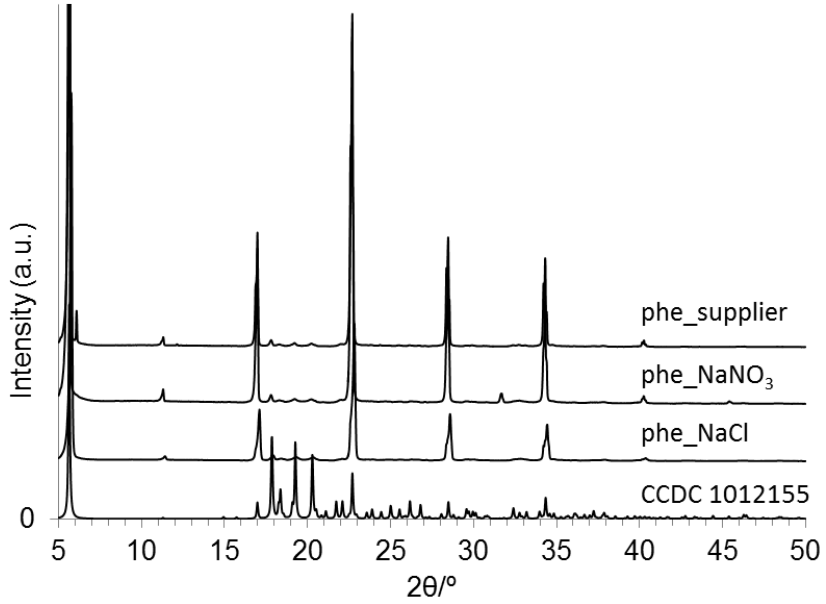

Figure C 4. Comparison of the experimental X–ray powder diffraction pattern of L-phenylalanine from supplier and L-phenylalanine samples obtained after crystallization with the NaCl and $NaNO_3$ salts solutions with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 1012155.

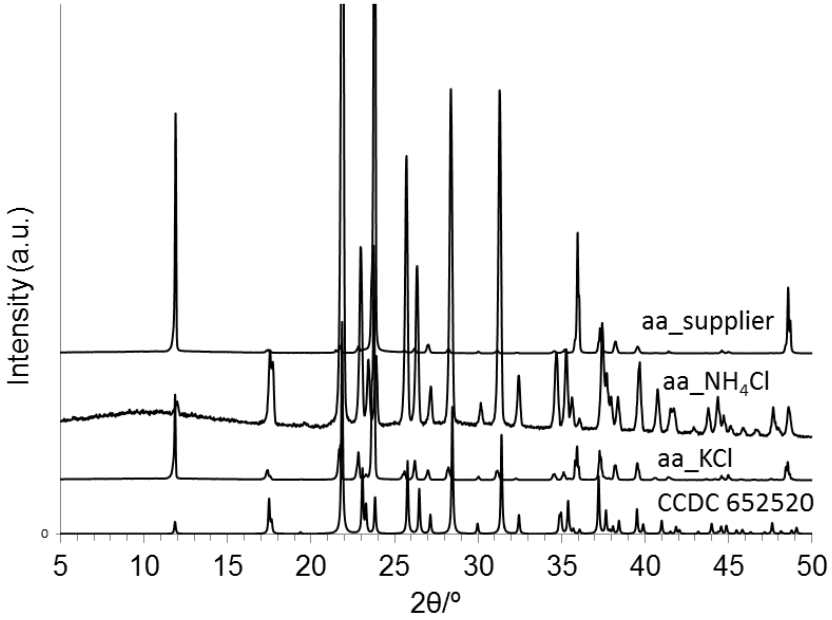

Figure C 5. Comparison of the experimental X–ray powder diffraction pattern of L-aspartic acid from supplier and L-aspartic acid samples obtained after crystallization with the KCl and NH_4Cl salts solutions with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 652520.

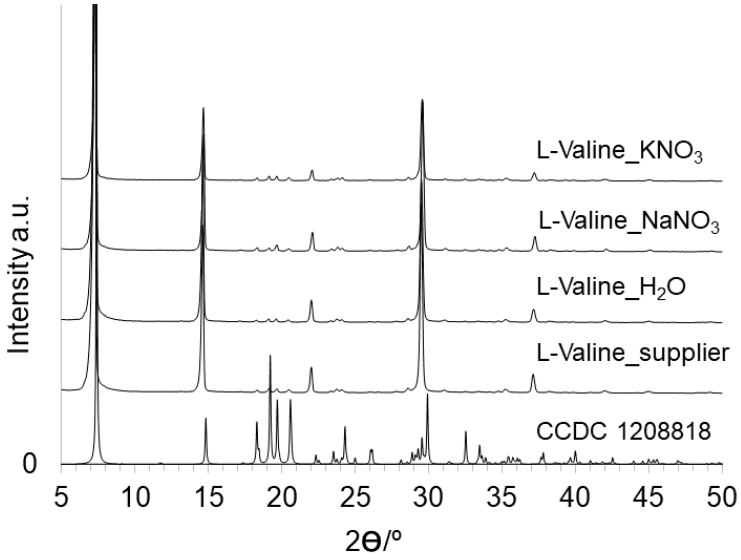

Figure C 6. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of L-valine (filtrated from aqueous solutions of $NaNO_3$ or KNO_3) with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 1208818.

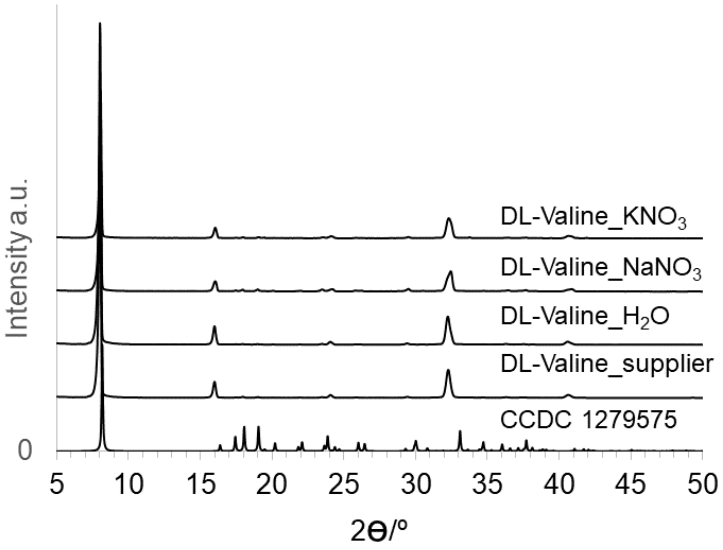

Figure C 7. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of DL-valine (filtrated from aqueous solutions of $NaNO_3$ or KNO_3) with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 1279575.

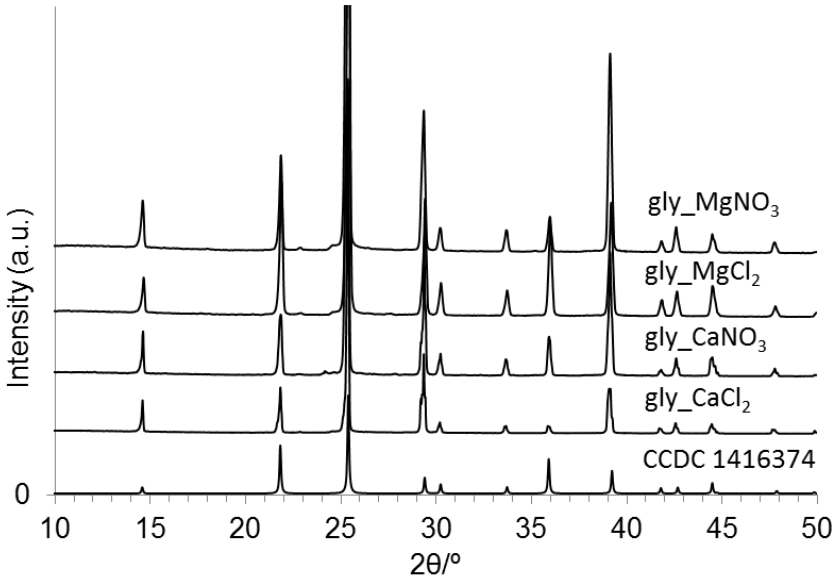

Figure C 8. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of glycine (from supplier and filtrated from aqueous solutions of chloride or nitrate salts of divalent (Mg^{2+} or Ca^{2+}) cations) with the powder pattern calculated from the single-crystal X-ray diffraction data 1416374 (γ form).

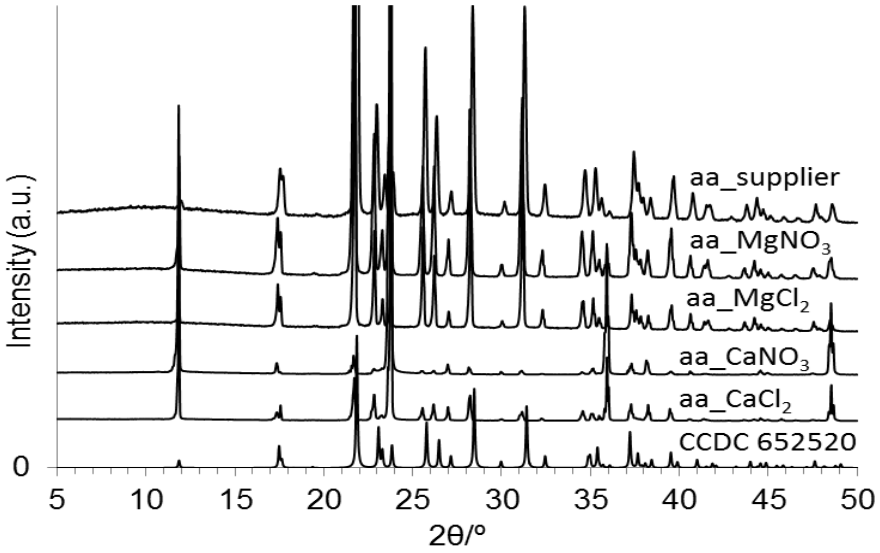

Figure C 9. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of aspartic acid (from supplier and filtrated from aqueous solutions of chloride or nitrate salts of divalent (Mg^{2+} or Ca^{2+}) cations) with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 652520.

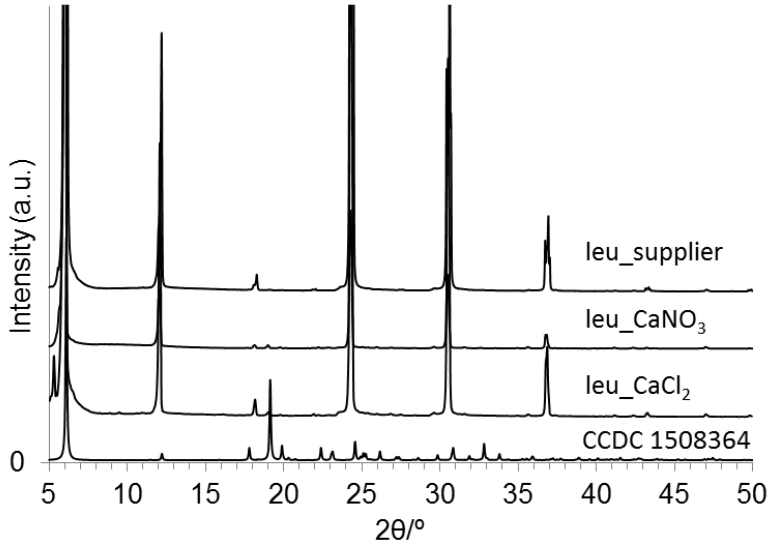

Figure C 10. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of leucine (from supplier and filtrated from aqueous solutions of calcium salts) with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 1508364.

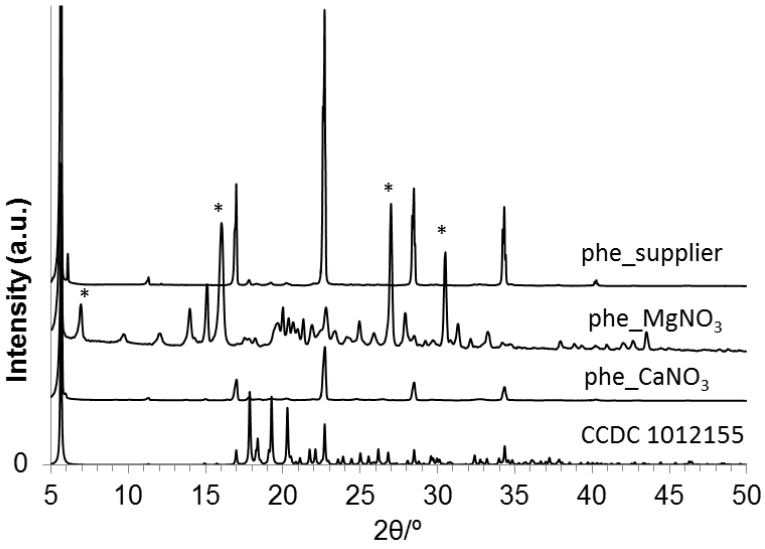

Figure C 11. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of phenylanine (from supplier and filtrated from aqueous solutions of nitrate salts of divalent (Mg^{2+} or Ca^{2+}) cations) with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 1012155 (the * denote a second phase present in the sample phe_$MgNO_3$, searching in ICDD database version 2022 was not possible to identified this second phase).

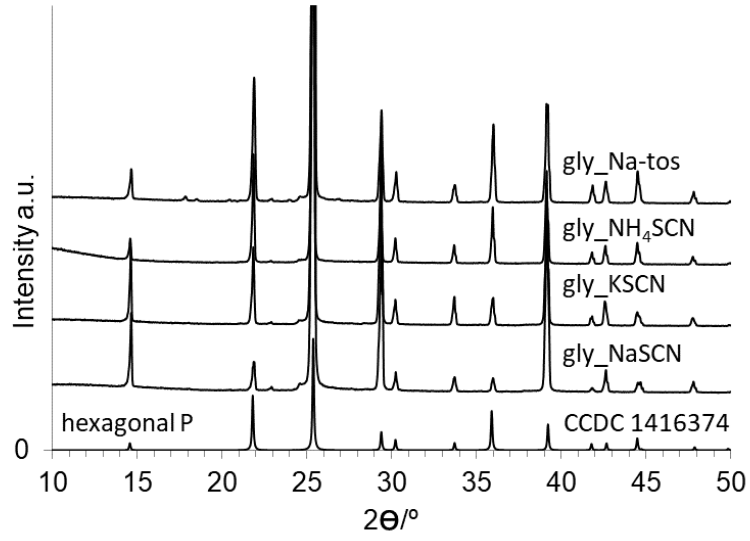

Figure C 12. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of glycine (filtrated from aqueous solutions of NaSCN, KSCN, and NH_4SCN salts or Na-tosylate) with the powder pattern calculated from the single-crystal X-ray diffraction data 1416374 (γ form).

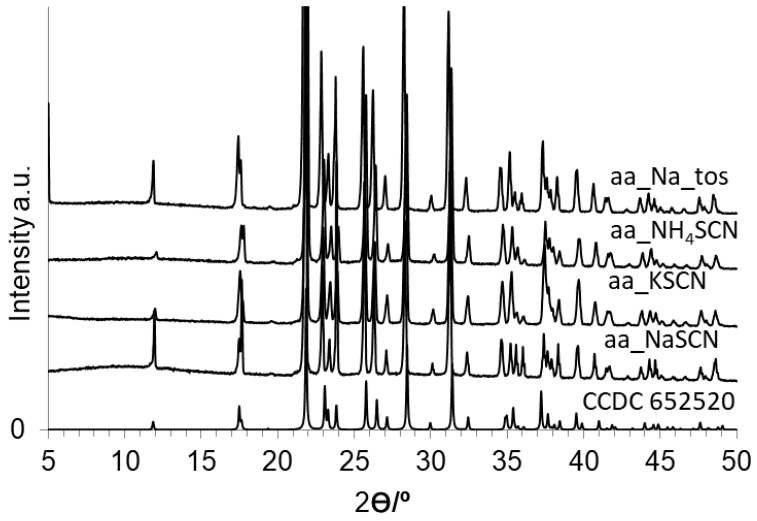

Figure C 13. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of aspartic acid (filtrated from aqueous solutions of NaSCN, KSCN, and NH_4SCN salts or Na-tosylate) with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 652520.

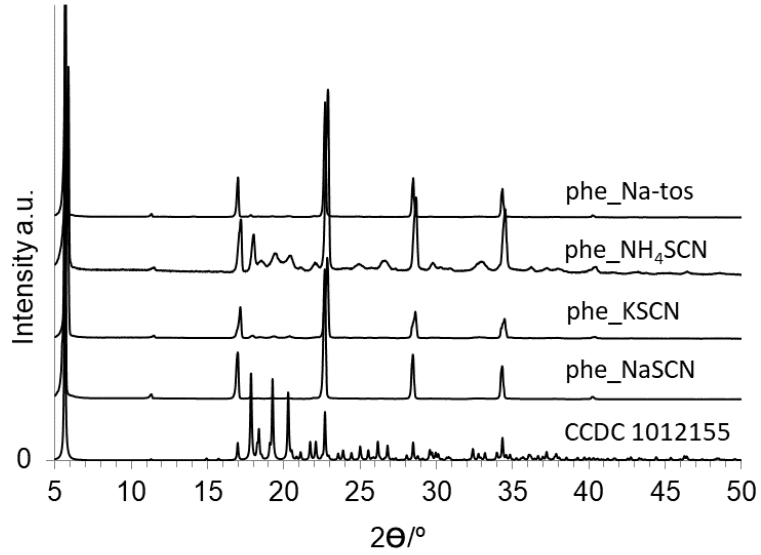

Figure C 14. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of phenylalanine (filtrated from aqueous solutions of NaSCN, KSCN, and NH_4SCN salts or Na-tosylate at 1.5 molal) with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 1012155.

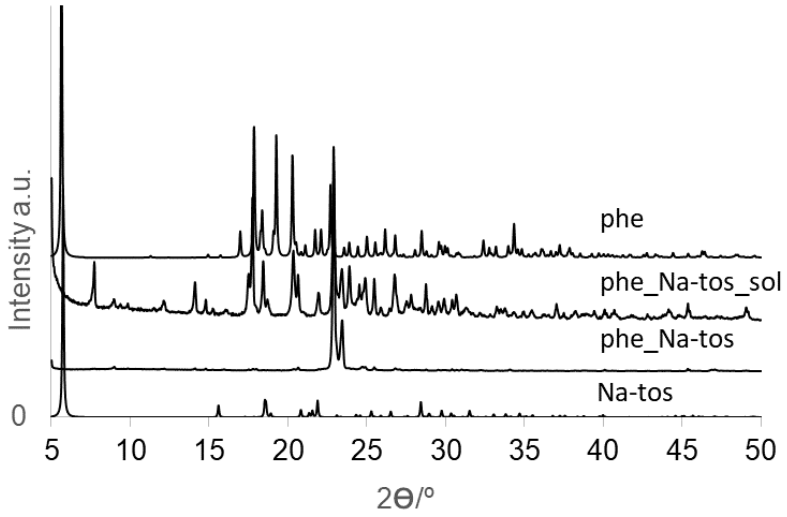

Figure C 15. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of phenylalanine (filtrated from aqueous solutions of Na-tosylate at 2 molal) with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 1012155.

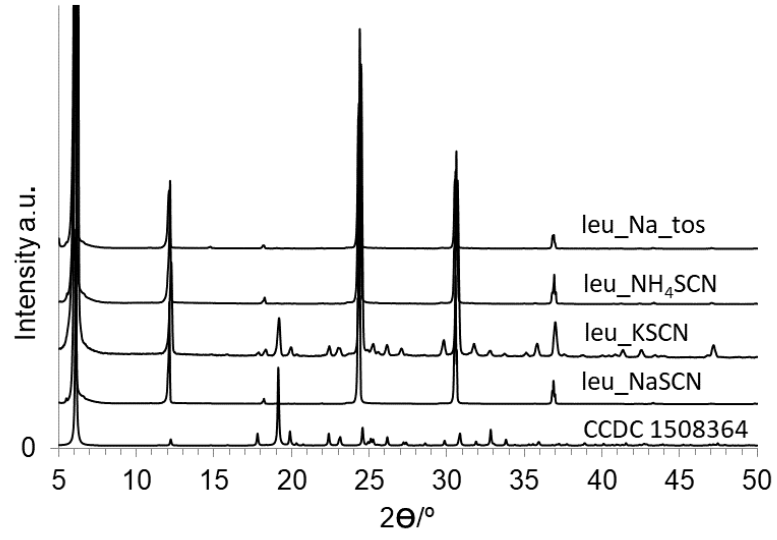

Figure C 16. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of leucine (filtrated from aqueous solutions of NaSCN, KSCN, and NH_4SCN salts or Na-tosylate) with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 1508364.

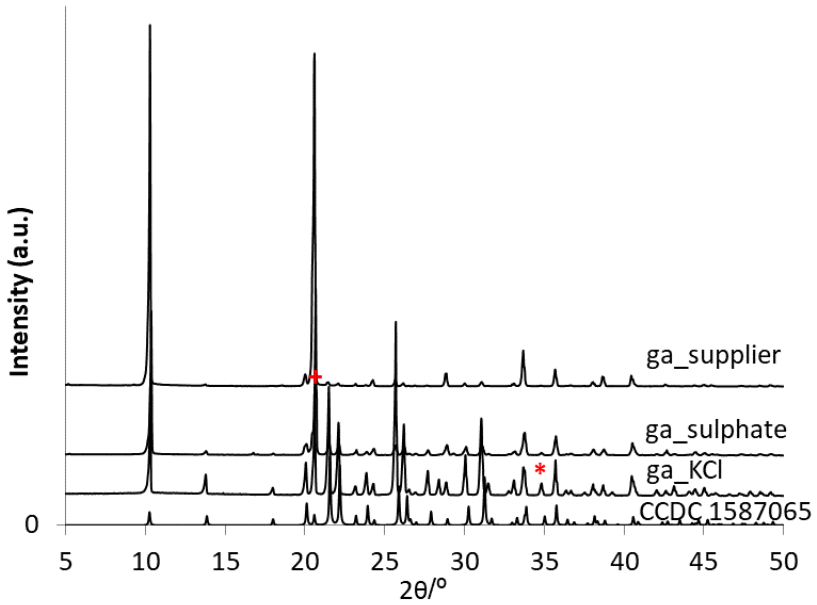

Figure C 17. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of L-glutamic acid (from supplier and filtrated from the ternary saturated solutions containing a 2 molal salt concentration) with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 1587065[165]. The symbols * and + locate the strong peaks of KCl (at around 35 2θ/º,[170]) and $NH_4(SO_4)_2$ (at around 21 2θ/º,[171]), respectively.

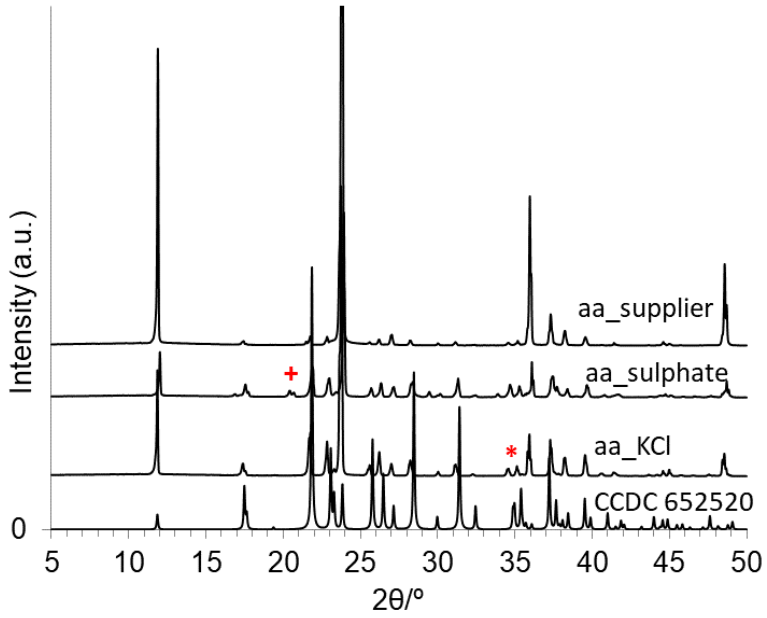

Figure C 18. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of L-aspartic acid (from supplier and filtrated from the ternary saturated solutions containing a 2 molal salt concentration) with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 652520[158]. The symbols * and + locate the strong peaks of KCl (at around 35 2θ/º,[170]) and $NH_4(SO_4)_2$ (at around 21 2θ/º,[171]), respectively.

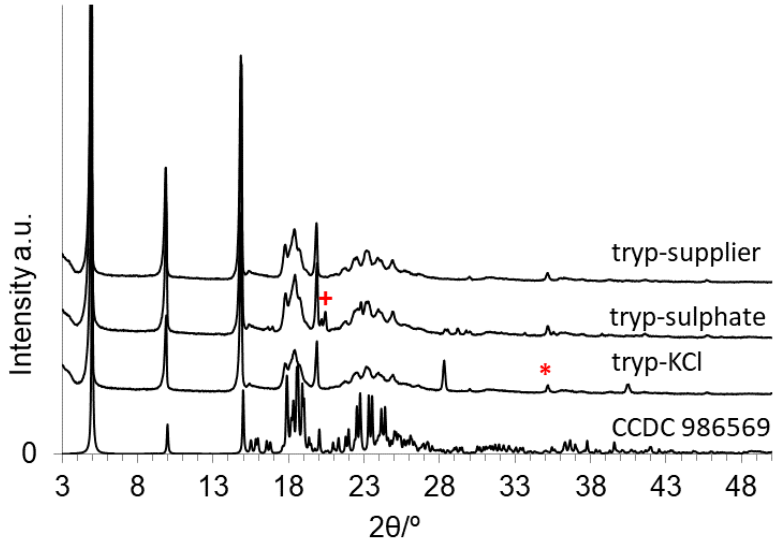

Figure C 19. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of L-tryptophan (from supplier and filtrated from the ternary saturated solutions containing a 2 molal salt concentration) with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 986569[166]. The symbols * and + locate the strong peaks of KCl (at around 35 2θ/º,[170]) and $NH_4(SO_4)_2$ (at around 21 2θ/º,[171]), respectively.

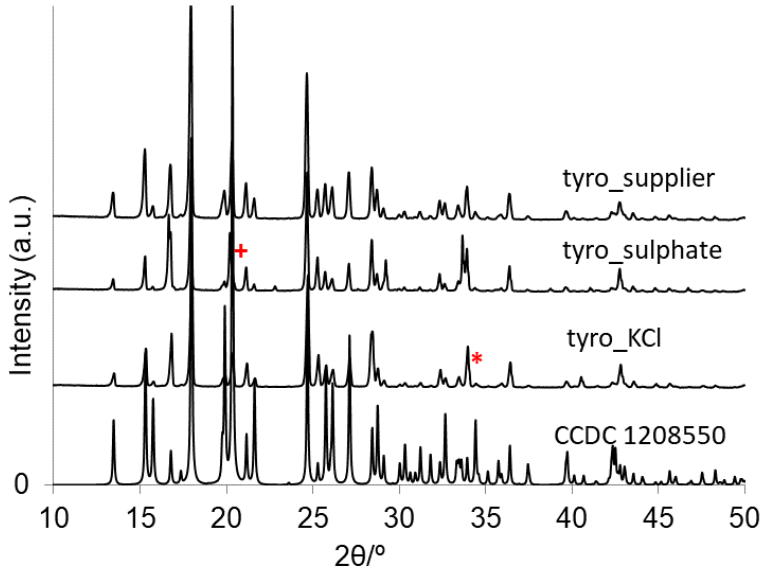

Figure C 20. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of L-tyrosine (from supplier and filtrated from the ternary saturated solutions containing a 2 molal salt concentration) with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 1208550[167]. The symbols * and + locate the strong peaks of KCl (at around 35 2θ/º,[170]) and $NH_4(SO_4)_2$ (at around 21 2θ/º,[171]), respectively.

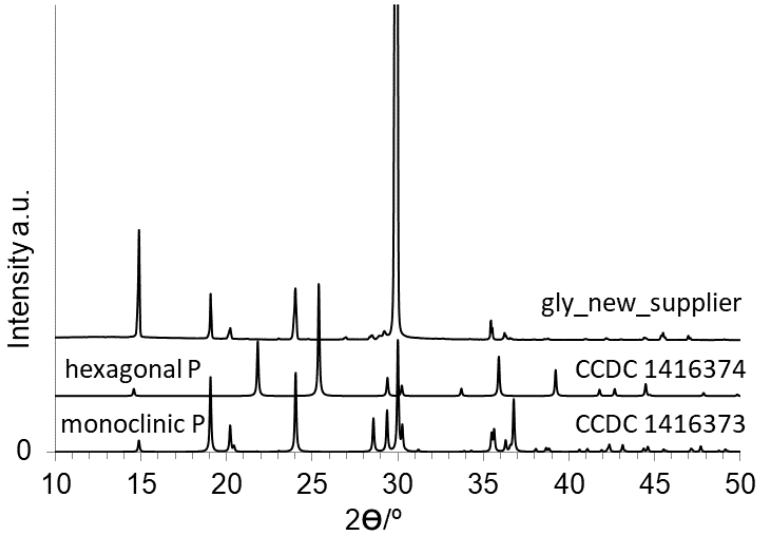

Figure C 21. Comparison of the experimental X–ray powder diffraction pattern of the solid phase samples of glycine (used in the analysis with Na-tosylate) from supplier with the powder pattern calculated from the single-crystal X-ray diffraction data CCDC 1416373 (α form) and CCDC 1416374 (γ form).

Appendix D – Calibration curve example

The following figure shows an example of a calibration curve obtained in the application of the refractive index measurement method.

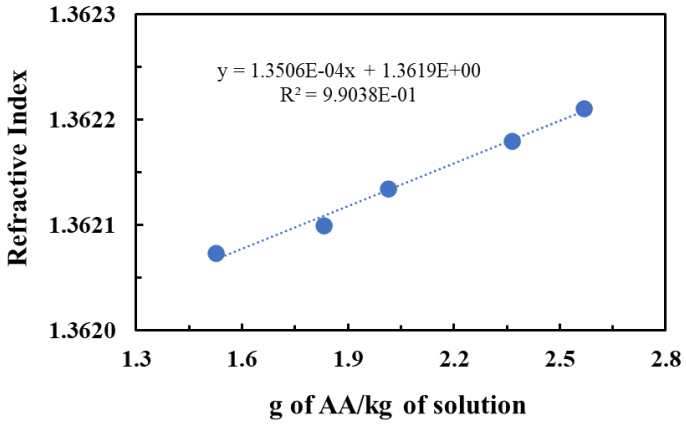

Figure D 1. Calibration curve of L-aspartic acid in aqueous Na-tosylate solution at 1 molal.

Appendix E – Effect of pH on the solubility of glycine and diglycine.

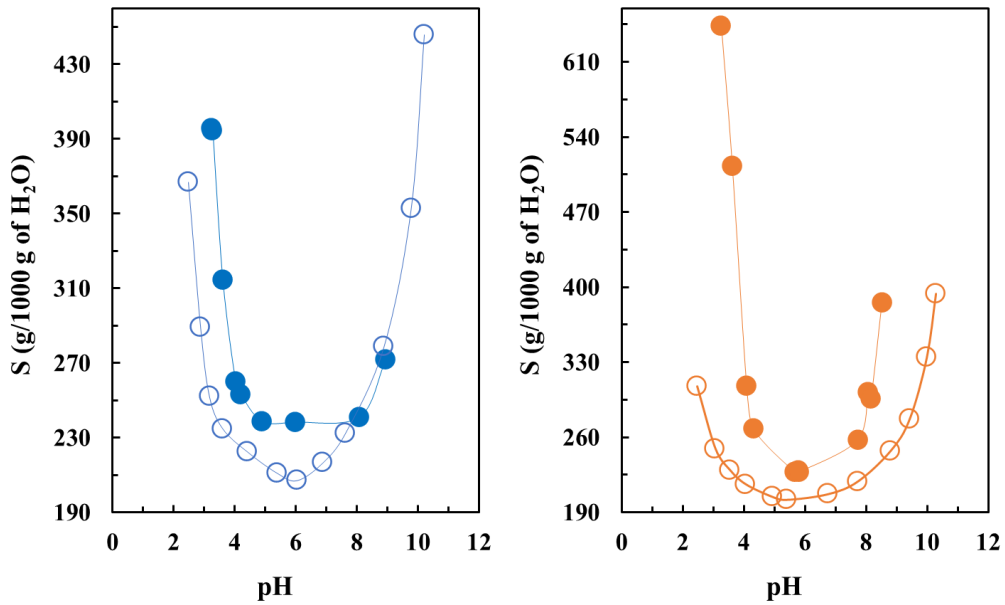

Figure E 1. Solubility of glycine: ●, (this work); ○,[43] and diglycine: ●, (this work); ○,[43] in water as a function of pH at 298.2 K.