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Research Article

Amino Acids and Biogenic Amines Evolution during the *Estufagem* of Fortified Wines

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The current study was focused on the impact of accelerated ageing (heating step) on the amino acid and biogenic amine profiles of fortified wines. In this sense, three Madeira wines from two commonly used grape varieties (one red and the other white) were analysed during the heating, at standard (45°C, 3 months) and overheating (70°C, 1 month) conditions, following a precolumn derivatization procedure using iodoacetic acid, *o*-phthaldialdehyde, and 2-mercaptoethanol, carried out in the injection loop prior to RP-HPLC-FLD detection. Eighteen amino acids were identified, with arginine being the most abundant. An important decrease of the amino acid levels was detected during the standard heating (up to 30%), enhanced up to 61% by the temperature increase. Cysteine, histidine, and asparagine revealed the greatest decreases at 45°C. Conversely, some amino acids, such as asparagine, slightly increased. Four biogenic amines were identified but always in trace amounts. Finally, it was observed that the accelerated ageing did not favour the biogenic amine development. The results also indicate that the heating process promotes the amino acid transformation into new ageing products.

1. Introduction

Amino acids are an important fraction (25–30%) of the nitrogenous source released in the crushing and pressing practices during winemaking, constituting a relevant source to yeast growing and vitality during the alcoholic fermentation [1, 2]. Among the primary amino acids found in musts, arginine and alanine are usually the most abundant. Free amino acids present in wine may have different origins, namely, from the degradation of grape proteins, metabolism of yeasts, and lactic acid bacteria, and from yeast and bacteria autolysis [3, 4]. Their profile and concentration in wines can be affected by several factors, such as grape variety (including rootstock), environment, and growing conditions (mainly nitrogen fertilization), and from the winemaking techniques employed, including the ageing procedure, such as biological ageing. Amino acids have motivated researchers to study their profile and influence in wine production, not only for their importance in the fermentation step but essentially due to their important effect on wine's flavour development (as metabolic precursors of higher alcohols), and also as criteria for differentiation and authenticity studies [5–11]. Their metabolism not only affects the aroma complexity but also can be responsible for the concentration increase of biogenic amines and ethyl carbamate precursors in wine.

In turn, the occurrence of amines in foods is usually associated to protein degradation through microbial activity, which cannot be easily extended to wines since the protein content is low. However, amine's occurrence in wines may be related to their natural presence in grapes, amino acid decarboxylation through the substrate-specific enzymes of yeast (during alcoholic fermentation) or spoilage bacteria (during

malolactic fermentation), such as *Lactobacillus*, *Pediococcus,* and *Leuconostoc* species, and aldehydes and ketones amination and/or transamination [12–14]. Inadequate sanitary conditions, at some stage of winemaking, can also contribute for their increase in wines [15]. It has been reported that several pharmacological reactions can occur after an excess intake of some biogenic amines, such as histamine and tyramine (most common in foods), like headaches, hypertension, and allergic reactions [1, 16]. Putrescine and cadaverine are not themselves toxic but are usually associated with wine's deficient sanitary conditions. Additionally, these amines can increase the toxicity of histamine, tyramine, and phenylethylamine, since they obstruct the removal of these toxic substances from the body. Several European countries have established informal legal limits for the histamine content in wines, namely, Germany (2 mg/L), Holland (3 mg/L), Belgium (5-6 mg/L), Finland (5 mg/L), France (8 mg/L), and Austria (10 mg/L) [17]. Switzerland has recently removed the legal limit previously imposed (10 mg/L) [18].

Most wines are aged at relatively low temperatures which is not the case of Madeira wines. These are fortified wines with alcoholic strengths between 17 and 22% (v/v), prepared at different styles (dry, medium-dry, medium-sweet, and sweet wines) according to a unique processing. The winemaking can include a thermal processing, known as *estufagem*, wherein the wine is usually heated to about 45°C for 3 months and finally undergoes a normal maturation process in wood casks, generally during 3 years. With the heating step a premature ageing takes place, originating the typical colour and bouquet of these wines. This accelerated ageing also contributes for its distinction among others fortified wines. Little is known about the amino acid profile and concentration in Madeira wines, in particular, from their evolution during the heating stage. Thus, the main purpose of this work was the determination of the amino acid profile and its behaviour in different styles of Madeira wines during the *estufagem* process. Additionally, was also performed the evaluation of amines present in these wines. To accomplish the purpose of the study, a previous developed methodology [19] was improved in order to derivatize cysteine, an amino acid that can be an important precursor of key-aroma compounds during wine ageing [20]. Then, Tinta Negra (TN) dry and sweet wines heated at 45°C during 3 months were analysed and compared with a sweet wine produced from Malvasia. For comparison purposes, the same wines were also baked at overheating conditions, 70°C during 1 month, to evaluate the temperature effect.

2. Materials and Methods

2.1. Wines. *Vitis vinifera* L. grapes, Tinta Negra (TN, red) and Malvasia (white), from 2007, were manually harvested and the corresponding wines were industrially elaborated in stainless steel tanks of a local Madeira wine producer, according to common practices. Firstly, 5% of potassium metabisulphite solution, pectins, and diammonium phosphate were added to free-run juice. The alcoholic fermentation was carried out by endogenous yeasts under controlled temperature (under 25°C), and without grape solids, known as the *bica aberta* fermentative process. The malolactic fermentation was not encouraged, once that any commercial bacteria were inoculated, according to the common practice followed in Madeira wine production. The production of two sweet wines, one from Malvasia grapes and the other from TN, was realized. Their fermentation was stopped by the addition of natural grape spirit (containing 95% (v/v) of ethanol), raising the alcohol content up to about 17% (v/v), when the must density attained 1019 and 1025 mg/L, remaining 96 and 115 g/L of reducing sugars, respectively.

A dry wine from TN grapes was also produced and the fortification was performed when the density reached 986 mg/L (reducing sugars of about 4 g/L). After fortification, all wines were clarified and stabilized through bentonite clays and Albuminocol gelatins. The wine pH ranged from 3.52 (TN sweet) to 3.45 (TN dry) while total acidity (expressed as tartaric acid equivalents) varied between 6.53 g/L (TN sweet) and 6.90 g/L (TN dry).

When postfermentation treatments were accomplished about 200 L of each wine was then placed in stainless steel vats fitted with stainless steel coils that allow the circulation of hot water inside the container, gradually releasing heat throughout the wine, up to about 45°C during 3 months. For comparison purposes, the wines were also baked at overheating conditions, 70°C during 1 month. The experiment is schematized in Figure 1 and it was carried out in a special pilot scale system equipped with 200 L stainless steel vats, designed for the careful and independent control of temperature by the circulation of hot raw water.

Samples were monthly collected and kept at −20°C before being submitted to the analytical determination. Three sample aliquots were always analysed.

2.2. Standards and Reagents. The amino acid, γ-aminobutyric acid (GABA), was supplied by Fluka BioChemika AG (Buchs, Switzerland) while the others were supplied by Sigma-Aldrich (St. Louis, MO, USA): aspartic acid (Asp), glutamic acid (Glu), cysteine (Cys), asparagine (Asn), serine (Ser), glutamine (Gln), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), tyrosine (Tyr), methionine (Met), tryptophan (Trp), valine (Val), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), and lysine (Lys). Histamine (Him), tyramine (Tym), phenylethylamine (Phm), isopentylamine (Ism), and cadaverine (Cad) were from Fluka BioChemika AG (Buchs, Switzerland) while tryptamine (Trm) was from Acros Organics (Geel, Belgium). All standards had a minimum assay of 98%.

Ultrapure water was obtained from a Milli Q-System (Millipore, Milford, MA, USA) while HPLC-grade methanol was obtained from Sigma-Aldrich (St. Louis, MO, USA). Tetrahydrofuran (99.5%), ethanol (99.9%), sodium hydroxide (98%), and sodium phosphate monobasic monohydrate (98%) as well as iodoacetic acid (IDA, 99%) were from Panreac Quimica SA (Barcelona, Spain). The derivatization reagent *o*-phthaldialdehyde (OPA, p.a.) and 2-mercaptoethanol (MCE, 99%) were supplied by Acros Organics (Geel, Belgium). Finally, hydrochloric acid (p.a.) was from

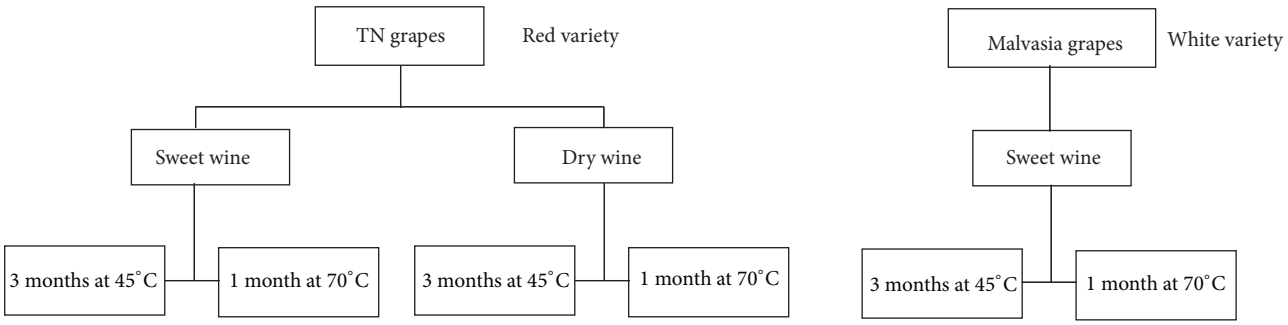

Figure 1: Scheme of the experience carried out: Tinta Negra (TN) dry and sweet wines were industrially elaborated and heated at 45°C during 3 months. Similarly, a sweet wine was also produced from Malvasia cultivar. For comparison purposes, all wines were baked at overheating conditions (70°C during 1 month).

Riedel-de Häen (Seelze, Germany) and boric acid (99.5%) from Merck Co. (Darmstadt, Germany).

The OPA/MCE solution was prepared by diluting 50 mg of OPA in 1.50 mL of ethanol and adding 400 mM borate buffer (pH 10.5) up to 10 mL. Finally, after the addiction of 200 μL of MCE reagent the solution settled down for 90 min before use. The IDA solution was prepared by adding 0.583 g of IDA to 10 mL of borate buffer.

2.3. Determination of Amino Acids and Biogenic Amines. A Waters HPLC system (Milford, MA, USA), consisting of separations module with autoinjector (Waters 2695) and a Multi λ Fluorescence detector (Waters 2475), was used for the simultaneous analysis of amino acids and amines. The Empower Pro software was used for data storage and integration. The methodology previously developed by Pereira et al. [19] was improved adding an initial step to include cysteine derivatization, by the carboxymethylation using iodoacetic acid (IDA). Briefly, the precolumn OPA/MCE derivatization was carried out according to a new sequence, in the sample injection loop: to 5 μL of filtered (0.45 μm) standard/sample (200 μL of sample/standard previously diluted in 1.5 mL of borate buffer solution) 5 μL of IDA solution and 10 μL of OPA/MCE solution were added. The remaining experimental design was maintained. As a consequence of the carboxymethylation step introduction in order to derivatize cysteine, Table 1 briefly presents the linearity parameters of the current methodology.

2.4. Statistical Analysis. Regular statistical analyses were performed with Microsoft Office Excel 2007, while Principal Component Analysis (PCA) was carried out with the computational platform MatLab (version 7.6, The Mathworks, Inc.).

3. Results and Discussion

3.1. Amino Acids. The amino acids found in the different wine samples at the initial stage (0 m) and after 1, 2, and 3 months of baking at 45°C are exposed in Table 2. The results of the experience at 1 month at 70°C are presented as well. The total concentration of amino acids was calculated as the sum of the concentrations of the individuals. In addition, the percentage of each compound in the samples was also determined relative to the total value.

As it can be seen in Table 2, only 18 amino acids were found in the current sample set. Arg was always the most abundant amino acid, followed by Ala in TN wines and by GABA in Malvasia wine. Similar results were also obtained by other researchers in white wines [21, 22]. Before the thermal procedure TN sweet wine presented the highest content in amino compounds, while the corresponding dry wine presented at least 3-fold less. As expected, this result confirms that extensive fermentations reduce the amino acid content. The Malvasia, which is also a sweet wine from a different variety presented a lower value. This result may be related with the fact that fermentation was slightly more extensive and, consequently, promoted higher amino acid consumption.

From the results it is also noteworthy that Arg seems to be the amino acid that the consumption, during the alcoholic fermentation, is more pronounced, given that its content in the TN dry is about 11-fold smaller than the corresponding sweet wine. The Arg consumption seems to be followed by the Thr and Ser. Amino acid consumption during fermentation is usually associated with formation of aromas, mostly alcohols, but other compounds can also be formed, and in turn the corresponding esters. Particularly, Arg consumption may be related with the production of proline, citrulline (precursor of ethyl carbamate), and other compounds, like amines. Ser depletion may be associated with the Cys formation, by condensation with homocysteine formed by yeast through sulphide sequestration, during fermentation [23]. Indeed, the levels of Cys are higher in the dry TN than in the sweet wines (less fermented), indicating that its occurrence might be essentially derived from the Ser decomposition.

Analysing Table 2, it is also observed that the total amino acid content decreased after 3 months at 45°C, about 12-13% in TN wines and 30% in Malvasia. The diminishment was more pronounced when the wines were submitted to overheating conditions (1 month at 70°C), falling down up to 61%, in the case of the wine made from Malvasia grapes. This result indicates that temperature seems to favour the consumption of some amino acids. Probably, their consumption is related with the formation of off-flavours, through Strecker

Table 1: Summary of the linearity parameters obtained for the in-loop IDA/OPA/MCE derivatization procedure occurring in the HPLC-FLD system. The working concentration range varied between 3 and 125 mg/L. LOQ was calculated on the basis of the linear regression $y = ax + b$, according to LOQ = $10\sigma/a$, where σ is the y-intercept standard deviation and a the slope.

Amino compound	Abbreviation	Linearity parameters ($y = ax + b$)			
		a	b	R^2	LOQ (mg/L)
Amino acids					
Aspartic acid	Asp	673980	302623	0.9998	1.52
Glutamic acid	Glu	635438	293410	0.9998	1.41
Cysteine	Cys	127643	33774	0.9999	1.90
Asparagine	Asn	449956	173267	0.9999	1.70
Serine	Ser	1036916	827421	0.9994	2.28
Glutamine	Gln	182147	456093	0.9994	2.21
Histidine	His	324410	218263	0.9998	1.38
Glycine	Gly	1629176	337283	0.9999	2.26
Threonine	Thr	742561	230380	0.9999	1.65
Arginine	Arg	521261	1797214	0.9992	3.88
Alanine	Ala	1177658	83468	0.9998	1.69
γ-Aminobutyric acid	GABA	992290	920153	0.9997	2.90
Tyrosine	Tyr	532135	230152	0.9998	1.29
Methionine	Met	778083	307865	0.9998	1.30
Tryptophan	Trp	931939	357251	0.9998	1.34
Valine	Val	509248	204224	0.9999	1.32
Phenylalanine	Phe	755146	407337	0.9999	1.43
Isoleucine	Ile	941243	257488	0.9998	1.36
Leucine	Leu	927913	580392	0.9999	1.46
Lysine	Lys	177571	−229832	0.9990	6.72
Biogenic amines					
Histamine	Him	851424	−284552	0.9996	2.39
Tyramine	Tym	897313	−196446	0.9997	2.67
Tryptamine	Trm	608809	−1013792	0.9993	3.95
Phenylethylamine	Phm	713629	−698822	0.9997	2.63
Isopentylamine	Ism	1127761	−1762345	0.9990	4.83
Cadaverine	Cad	518226	−4898236	0.9955	11.4

degradation, as it was suggested by Escudero et al. [24] and Marchand et al. [25]. Although there is little supportive evidence that this kind of reaction plays an important role during the *estufagem* of Madeira wines (about pH 3.5, 6 g/L of acidity and 18% of ethanol) it cannot be neglected that Maillard reactions can occur.

The oxidation effect upon sulphur-containing compounds has been depreciated [26]. However, sulphur-containing amino acids, such as Cys, Met, Phe, and Thr, have been related with the formation of volatile compounds associated with oxidative phenomena. Cys and Met can play an important role in wine aroma since they are usually related with the formation of several volatile compounds with low olfactory threshold, namely, off-odours such as methional [24, 27]. Cys has been pointed out as the most interesting sulphur amino acid that through Maillard and Strecker reactions is transformed into many products including heterocyclic groups in their structure revealing strong-smelling notes, namely, of sulphur, popcorn, hazelnut, toasted, roasted, and ripe fruits [28, 29]. In turn, the oxidative degradation of Phe and Thr is associated with the formation of phenylacetaldehyde and sotolon, respectively, both oxidation wine markers [30, 31].

In the current study, Cys, His, and Asn revealed an evident decrease (up to 100%) when the wines were heated at standard conditions, as well as when the heating was accomplished in 1 month at 70°C. At overheating, the decrease was more expressive and at least 7 amino acids reduced more than 50%, especially Thr up to 100%. It is interesting to notice that higher amounts of Thr were transformed during the ageing of sweet wines at 70°C, especially in TN. Nonetheless, Thr did not decrease so significantly at standard heating. Regarding Phe, a similar behaviour is observed; that is, the temperature increase favoured its transformation (up to 39%), mostly in sweet wines. Thus, these facts suggest that both might be involved in Strecker degradation reaction at higher temperatures. Met was not found in the current samples.

Other amino acids slightly increased during the heating period, especially Asp, Gly, and Ile, mostly at overheating conditions wherein Asp grew up to 3-fold its initial amount. During the traditional elaboration of sparkling

Table 2: Contents of the amino acids found in the current Madeira wines at different wine stages: initial (0 m), after 1, 2, and 3 months at 45°C (standard heating), and after 1 month at 70°C (overheating). The results are expressed in mg/L as mean ± standard deviation (SD). The percentage of decrease (% Dec) of each compound relative to the initial value is also presented.

Amino acid			Standard heating									Overheating		
	0 m		1 m, 45°C			2 m, 45°C			3 m, 45°C			1 m, 70°C		
	Mean	SD	Mean	SD	% Dec	Mean	SD	% Dec	Mean	SD	% Dec	Mean	SD	% Dec
Tinta Negra sweet														
Asp	22.1	0.1	16.7s	0.1	24	23.4s	0.0	−6	25.4s	0.1	−15	24.4s	0.3	−10
Glu	32.0	0.1	17.1s	0.1	47	18.0	0.0	44	14.2s	0.1	55	2.66s	0.02	92
Cys	n.d.		n.d.			n.d.			n.d.			n.d.		
Asn	7.12	0.10	4.78s	0.05	33	5.64s	0.02	21	5.19s	0.08	27	1.90s	0.01	73
Ser	19.2	0.1	13.6s	0.1	29	18.4s	0.1	4	19.1ns	0.1	0	13.7s	0.0	28
Gln	n.d.		n.d.			n.d.			n.d.			3.80s	0.09	−100
His	4.04	0.17	1.65s	0.08	59	2.50s	0.12	38	2.06s	0.12	49	5.76s	0.03	−43
Gly	4.03	0.09	3.41s	0.04	15	4.85s	0.10	−20	5.40s	0.10	−34	5.40s	0.11	−34
Thr	26.8	0.1	18.5s	0.1	31	23.9s	0.3	11	24.9s	0.3	7	14.2s	0.0	47
Arg	356	1	254s	1	29	306s	1	14	302s	4	15	172s	0	52
Ala	84.3	0.3	61.5s	0.1	27	82.3s	0.41	2	84.2ns	0.4	0	74.5s	0.3	12
GABA	37.7	0.6	25.2s	0.2	33	33.3s	0.8	12	32.9s	0.8	13	16.5s	0.2	56
Tyr	14.4	0.02	10.3s	0.1	28	13.8s	0.1	4	14.0s	0.2	3	11.4s	0.0	21
Trp	7.72	0.02	5.43s	0.02	30	7.52s	0.00	3	7.70ns	0.07	0	6.90s	0.06	11
Phe	7.96	0.07	5.59s	0.03	30	7.63s	0.04	4	7.75s	0.08	3	5.96s	0.04	25
Ile	4.52	0.02	3.17s	0.01	30	4.45s	0.01	2	4.55ns	0.04	−1	3.87s	0.02	14
Leu	8.19	0.04	5.76s	0.02	30	7.91s	0.02	3	8.02s	0.09	2	6.06s	0.02	26
Lys	8.00	0.11	d-n.q.			7.88ns	0.22	2	7.63ns	0.13	5	6.63s	0.16	17
Total	**644**		**447**		**31**	**568**		**12**	**565**		**12**	**376**		**42**
Tinta Negra dry														
Asp	11.2	0.1	13.1s	0.1	−17	15.6s	0.1	−39	17.3s	0.0	−54	30.3s	0.1	−171
Glu	21.8	0.0	16.8s	0.2	23	14.3s	0.0	35	11.7s	0.0	47	2.70s	0.01	88
Cys	5.74	0.15	d-n.q.		100	d-n.q.		100	d-n.q.		100	d-n.q.		100
Asn	5.95	0.17	5.66s	0.07	5	5.44s	0.03	9	4.11s	0.02	31	1.95s	0.02	67
Ser	4.49	0.03	4.54ns	0.06	−1	5.14s	0.11	−14	5.02s	0.03	−12	5.89s	0.05	−31
Gln	n.d.		n.d.			n.d.			n.d.			n.d.		
His	1.76	0.05	d-n.q.		100	d-n.q.		100	d-n.q.		100	d-n.q.		100
Gly	7.01	0.03	6.97ns	0.09	1	7.39s	0.08	−5	7.58s	0.08	−8	7.72s	0.01	−10
Thr	4.93	0.19	4.82ns	0.21	2	4.94ns	0.10	0	4.86ns	0.07	1	3.22s	0.04	35
Arg	31.9	0.1	30.2s	0.5	5	30.0s	0.1	6	27.1s	0.1	15	23.8s	0.4	25
Ala	28.7	0.1	27.7s	0.3	3	28.4ns	0.1	1	27.8s	0.1	3	28.4ns	0.1	1
GABA	15.8	0.1	14.5s	0.1	8	14.4s	0.2	9	13.6s	0.1	14	8.97s	0.08	43
Tyr	6.06	0.06	5.89s	0.06	3	6.09ns	0.08	0	5.99ns	0.02	1	6.13ns	0.01	−1
Trp	4.02	0.01	3.92s	0.04	2	4.16s	0.03	−3	3.90s	0.01	3	4.41s	0.02	−10
Phe	5.75	0.00	5.68ns	0.06	1	5.88s	0.06	−2	5.59s	0.02	3	5.65ns	0.03	2
Ile	2.66	0.01	2.65ns	0.03	0	2.84s	0.03	−7	2.81s	0.01	−6	3.17ns	0.02	−19
Leu	8.72	0.02	8.49s	0.08	3	8.83s	0.06	−1	8.43s	0.05	3	8.65ns	0.04	1
Lys	11.1	0.5	11.1ns	0.4	0	10.5ns	0.6	5	9.52s	0.03	14	7.19s	0.25	35
Total	**178**		**162**		**9**	**164**		**8**	**155**		**13**	**148**		**17**
Malvasia														
Asp	6.13	0.26	5.63ns	0.05	8	5.94ns	0.07	3	6.21ns	0.07	−1	6.53ns	0.32	−7
Glu	12.7	0.1	9.32s	0.06	27	7.82s	0.08	39	6.37s	0.08	50	2.03s	0.02	84
Cys	d-n.q.		n.d.			n.d.			n.d.			n.d.		
Asn	2.40	0.06	1.87s	0.08	22	d-n.q.		100	d-n.q.		100	n.d.		100
Ser	5.48	0.13	5.94s	0.08	−8	5.32ns	0.07	3	5.07s	0.06	7	3.49ns	0.03	36

Table 2: Continued.

Amino acid	0 m		Standard heating 1 m, 45°C			2 m, 45°C			3 m, 45°C			Overheating 1 m, 70°C		
	Mean	SD	Mean	SD	% Dec	Mean	SD	% Dec	Mean	SD	% Dec	Mean	SD	% Dec
Gln	n.d.		n.d.			n.d.			n.d.			n.d.		
His	3.45	0.18	2.27s	0.11	34	1.57s	0.05	54	d-n.q.		100	1.66s	0.03	52
Gly	3.48	0.09	3.97s	0.02	−14	4.10s	0.08	−18	4.47s	0.05	−28	3.65ns	0.08	−5
Thr	5.91	0.07	5.31s	0.05	10	4.85s	0.04	18	4.22s	0.04	29	d-n.q.		100
Arg	112	4	91.9s	2.2	18	82.9s	1.3	26	73.6s	0.6	34	37.1s	0.3	67
Ala	17.0	0.2	16.0s	0.1	6	16.4s	0.2	4	16.0	0.1	6	12.8s	0.2	24
GABA	30.8	0.4	25.4s	0.4	18	24.7s	0.4	20	22.6s	0.1	27	8.83s	0.02	71
Tyr	1.96	0.05	1.82s	0.03	7	1.79s	0.03	9	1.68s	0.02	14	d-n.q.		100
Trp	2.86	0.04	2.73ns	0.01	5	2.75ns	0.04	4	2.69s	0.04	6	2.11s	0.11	26
Phe	2.69	0.08	2.44s	0.03	9	2.43s	0.05	10	2.28s	0.03	15	1.65s	0.07	39
Ile	d-n.q.		d-n.q.			d-n.q.			d-n.q.			d-n.q.		
Leu	3.27	0.06	2.99ns	0.07	9	2.99ns	0.11	9	2.77ns	0.04	15	1.92s	0.07	41
Lys	d-n.q.		d-n.q.			d-n.q.			d-n.q.			d-n.q.		
Total	**210**		**178**		16	**164**		**22**	**148**		**30**	**82**		**61**

Compounds never detected are not presented.
n.d.: not detected; d-n.q.: detected, not quantified.
s: $p < 0.050$ significant difference when compared with the initial state; ns: $p > 0.050$ no significant difference when compared with the initial state.

wines the amino acid increase was also observed [32], being related with the yeasts autolysis that occurs during its ageing. Indeed, peptides and amino acids are considered the major compounds released into wine during autolysis. However, in the case of the Madeira wine production, yeasts autolysis probably occurs before the heating step. The application of the thermal processing (especially at high temperatures) can eventually promote the hydrolysis of the wine peptides and consequently enhance some amino acids. It was also verified that the amino acid evolution during *estufagem* was not so linear, probably due to the balance between release and consumption.

3.2. Biogenic Amines. Regarding biogenic amines, neither OIV (*Office International de la Vigne et du Vin*) nor European Union, including Portugal, impose any legislation setting the maximum limits for biogenic amines in wines. However, some authors suggest [15] that biogenic amine contents lower than 8 mg/L should be reasonable. Among the 6 amines analysed only 4 were detected in the studied wines, namely, Him, Phm, Ism, and Cad, but all under their LOQ (in average 5 mg/L). Considering that Madeira wine's vinification does not favour the development of malolactic fermentation, the biogenic amine levels found were very low. The biogenic amine occurrence in wines is normally associated with this type of fermentation. The *estufagem* process did not induce or enhance the biogenic amine content as the levels were below the quantification limit. Moreno and Azpilicueta [33] also showed that the natural oak ageing of red wines did not have any influence on the accumulation of biogenic amines.

The obtained results lead us to conclude that these wines do not raise concerns in regard to the biogenic amine effect, since only vestigial levels were found.

3.3. Multivariate Analysis. In previous sections how amino acids evolve during the *estufagem* process was analysed, namely, the identification and quantification of the amino acids which suffer important decreases and those whose concentration increases during *estufagem*. However, this quantitative analysis does not clearly disclose if the changes observed compromise the typical wine features, that is, if *estufagem* leads to some loss of identify of each style of wine. In this sense, the Principal Components Analysis (PCA) [34–36] was applied and biplot tools were chosen in order to simultaneously analyse how samples cluster together in the reduced PCA subspace (groups of varieties or according to time of *estufagem*) and which variables contribute more to the observed separation patterns. To carry out PCA, the data matrix of samples (45) by variables (18) was scaled, that is, each variable was centred in its mean and divided by its sample standard deviation. The PCA model selected, formed by the first three components, explain about 90% of all data variability.

Figure 2 depicts the first two dimensions of the PCA model, from which it is possible to identify three different clusters, each one representing a different type of wine (TN dry, TN sweet, and Malvasia wines). Across the first PC, the clusters formed appear to be in agreement with the grape variety, while the second PC contributes to make the distinction among sweet and dry wines. This arrangement suggests that the variability observed during the heating process is not significant when compared with those observed between wines, suggesting that *estufagem* does not lead to significant changes in wine typical profile. On the other hand, this suggests that the effect of *estufagem* should be evaluated individually for a specific type of wine, comprising a larger number of samples in order to avoid that the impact of

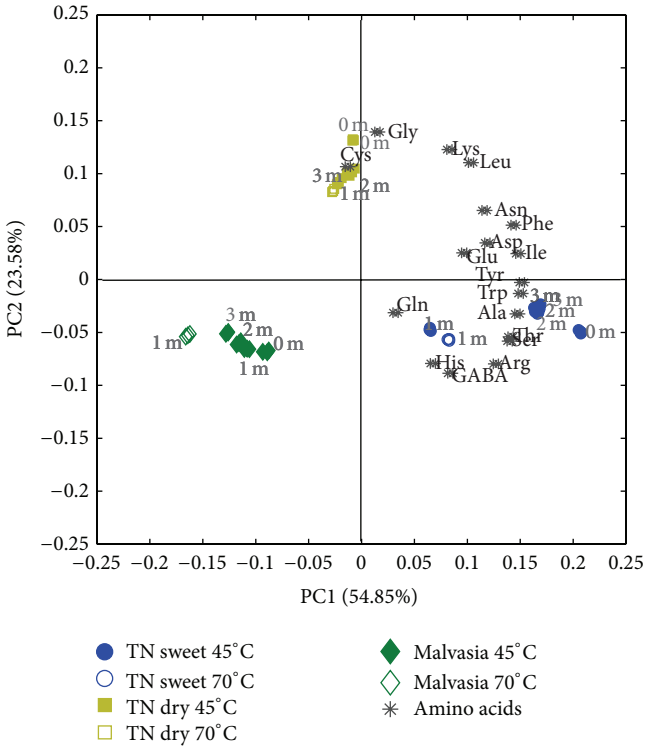

Figure 2: PC1 versus PC2 biplot of the Madeira wine samples submitted to *estufagem* in order to visualize the different clusters.

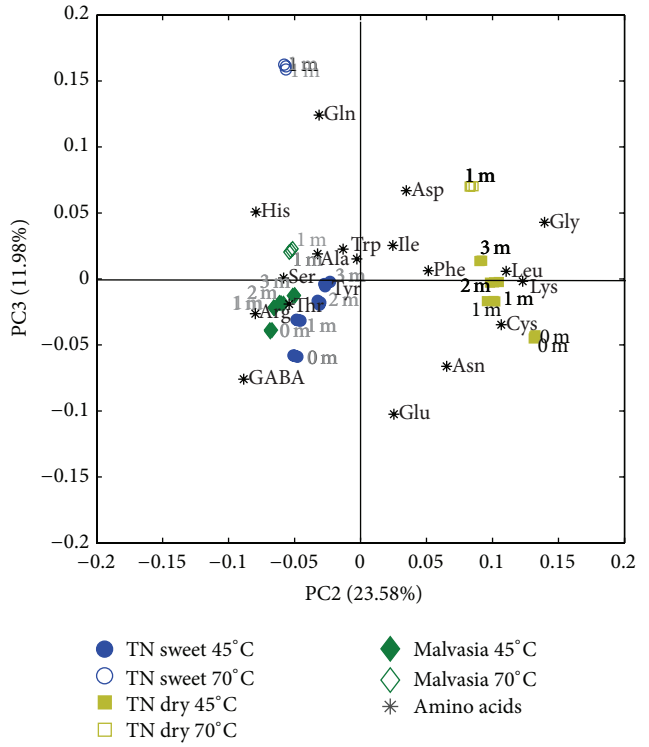

Figure 3: PC2 versus PC3 biplot of the Madeira wine's sample set in study, in order to reveal how wines evolve during the *estufagem* at standard (45°C) and overheating (70°C) conditions.

the intrinsic characteristics does not overlap the observed variability. Nevertheless, this analysis allowed the identification of the characteristics of wine amino acids, namely, the analysis of the loading information. With exception of Cys and Gly we can conclude that the remaining amino acids are closely related to the distinction found across the first PC and correlated positively among them. On the other hand, Cys and Gly correlate positively on the second PC, indicating their importance in sweet and dry wines distinction. Also, GABA, Arg, and His are prominent compounds in that differentiation. The amino acids Arg, GABA, His, Ser, Thr, Ala, Trp, Tyr, and Gln are associated with TN sweet wines group. According to PCA subspace analysis, a differentiation can be seen according to the fermentation extension (sweet and dry wines), since the same variety produced under different conditions was well differentiated, essentially due to the content of the amino acids Gly, Lys, Cys, Leu, GABA, Arg, and His. Moreover, PCA also enabled distinguishing the two varieties in spite of both being sweet wines (closer fermentation times). PCA results suggest that the difference observed between both sweet wines is a consequence of the intrinsic characteristic of both grape varieties; otherwise a similar behaviour on the principal component subspace was expected for both sweet wines, if the fermentation extension was determinant for their amino acid content. Indeed, PCA analysis indicates that there are strong evidences that Malvasia grape variety could already offer a smaller amount of this kind of nitrogen source, associated to the fact that only the endogenous yeast strains were used. Moreover, this result can also justify the versatility of TN variety, which is broadly used for the production of wines with different sweetness levels, whilst Malvasia is almost exclusively used for sweet wines.

The evolution trend during the heating process can be analysed in Figure 3. It is observed that all wines follow the same trend along the positive PC3. The most relevant amino acids that are involved in this process are mainly Gln for TN sweet at 70°C, His for Malvasia at 70°C, and Asp and Gly for TN dry at 70°C. Also, from this analysis it can be concluded that higher temperatures lead to significant changes comparative to standard heating, even for longer periods.

4. Conclusions

The evaluation of the profiles of sweet and dry Madeira wines during the heating, at standard (45°C, 3 months) and overheating (70°C, 1 month) conditions, was achieved. Eighteen amino acids were identified, with arginine as the most abundant.

The TN variety presented the highest content in total amino acids, in particular the sweet style. As expected, it was demonstrated that the amino acid decreases with fermentation (the amino acid content of the initial TN dry wine was 3-fold lower than that of the TN sweet).

The accelerated ageing (45°C during 3 months) decreases the concentration of specific amino acids, namely, cysteine,

histidine, and asparagine. This diminishment was probably related to their involvement in Maillard reactions. The decrease of total amino acids was more pronounced in sweet wines, which can be related with the development of the bouquet with ageing and explain the lower evolution of dry wines during the heating process. This evidence also shows that the conditions for heating sweet and dry wines are not necessarily the same, as it is actually being done. Moreover, this also fosters the fact that most changes observed during heating are related with sugar degradation, particularly Maillard reactions involving specific amino acids, controlled by residual sugars. However, it was verified that in spite of the fact that there is a variation in amino acid content during *estufagem*, that did not lead to the loss of wine identity, since it was verified that the variability observed during the 3 months of heating is small when compared with the differences observed between different styles of wine produced from the same grape variety.

Final remarks can be made: if there is consumption of amino acids during heating it means that they can play an important role in the development of Madeira wine typical features and that accelerated ageing does not foster the development of biogenic amines.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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