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HPLC analysis and Study of the evolution of the amino acids contents in sugar beet pulp during silage

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ABSTRACT: The production of sugar from the sugar industry generates by-products such pulp which is directly valued in animal alimentation. Knowing that pulp is a perishable food it's conservation as silage is the best solution from the economic, social and environmental side on condition to know well I's nutritional value to accommodate the dietary needs of animals. The objective of the present work was to evaluate amino acid composition of silage produced from sugar beet pulp during 3 periods of fermentation by high-performance liquid chromatography using pre-column derivatization. A fluorometric analytical system is utilized and the fluorogenic reaction of o-phthalaldehyde (OPA) was applied to quantify amino acid content. The reaction was measured by a single fluorescence sensing system with excitation and emission wavelengths (350/450 nm). The results obtained show that there is an elevation of the amino acids contents: methionine, lysine, threonine and cysteine from sugar beet pulp during storage.

KEYWORDS: Amino acids; HPLC; OPA; sugar beet pulp ; silage.

1 INTRODUCTION

Thanks to its high nutritional value and excellent palatability, sugar beet pulp has an important place in the animal feed sector. It has a relatively stable composition but it can vary during the breakout campaign, according to the maturity of the beets, geographical origin, variety and by mode of conservation either by drying or silage that is technical food conservation by controlled anaerobic acidification.

In animal food and especially for livestock, we attribute a great importance to the nitrogen fraction of the ingested food ration whatever the nature of the food (fresh, dry ...) and the method of preservation (silage, drying ...)..

In this work we were limited to the study of the sugar beet pulp silage and the effect of the fermentation period on the amino acid content.

Research on the evolution of the distribution of amino acids during the fermentation process is relatively incomplete. The feed proteins undergo changes more or less profound depending on whether conservation is good or bad, and besides some studies have pointed out [1][2][3][4]. Also the storage conditions (pH, dry matter content, temperature ...) where the amino

nitrogen silage may undergo profound changes, which can affect more or less seriously the value of protein presented to livestock.

Establishing a biochemical study in amino acid is essential for the development of a feed for animals.

It is known that proteins with the polysaccharides and nucleic acids are of the three classes of biopolymers occurring in the structure and functioning of all living organisms. Their total hydrolysis then leads to the amino acids that characterize them [5]. These amino acids have an identical basic structure: the acid is a carboxylic acid functional group carried by the same carbon as the amine function. The amine function is in α to the acid function where his name: α-amino acids.

Amino acids are divided into two categories: the essential amino acids and non-essential amino acids. Although both classes are essential for the production of proteins, these descriptions have been allocated according to the physiological ability of the animal to manufacture. The animal is unable to manufacture the essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) except from metabolites from these same amino acids, so that it has the metabolic pathways for synthesis of non-essential amino acids (alanine, aspartate, asparagine, cysteine, glutamate, glutamine, glycine, proline, serine, tyrosine). [6]

Diverse analytical methods have been proposed for the analysis of amino acids including thin layer chromatography [7][8], capillary electrophoresis [9][10] gas chromatography [11][12], and high-performance liquid chromatography (HPLC) [13][14][15][16][17].

HPLC is the most widely used and accepted technique due to its high resolution, sensitivity, great versatility, and simple sample treatment. Owing to the absence of a strong chromophore or fluorophore, chemical derivatization or labeling is usually employed for amino acids detection, and it becomes a necessary procedure to transform the analytes into derivatives that can be easily isolated, separated, and detected [18][19]. The analysis of amino acid in food samples is currently done by HPLC following precolumn derivatization using various reagents including 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), o-phthalaldeyde (OPA), phenyl isothiocyanate (PITC), 9-fluorenylmethylchloroformate (Fmoc-Cl), 2,4-dinitrofluorobenzene (DNFB).[20]

In the present work, we studied the evolution in time of 4 amino acids: lysine, methionine, threonine and cysteine from sugar beet pulp from its wet stage (time T0) until 6 months of silage (time T6) in bags of 50 kg based on the HPLC method after pre-column derivation with OPA.

2 MATERIALS AND METHODS

2.1 SUGAR BEET PULP

Sugar beet pulp was collected from Cosumar (SUTA) company in Beni Mellal zone in Moroco in May, August and November 2014

2.2 EXTRACTION METHOD

The method is based to measure alpha amino acids in beet pulp after defecation lead. This method is officially adopted as the reference method by the ICUMSA (International Commission for uniform sugar Methods of analysis).

After extraction, the extract undergoes a Millipore filtration and reacted with OPA before being injected in liquid chromatography for representing the amino acid as a fluorescent molecule.

2.3 AMINO ACID DERIVATIZATION

The extract and amino acid calibration mixtures were derivatized with OPA. Aliquot (5µl) followed in order by 25µl deionized water, 25µl of saturated sodium borate buffer (pH 9.5), 12.5 µl of OPA-derivatizing reagent, and 62.5µl of methanol. The vial was thoroughly mixed on a vortex mixer after each addition. The mixture was normaly kept at room temperature for at least 2min (but not more than 10 min), after they was injected into the HPLC system. [21]

The OPA-derivatizing reagent was stable up to one week and consisted of 25mg of OPA, 50 µl of Thiol reagent(2 mercaptoethanol), 0.5 ml of saturated borate buffer (pH 9.5), and 4.5ml of methanol.

Chromatographic conditions: Column: C18 (30 cm 3.9 mm id)

Fluorecsence detector: Excitation 350 nm; Emission 450 nm

Mobile phase: (A: Acetonitrile. -B : Buffer: 0.01 M Na2HPO4 (pH 7.4)) and Injection volume: 2 µl.

The chromatographic separation of mixture of standard amino acids, Lys, Met, Thr, and Cys is carried out in gradient mode that is illustrated in the following table 1.

Table 1. Elution mode

	Time (min)					
Solvent	0	2	35	55	56	56.1
% CH_3CN	9	20	32	80	80	9
% Buffer	91	80	68	20	20	91

2.4 STATISTICAL ANALYSIS

Data analysis was performed with SPSS statistical software (version 19) and excel. All data was subjected to analysis of variance one way ANOVA for descriptive and comparative analysis and using Tukey test ($P < 0.05$) for significant difference values between amino acids in different periods.

3 RESULTS AND DISCUSSION

The different amino acids have been identified by comparison to retention times for amino acid stock solutions. To determine the retention times, the reference standards were injected as a mixture. A peak table of mixture of amino acids standards is shown in table 2.

Table 2. Peak Table of mixture amino acids standards

Name	**Retention Time (min)**	**Area**	**Area %**
Threonine	10.315	27779157	28.574
Cysteine	15,396	661991	0.681
Methionine	19.601	38719201	39.828
Lysine	42.060	16004642	16.463

The analysis was done on the basis of retention times peak areas (A) and according to the external calibration method that comprises the sequential injection of an equal volume of a standard solution (with a known concentration of the element to be assayed C_{ref}) and the test solution (unknown concentration C_i), then comparing chromatograms to deduce the concentration $C_i = C_{ref}.A_i/A_{ref}$

A right calibration was performed by injecting several standard solutions at different concentrations.

The results corresponding to peak table of amino acids from sugar beet Pulp silage are shown in table 3.

Table 3. Peak Table of amino acids from sugar beet pulp

Name	**Retention Time (min)**	**Area**	**Area %**
Threonine	**9.866**	**31208667**	**68.704**
Cysteine	**42.801**	**636256**	**1.401**
Methionine	**17.943**	**8062511**	**17.479**
Lysine	**41,293**	**3310904**	**7.289**

The analysis results of amino acid content during storage period are shown in Table 4. T0 is a period before silage,

T3 is a period after 3 months silage and T6 is after 6 months silage.

Table 4. Analysis result from the beet pulp of silage

Amino Acids	Period	Average	SD*	SE**	Minimum	Maximum
Threonine (g /Kg DM)	T0	0.2765	0.065957	0.014748	0.2	0.4
	T3	0.423	0.25913	0.057943	0.1	0.9
	T6	0.9915	0.19258	0.043062	0.71	1.23
Cysteine (g/Kg DM)	T0	0.3845	0.146986	0.032867	0.22	0.72
	T3	0.6955	0.374468	0.083734	0.24	1.46
	T6	0.647	0.171068	0.038252	0.49	1.05
Methionine (g/Kg DM)	T0	0.198	0.037077	0.008291	0.1	0.3
	T3	0.1985	0.074571	0.016675	0.12	0.36
	T6	0.255	0.073664	0.016472	0.14	0.37
Lysine (g/Kg DM)	T0	0.0995	0.061855	0.013831	0.03	0.3
	T3	0.2235	0.148653	0.03324	0.09	0.55
	T6	0.2955	0.107139	0.023957	0.17	0.56

** SD: Standard deviation, **SE: Standard error.*

It is noted in the graph in the Figure 1 (Fig.1) that there is a significant change in amino acids content analyzed as follows: Lysine Methionine and Threonine depending on the duration of silage from T0 time to T6 time.

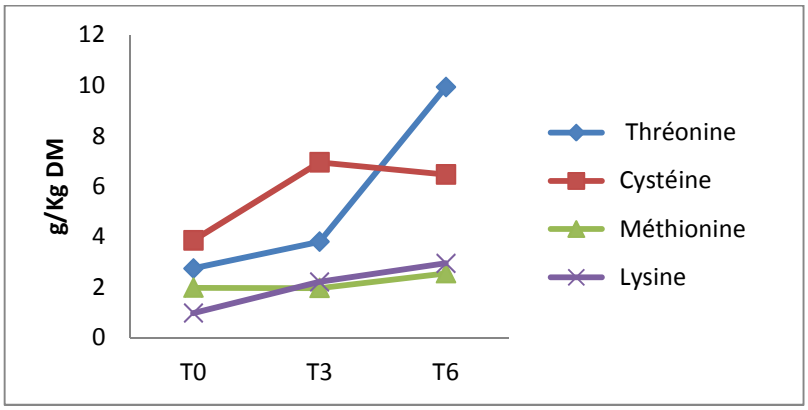

Fig. 1. Evolution of the amino acid content according to the storage time.

Lysine was 0.98 g / kg at T0 time immediately after pressing and prior to silage bag, and then it is increased to a higher value that is equal to 2.22 g / Kg after 3 months of silage and then increased for a second time after 6 months of silage and reaches the value of 2.95 g / Kg.

Methionine was 1.98 g / kg at T0 time, it remained stable after 3 months silage then it increased at T6 time to reach the value of 2.55 g / Kg.

Cysteine was 3.86 g / Kg at T0 time then it increased to a maximum that is equal to 6.95 g / Kg silage after 3 months. After 6 months of silage there was a slight decrease in cysteine which reached the value of 6.47 g / Kg.

Threonine was 2.75 g/Kg at T0 before silage, then it increased to a value that is equal to 3.81 g/kg after 3 months of silage then she experienced a very large increase after 6 months of silage and reaches the value of 9.93 g / Kg.

The elevation of the amino acids contents of silage beet pulp is due to the phenomenon of proteolysis. Generally, proteins are degraded by proteolytic enzymes, proteases, until stage amino acids. Proteolysis continues until the pH decreases below pH4 **[22].** Aim of silage fermentation is to induce a rapid pH drop to halt fermentation and preserve drilling material **[23].**

For the statistical analysis ANOVA revealed a highly significant effect of the period for all the amino acids considered, see table 5. The significant result of this test indicates that some groups of samples taken in pairs are significantly different; multiple comparison of the means provided by the post-hoc test (Tukey) indicates that, for the T0 periods and T3 the

difference is very highly significant (p <0.001) for cysteine, highly significant (p <0.01) for lysine, significant (p <0.05) for threonine and not significant (p> 0.05) for methionine. For periods T0 and T6 the difference is highly significant to threonine and lysine (p <0.001), highly significant for cysteine (p <0.01) and significant for methionine (p <0.05). Finally the differences for T3 and T6 periods are highly significant for threonine (p <0.001), significant for methionine but they are insignificant for cysteine and lysine.

Table 5. ANOVA results data of 4 amino acids studied

Variables	ddl	MS	F	P
Threonine	2	2.853	78.821	0.000
Cysteine	2	0.560	8.790	0.000
Methionine	2	0.021	5.211	0.008
Lysine	2	0.197	15.768	0.000

Pearson correlations presented in Table 6 between the different amino acids: lysine, methionine, threonine and cysteine show that there is a positive and significant correlation between threonine and methionine (r = 0.37 **), between lysine and threonine (r = 0 .555 **) and between lysine and methionine (r = 0.698 **), but there is a negative correlation is weakly significant between cysteine and methionine (r = -0086), for lysine and cysteine no significant correlations (r = 0.205).

Table 6. Results of correlations between the different amino acid studied

Correlations					
		Threonine	**Cysteine**	**Methionine**	**Lysine**
Threonine	**Correlation de Pearson**	**1**	**0.232**	**0.370****	**0.555****
	Sig. (bilateral)		**0.074**	**0.004**	**0**
Cysteine	**Correlation de Pearson**	**0.232**	**1**	**-0.086**	**0.205**
	Sig. (bilateral)	**0.074**		**0.513**	**0.117**
Methionine	**Correlation de Pearson**	**0.370****	**-0.086**	**1**	**0.698****
	Sig. (bilateral)	**0.004**	**0.513**		**0**
Lysine	**Correlation de Pearson**	**0.555****	**0.205**	**0.698****	**1**
	Sig. (bilateral)	**0**	**0.117**	**0**	

*** The correlation is significant at the 0.01 level (bilateral).*

Comparison of the results obtained on the amino acid composition of the beet pulp from Beni Mellal zone (BM) with those found on Animal Feed Resources Information System (AFRSI) about beet pulp (BP), alfalfa silage (AS), coffee pulp (CP) and corn silage (CS) presented in Table 7 shows that there is no significant difference in methionine from the stage fresh pulp silage up to 3 months for the BP. However, the results are superior to BP, AS and PC after 6 month of silage. For lysine (BM) is 1.5 to 2 times lower than the BP, CP and AS, slightly less compared to CS throughout the storage period. In addition increasing the threonine content along silage of BM allowed to have a rate 2 fold greater than BP, AS and CS and 10 fold greater than CP.

Table 7. Amino acid composition of the beet pulp Tadla Azillal (TA). beet pulp (BP). silage alfalfa (AS). coffee pulp (CP) and corn silage (CS) (BP.AS.CPand CS was provided by AFRSI)

Amino acids (g/Kg DM)	Pulp silage in Beni Mellal (PSBM)			Animal Feed Ressources Système d'information AFRSI (INRA Cirad AFZ et de la FAO 2012-2015) [24]*			
	T0	T3	T6	BP	AS	CP	CS
Lysine	0.98	2.22	2.95	3 to 5.41	6.49	3.84	3.07
Methionine	1.98	1.97	2.55	1.54 to 1.97	1.33	0.3	Nd
Cysteine	3.86	6.95	6.47	Nd**	Nd	Nd	Nd
Threonine	2.75	3.81	9.93	4.21	4.58	0.1	2.99

** The AFRSI value have been converted into g / Kg*

***not determined*

4 CONCLUSION

The results of the present work confirm that the conservation of the sugar beet pulp as silage is a technique that preserves the quality of compounds of interest like amino acids and can maintain the value of food products for animals as similar as possible to the fresh products.

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