Effects of Extraction Solvents on the Quantification of Free Amino Acids in Lyophilised Brewer's Yeast

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Abstract

The aim of this work was to test some solvents in order to improve the free amino acids extraction from lyophilised brewer's yeast. The brewer' yeast was treated with four types of extraction solvents: Solvent I – acetonitrile 25%/HCl 0.01M (ACN); Solvent II – ethanol 80%; solvent III – HCl 0.05M/deionized water (1/1 volume); Solvent IV – HCl 0.05M/ethanol 80% (1/1 volume). The supernatants were analysed by HPLC-DAD-ESI-MS method. Acetonitrile provided the less quantities and number of amino acids extracted due to its weaker polarity. Solvent II and IV (ethanol, respectively acidified ethanol), which have an increased polarity, extracted 15 amino acids due to the addition of HCl in solvent IV. Solvent III (acidified water) proved to be the best extraction solvent for the amino acids from brewer's yeast providing the separation of 17 compounds: GLN, ASN, SER, GLY, ALA, ORN, PRO, HIS, LYS, GLU, TRP, LEU, PHE, ILE, AAA, HPHE, TYR.

Keywords: amino acids, brewer yeast, HPLC-MS, solvent extraction polarity

Introduction

The brewing industry generates quite large amounts of by-products and wastes but the yeast is one of the most common. The yeast is used by the brewer several times and taken from a fermentation to begin the next one. Nevertheless, brewer's *Saccharomyces* yeast biomass is the second major by-product from brewing industry (after brewer spent grain). Its use is still limited, basically in animal nutrition. It can be of value as a raw material with different uses. Several attempts have been made to use it in biotechnological processes, as for example in fermentative processes for the production of value-added compounds such as ethanol; as substrate for microorganisms' cultivation, or simply raw material for extraction of compounds (Ferreira et al., 2010; Wang and Chen, 2009). The yeast biomass has been successfully used as biosorbent for removal of Ag, Au, Cd, Co, Cr, Cu, Ni, Pb, U, Th and Zn from aqueous solution. Most of yeasts can bond a wide range of metal ions or be strictly specific in respect of only one metal ion. A number of literatures have proved that *S. cerevisiae* can remove toxic metals, recover precious metals and clean radionuclides from aqueous solutions to various extents. Metal uptake by non-living cells is mainly a passive biosorption and consists in an adsorption of metal ions to the

cells surface by interactions between metal and functional groups displayed on the surface of the cells (Özer and Özer 2003; Zouboulis et al., 1999; Mahmood, 2010; Malik 2004).

Yeast contains about 50-55% protein, has higher protein-carbohydrate ratio than forages, a good balance of amino acids and is rich in β–complex vitamins. Protein is made up of amino acids and nutritional yeast has 18 amino acids including all of essential amino acids (Walker 1998). Due to the chemical composition and good digestibility, brewer's yeast is still used as nutritional supplement in animal and human feed (Fărcaș et al., 2015). Brewer's yeast is an excellent source of high-quality protein comparable in value to soy protein. Approximately 40 percent of the weight of dried brewer's yeast consists of protein that includes all the essential amino acids. Plus, like soybeans, brewer's yeast is rich in lysine, which makes both of these foods excellent supplements to cereals, whose proteins are generally low in lysine (Barnett, 2003). After consumption of yeast supplement, free amino acids are rapidly absorbed, while proteins are easily hydrolysed into peptides and amino acids, which in turn are also absorbed. The most common method to analyse free amino acids in food matrices has been reverse phase-high performance chromatography (RP-HPLC) with a precolumn derivatization step (Jimenez-Martin et al., 2012). Also, a very used method is gas chromatography coupled with mass spectrometry (GC-MS), when the sample amounts are limited and a good sensitivity is necessary. The GC method has higher resolution and speed of analysis compared with HPLC method (Silva et al., 2003).

A previous extraction of free amino acids is required before their derivatization and further analysis. The solvents for free amino acids extraction involve the use of HCl diluted in water or ethanol (Silva et al., 2003; Perez-Palacios et al., 2010; Duncan and Poljak 1998). Other solvents for the extraction of free amino acids have been described in literature, such as ethanol (Mustafa et al., 2007), mixtures of water/acetonitrile (50:50,v/v) (Gokmen, Serpen, and Mogol, 2012) or 0.1% (v/v) formic acid in 20% (v/v) methanol (Nimbalkar et al. 2012). The homogenization step is essential for the amino acids better extraction such as stirring (Silva et al. 2003), ultraturrax (Erkan and Özden, 2007), stomacher (Jimenez-Martin et al., 2012), omnimixer (Perez-Palacios et al., 2010), rotary mixer at 50 °C (Mustafa et al., 2007), vortex (Nimbalkar et al., 2012), and a heating block at 40 °C with stirring (Becalski et al., 2004). Then, centrifugation is usually carried out, followed by the collection of the supernatant and its filtration through glass wool (Jimenez-Martin et al., 2012; Perez-Palacios et al., 2010), nylon membrane (Nimbalkar et al. 2012), or Whatman 42 paper (Erbas et al., 2005).

The aim of this study was to test the extraction capacity of brewer's yeast free amino acids using four specific extraction solvents.

Materials and methods

Saccharomices cerevisae biomass was supplied as a lyophilised by-product from industrial ethanol production. The biomass was pretreated in order to remove fine particles and to displace any metals already bound to the sorption sites. The waste biomass as washed with deionized water by stirring followed by centrifugation at 3000 rpm for 20 minutes. The supernatant was discarded and the pellet was kept for further analysis.

Amino acids extraction from brewer yeast pellet

The brewer' yeast pellet (1 g) washed with deionized water and dried was treated with 10 ml of four types of extraction solvents: Solvent I – acetonitrile 25% dissolved in HCl 0.01M (ACN); Solvent II – ethanol 80%; solvent III – mixture HCl 0.05M/deionized water (1/1 volume); Solvent IV – mixture of HCl 0.05M/ethanol 80% (1/1 volume). The samples were sonicated for 30 minutes and kept for 24 h for extraction. After centrifugation at 3000 rpm the supernatant was analysed by chromatographic method.

HPLC-DAD/-ESI-MS identification and quantification of amino acids extracted from brewer yeast

HPLC analysis was performed on a Agilent 1200 system equipped with a binary pump delivery system LC-20 AT (Prominence), a degasser DGU-20 A3 (Prominence), diode array SPD-M20 A, UV–VIS detector (DAD). Amino acids (100 μl) were identified using an EZ:Faast Kit for free amino acids, provided by Phenomenex (USA).

The EEZ-fast amino acid analysis procedure consists of a solid phase extraction step followed by a derivatization and a liquid/liquid extraction step. Derivatized samples are quickly analysed by liquid chromatography-mass spectrometry.

The solid phase extraction is performed via a sorbent packed tip that blinds amino acids while allowing interfering compounds to flow through. Amino acids on sorbent are then extruded into the sample vial and derivatized with reagent at room temperature in aqueous solution. Derivatized amino acids migrate to the organic layer for additional separation from interfering compounds. Organic layer is then removed, evaporated and redissolved in aqueous mobile phase on a LC/MS system. The mobile phase was a mixture of A: 10mM ammonium formate in water and B: 10mM ammonium formate in methanol. The column included in the kit was an EZ:faast AAA-MS column 250x3.0 mm. The gradient elution system started with 68% B, increased to 83% for 13 min, decreased to 68% B at 13.01 min and continued at that concentration till the 17 min.

In-line MS data were recorded by directing the LC flow to a Quadrupole 6110 mass spectrometer (Agilent Technologies, Chelmsford, MA) equipped with an ESI probe. Flow rate was 0.5 ml/min and the column temperature was maintained at 35 °C. The measurements were performed in the positive mode with an ion spray voltage of 3000 V, and a capillary temperature of 350 °C. Data were collected in full scan mode within the range 100 to 600 m/z. Identification of amino acids was carried out based on molecular mass determination, masses and occurrence of fragments, elution order and literature data reported previously.

For quantification of amino acids there were prepared aliquots of amino acids standard mixtures following the sample preparation by solid phase extraction and derivatization procedure described above. The concentrations of internal standard HARG (homoarginine) in calibrators and samples prepared for chromatographic analysis was 200 nmoles/ml. Calculations were performed by the Data Analysis portion of the software controlling the analytical instrument. Calculations and calibration are based on internal standard.

Statistical analysis

All measurements were performed in three replicates and the values were averaged and reported along with the standard deviation (±S.D). The effect of the extraction methods on the content of each detected amino acid was analysed by the Duncan's test for independent samples. For all 4 extraction solvents there were significant statistical differences ($P<0.05$) regarding the amount of extracted amino acids.

Results and discussions

HPLC-DAD/-ESI-MS identification and quantification of the free amino acids in different solvents

The extraction procedure utilized to quantitatively extract the physiological amino acids had to meet some criteria: all of the free amino acids had to be released from tissue cells; amino acids had to be soluble in the extracting solvent; the extracting solvent could not cause modification of amino acids; the extraction conditions had to minimize non-specific or specific binding of free amino acids to tissue or macromolecules as well as prevent enzymatic modifications of amino acids to occur. Water soluble organic solvents as 50% 2-propanol or 70% methanol, yielded reduced levels of physiological amino acids, probably due to the reduced solubility of many acids in high concentration of alcohols (Fish, 2012). Peak assignments of identified amino acids are shown in Table 1.

Most extraction methods for amino acids in food products involve using perchloric acid or hydrochloric acid (HCl) diluted in water or in ethanol (Perez-Palacios et al., 2010). Other solvents for the extraction of amino acids have also been described in the scientific literature, such as water/acetonitrile (50:50, v/v) (Pérez-Palacios et al., 2014) or 0.1% (v/v) formic acid in 20% (v/v) methanol (Nimbalkar et al., 2012).

The present study was aimed at improving the extraction conditions to provide an accurate assessment of free amino acids present in lyophilised brewer's yeast. The separation and identification of amino acids were performed on brewer yeast at pH=6 using four types of solvents for extraction. The results of the quantification of amino acids in different solvents are summarized in the Table 2.

Application of the chromatographic analysis described above to brewer's yeast samples demonstrated that the method adequately separated amino acids to allow their individual quantification. The effectiveness of each extractant varied with the amino acid chemical structure. Amino acids possessing both acidic and basic properties, within the same molecule, are dipolar compounds, with either zwitterionic species formation or anionic/cationic species, depending

Table 1. Amino acids identified in brewer yeast

Solvent	Peak assignment	RT [min]	MS fragment m/z	Amino acids identified
Solvent I (ACN)	10	3.5	275.1611	GLN
	16	4.7	204.1246	GLY
	24	7.2	347.2188	ORN
	31	9.4	361.2355	LYS
	32	9.5	370.2005	HIS
	34	10.0	318.1925	GLU
	40	12.0	260.1872	LEU
	42	12.7	260.1864	ILE
	48	14.6	308.1868	HPHE
	52	16.1	396.2036	TYR
Solvent II (Ethanol)	14	4.6	204.1237	GLY
	15	5.2	234.1127	SER
	17	5.8	218.1396	ALA
	21	7.1	347.2188	ORN
	26	8.3	244.1561	PRO
	28	9.3	361.2346	LYS
	29	9.4	370.1988	HIS
	30	9.7	246.1708	VAL
	31	10.0	318.1922	GLU
	32	10.3	333.1817	TRP
	36	12.0	260.1869	LEU
	37	12.2	294.1714	PHE
	38	12.6	260.1864	ILE
	42	14.5	308.1868	HPHE
	45	16.0	396.2038	TYR
Solvent III (Acidified water)	8	3.5	275.1613	GLN
	12	4.1	243.135	ASN
	14	4.6	204.1241	GLY
	15	4.9	234.112	SER
	18	5.8	218.1401	ALA
	22	7.8	347.2196	ORN
	23	8.3	244.157	PRO
	26	9.4	370.1997	HIS
	27	9.7	361.2346	LYS
	28	9.9	318.1935	GLU
	29	10.3	333.1817	TRP
	33	12.0	260.1872	LEU
	34	12.2	294.1714	PHE
	36	12.6	260.1865	ILE
	37	13.2	332.2075	AAA
	40	14.5	308.1867	HPHE
	43	16.0	396.2038	TYR
Solvent IV (Acidified ethanol)	11	4.1	243.1348	ASN
	13	4.6	204.124	GLY
	15	5.2	234.1128	SER
	17	5.8	218.1399	ALA
	21	7.8	347.2194	ORN
	22	8.3	244.1566	PRO
	24	9.3	361.2346	LYS
	25	9.7	246.171	VAL
	26	9.9	318.193	GLU
	27	10.3	333.1817	TRP
	30	11.9	260.1866	LEU
	32	12.6	260.1864	PHE
	33	13.2	332.2074	AAA
	36	14.5	308.1865	HPHE
	38	16.0	396.204	TYR

Table 2. Free amino acids quantification in brewer's yeast using different extraction solvents (nmol/ml)

Solvent / Amino acid	Solvent I	Solvent II	Solvent III	Solvent IV
Serine (SER)	-	211.96^{d} ±1.86	260.16^{f} ±1.97	248.62^{f} ±1.28
Glycine (GLY)	45.94^{h} ±1.21	69.36^{i} ±1.39	483.92^{c} ±2.06	136.09^{h} ±1.95
Glutamic acid (GLU)	97.94^{f} ±1.95	91.56^{g} ±1.40	202.58^{f} ±2.46	794.38^{c} ±1.71
AAA (α-Aminoadipic acid)	-	-	36.84^{n} ±0.60	25.50^{o} ±0.58
Leucine (LEU)	385.48^{a} ±1.50	171.44^{e} ±1.32	322.41^{e} ±1.73	150.63^{g} ±0.80
Isoleucine (ILE)	201.05^{d} ±1.80	77.47^{h} ±0.81	87.29^{k} ±0.73	-
Ornithine (ORN)	97.84^{f} ±1.18	10.37^{n} ±0.38	400.21^{d} ±1.12	80.17^{i} ±0.72
Proline (PRO)	-	855.53^{a} ±2.43	1203.61^{a} ±2.16	959.96^{a} ±1.95
Lysine (LYS)	51.94^{k} ±1.51	38.51^{l} ±0.87	64.61^{m} ±1.13	54.09^{k} ±1.11
Histidine (HYS)	215.39^{c} ±1.39	27.26^{m} ±0.61	181.38^{h} ±1.38	-
Phenylalanine (PHE)	-	115.55^{f} ±0.87	191.26^{g} ±1.87	72.40^{j} ±1.17
Homophenylalanine (HPHE)	92.97^{g} ±0.98	40.15^{l} ±0.62	106.90^{j} ±0.93	34.94^{n} ±0.85
Tyrosine (TYR)	60.56^{e} ±0.80	-	819.61^{b} ±0.96	839.34^{b} ±1.61
Glutamine (GLN)	24.69^{i} ±0.39	-	14.05^{o} ±0.45	-
Asparagine (ASN)	-	-	110.56^{i} ±0.97	466.18^{d} ±1.43
Tryptophan (TRP)	-	42.20^{k} ±1.11	70.59^{l} ±0.62	44.40^{m} ±0.68
Alanine (ALA)	-	347.42^{c} ±0.55	838.01^{b} ±1.96	459.16^{e} ±0.84
Valine (VAL)	-	67.22^{j} ±0.86	-	195.38^{f} ±1.78

Note: values followed by the same letters not significantly differ at P≤0.05, according to Duncan's test.

on the existing solvent. Since the charged form of amino acid would be dominant, it was assumed that these compounds exhibit a high solubility in water and relatively insolubility in non-aqueous system, due to the inability of the solvent to interact with charged molecules.

Variation in solubility characteristics of amino acids can be attributed to differences in their chemical structure. Glycine could be considered the basic common structural unit in each amino acid and could be used as standard in comparing solubility differences of the other amino acids. It was demonstrated that an increase in the number of carbon atoms decreases solubility and the total amino acid solubility is dependent on both the polar and nonpolar parts of the molecule (Nozaki and Tanford, 1971).

The addition of a semi-polar liquid to the aqueous solvent to form a binary solvent system would be expected to cause a change in the solubility behaviour of the amino acid by providing an environment of continuous decreasing polarity. The polarity of the system is changed by increasing the alcoholic content. The majority of α-amino acids are less soluble in ethanol, ethanol/water system than water and as the side chains lengthens the solubility in ethanolic solution increases. The relative differences in solubility between amino acids are due to the effect of the non-polar portion of the molecule (Orella and Kirwan, 1991). The addition of acid or base to an amino acid in aqueous solution at the point of neutral solubility produces a change in the species present. It would seem that at the low concentration of acid added in solution, the chloride ions would provide the major attractive force by the predominantly charged amino acids (Pinho, Silva, and Macedo, 1994). The chloride or sodium ions which are added to different solvent systems can be attracted to the dipolar ions in the form of cations or anions to form the amino acid salts or can form associations with the hydrogen

or hydroxyl group ion with which were added. This increased attraction, as the polarity of the solvent system is reduced, would seem to explain the greater efficiency of salt formation. The increase in total solubility of amino acids could be attributed to the salt formation. As the non-polar portion of the amino acid molecule increases, the anion or cation species are more readily available to salt formation, leading to the increasing of the amino acid solubility.

Our experimental results are in line with the previous researches. The solvent III and IV (mixture HCl 0.05M/deionized water, respectively HCl 0.05M/ethanol 80%) provided the greater number of free amino acids extracted from brewer's yeast and their profiles are in accordance with their solubility in the solvents due to the chemical structure.

The chromatographic profile of amino acids detected in brewer's yeast using solvent I for extraction are shown in Fig.1 and there were separated 10 compounds: glutamine, glycine, ornithine, lysine, histidine, glutamic acid, leucine, isoleucine, homophenylalanine and tyrosine. This was the less polar solvent used leading to a weak amino acids extraction.

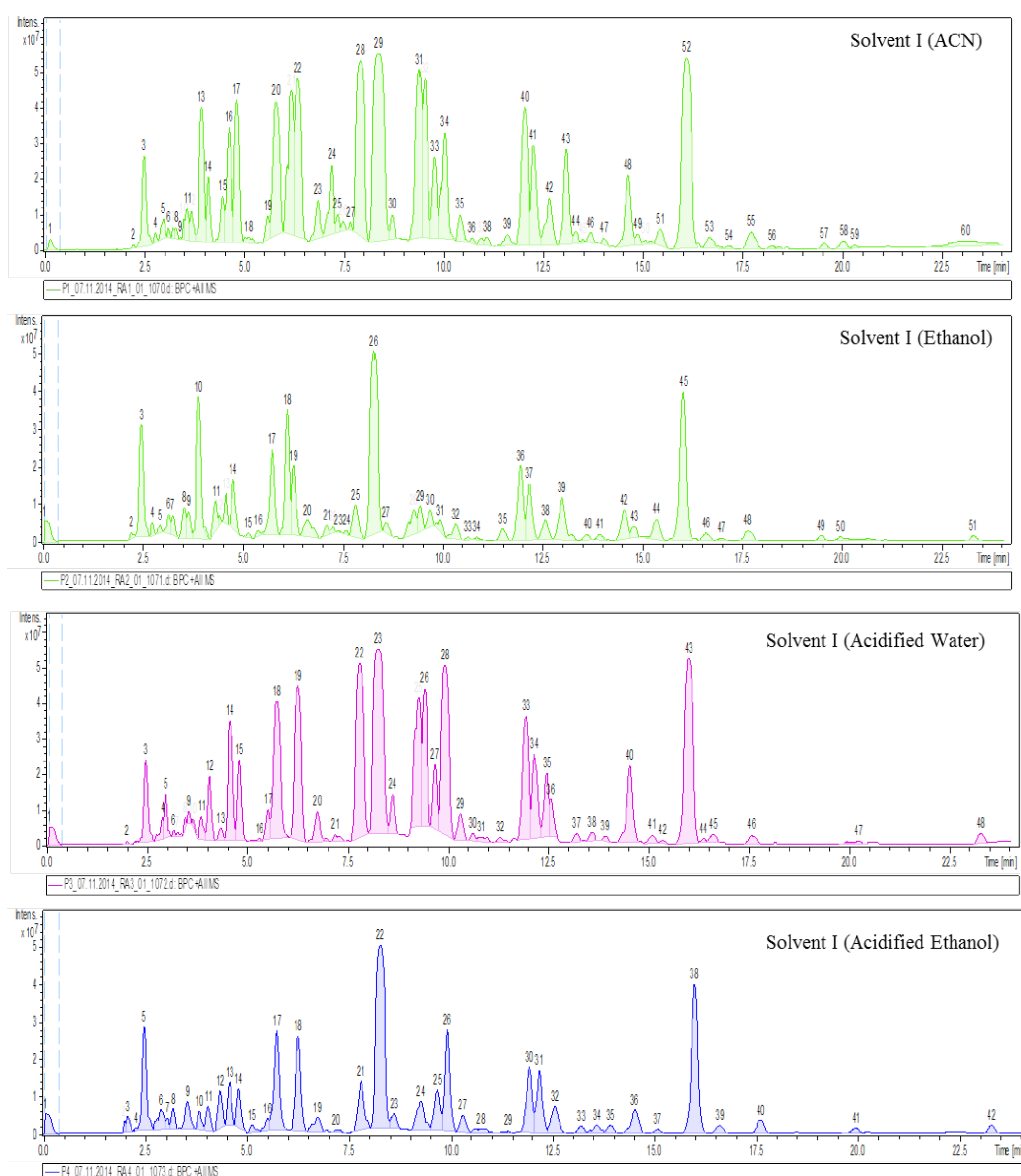

Figure. 1. HPLC chromatogram of amino acids extracted with all solvents analysed with LC/MS-positive ESI

Acetonitrile provides lesser quantities of the amino acids comparing with the other solvents. Leucine, isoleucine and histidine were extracted with good results in the same solvent compared with others amino acids due to their non-polar chemical structure and the concentrations ranged between 385.4 nmol/ml for leucine in ACN solvent to 150.63 in acidified ethanol; for isoleucine the concentrations ranged from 201.5 nmol/ml in ACN to 77.47 nmol/ml in ethanol to non-quantified in solvent IV; for histidine the concentration ranged between 215.39 nmol/ml in ACN solvent and 181 nmol/ml in acidified water to non-quantified in acidified ethanol.

Using solvent II for extraction it was achieved the separation of 15 amino acids. With glutamine exception there are the same amino acids like in the first extraction, but appear other four amino acids: serine, alanine, proline and valine due to a better solubility in ethanol. Serine, proline and alanine were obtained with better results comparing with the other amino acids in the same extraction solvent. If these results are compared with those obtained in solvent IV, which is acidified ethanol, it could be concluded that the addition of the HCl improved the solubility of the amino acids specified above. The concentration of serine in ethanol was 211.96 nmol/ml compared with 248.62 nmol/ml obtained in ethanol/HCl mixture. Same results were obtained for alanine which varied from 347.42 nmol/ml in solvent I to 459.1 in solvent IV; proline varied from 855.5 nmol/ml in solvent II to 959.6 nmol/ml in solvent IV.

Solvent III (mixture HCl 0.05M/deionized water – 1/1volume) provides 17 amino acids. Comparing the chromatographic profiles observed in Fig. 1 it can be seen those two different compounds appeared (AAA – alpha aminoadipic acid and ASN – asparagine), which were not present before. These amino acids had pronounced polar structures which lead to an increased solubility in a polar solvent. The addition of acid to an amino acid in aqueous solution at the point of neutral solubility produces a change in the species present. It would seem that at the low concentration of acid added in solution the chloride ions would provide the major attractive force by the predominantly charged amino acids. Acidified water proved to be the best extraction solvent for serine, glycine, leucine, ornithine, proline, histidine, homophenylalanine, tyrosine and alanine compared with others extraction solvents. The concentration of glycine decreased from 483.92 nmol/ml in acidified water to 45.94 nmol/ml in acetonitrile; ornithine varied from 400.21 nmol/ml in acidified water to 10.37 nmol/ml in ethanol; proline concentration decreased from 1203 nmol/ml in acidified water to 855.5 nmol/ml in ethanol and was unquantified in acetonitrile; alanine concentration varied from 838.01 nmol/ml in acidified water to 347.42 in ethanol and was also unquantified in acetonitrile.

Solvent IV provided 15 amino acids and the chromatographic profile showed that the addition of HCl increased the solubility of some amino acids like α-aminoadipic acid, tyrosine and asparagine which weren't identified in ethanol. Significant increases in concentrations were observed in the case of glutamic acid, asparagine and valine. The concentration of glutamic acid in acidified ethanol was 794.3 nmol/ml compared with 202.5 nmol/ml in acidified water; asparagine concentration varied from 466.1 nmol/ml in acidified ethanol to 110.5 nmol/ml in acidified water and wasn't quantified in the other two extraction solvents. Same results were obtained for valine which wasn't quantified in ACN and acidified water, and whose concentrations decreased from 195.38 nmol/ml in acidified ethanol to 67.22 nmol/ml in ethanol, in accordance with literature data (Pinho, Silva and Macedo, 1994; Thiele et al., 2008).

Conclusion

Due to the chemical composition and good digestibility, brewer's yeast is still used as nutritional supplement in animal and human feed. Brewer's yeast is an excellent source of high-quality protein which includes essential amino acids and also free amino acids.

The aim of this work was to test some solvents in order to improve the free amino acids extraction from lyophilised brewer's yeast. Solvent I, acetonitrile, provided the less quantities and number (10) of amino acids extracted comparing with the other solvents due to its weaker polarity. Solvent II (ethanol) led to the extraction of 15 amino acids due to its greater polarity. Solvent III (water/HCl) proved to be the best extraction solvent for the amino acids from brewer's yeast providing the separation of 17 compounds. Using solvent IV (ethanol/HCl) conducted to the extraction of 15 amino acids which differ from

those extracted only in ethanol regarding their profiles and quantities.

These preliminary results demonstrated that increasing the polarity of the solvents led to the improvement of amino acids extraction.

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