Technical paper

Amino Acid Quantification in the Presence of Sugars using HPLC and Pre-Column Derivatization with 3-MPA/OPA and FMOC-Cl

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Abstract

Determination of free amino acids (AA) in musts and young wines using pre-column derivatization with 3-MPA/OPA for primary AA and FMOC-Cl for secondary AA was studied. As samples differ in their sugar content considerably, the influence of sugars on derivatization was investigated using two internal standards: (i) primary AA norvaline and (ii) secondary AA sarcosine, in real samples and in model fructose and glucose solutions. A strong matrix effect was observed, especially when concentrations of sugars exceeded 50 g/L. The influence of sugars on derivatization of secondary AA sarcosine was more pronounced than on the primary AA norvaline. For quantitative evaluation of AA in samples with variable but high sugar content we propose to use external calibration in combination with internal standard method, where the response factors for individual AA are corrected with respect to that of internal standards.

Keywords: Amino acids, sugars, 3-MPA/OPA, FMOC-Cl, wine, must

1. Introduction

Determination of amino acids (AA) in different matrices, such as food and feed, is of high interest. Among the variety of available techniques, liquid chromatography after pre-column derivatization of AA with different reagents: *o*-phthaldehyde (OPA), 9-fluoroenylmethyl-chloroformate (FMOC-Cl), *N*-hydroxysuccinimidyl-α-naphthylacetate (SINA), diethyl ethoxymethylene malonate (DEMM), 4-dimethyl-aminoazobenzene-sulfonyl chloride (DABS-Cl), 6-*N*-aminoquinolyl-*N*-hydroxysuccimidyl carbamate (ACQ), phenylisothiocyanate (PITC), dansyl chloride (Dns-Cl), dabsyl chloride (Dbs-Cl), ninhydrin[1] and naphthalene-2,3-dicarboxaldehyde (NDA)[2] are frequently used. Combinations of reagents are also used, such as OPA for primary AA and FMOC for secondary AA.[3–5]

As food samples are usually complex, matrix effect must be given due consideration. The effect of salts, buffers and surfactants on pre-column derivatization of AA with FMOC, PITC and NDA have already been studied and the influence of different compounds on AA derivatization was observed.[2]

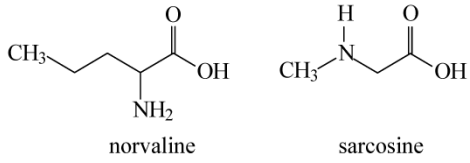
In food analysis, sugars frequently represent one of the major matrix components. This issue is especially important when analyses of samples with various sugar contents are considered. Frequently, the influence of sugars on AA determination in wines and musts is overlooked. In the literature, it was already observed that high sugar content influences determination of AA with PITC[6] and DEEM[7] derivatizing reagents. In our case, we monitored AA content during fermentation of must to wine and our task was to examine the effect of sugars in musts and young wines on free AA determination, in detail. We used the two-step derivatization with 3-mercaptopropionic acid

(3-MPA)/OPA for primary AA and FMOC-Cl for secondary AA. This kind of derivatization was already used for AA determination in musts and port wine,[8] but the influence of sugar content on OPA/FMOC derivatization was not described. According to our results, we suggest a new procedure using internal standards for determination of AA in food samples with high sugar content.

2. Experimental

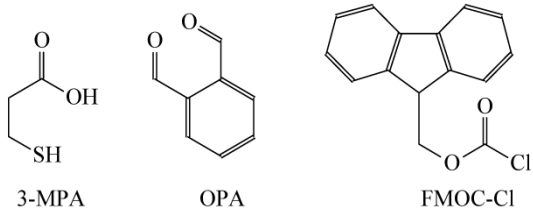
2. 1. Reagents

The 22 amino acid standards (aspartic acid, glutamic acid, asparagine, serine, glutamine, histidine, glycine, threonine, alanine, arginine, cysteine, cystine, valine, methionine, phenylalanine, isoleucine, leucine, lysine, tyrosine, tryptophan, 4-hydroxyproline and proline) were obtained from Fluka (Buchs, Switzerland). AA internal standards norvaline and sarcosine (Figure 1) plus derivatization reagents 3-MPA, OPA and FMOC-Cl (Figure 2) were from Sigma Aldrich (Steinheim, Germany). Solvents for mobile phase acetonitrile and methanol (both gradient HPLC grade) were from Scharlau (Barcelona, Spain). Water used for dilutions and for the mobile phase was additionally purified using the Milli-Q water purification system (Millipore, Molsheim, France). Glucose was from Kemika (Zagreb, Croatia), fructose and H_2SO_4 from Merck (Darmstadt, Germany).



norvaline sarcosine

Figure 1: AA internal standards.



3-MPA OPA FMOC-Cl

Figure 2: Reagents for AA derivatization.

2. 2. AA Solutions

A mixed AA standard solution was prepared in water with all 22 AA and the two AA internal standards with a concentration of 1 mM of individual AA. The mixed AA internal standards (IS) solution was prepared by dissolution of 124 mg of norvaline and 37.6 mg of sarcosine in 5 mL of water.

2. 3. Sugar Solutions

Glucose and fructose solutions in water were prepared in the concentration range from 0.5 to 200 g/L. To 1 mL of the described solutions 20 μL of the mixed AA internal standard solution was added.

2. 4. Sample Preparation

All wine and must samples were stored at –20 °C in polystyrene vessels. Prior to AA and sugar determination, samples were equilibrated at 20 °C, centrifuged for 10 min at 14000 r.p.m. and filtered through 0.45 μm acetate membrane filters (Sartorius). Before AA determination 10 μL or 20 μL of the mixed AA internal standard solution was added to 1 mL of samples.

2. 5. AA Derivatization

For derivatization of AA, the following reagents were used: 0.1 M borate buffer in water with pH 9.9; 3-MPA/OPA reagent (pH 9.3): 10 mg/mL of OPA was dissolved in 0.02 M borate buffer (pH 9.9) with 0.8% of 3-MPA, then pH was adjusted with 10 M NaOH and FMOC-Cl reagent: 5 mg/mL in acetonitrile.

Derivatization was performed using the automatic injector: successive sampling of 2.5 μL of borate buffer and 0.5 μL of sample, then mixed two times and wait time of 0.5 min. Subsequently, 0.5 μL of 3-MPA/OPA reagent was added. After mixing six times, 0.5 μL of FMOC-Cl was taken up, mixed six times. After that, 32 μL of water was added, mixed two times and finally 20 μL of the mixture was injected.[9]

3. Instrumentation

3. 1. AA Determination

Analyses were performed using the Agilent HPLC system 1100 (Palo Alto, USA), equipped with degasser, quaternary pump, autosampler, column thermostat set at 40 °C and a diode-array detector set at 338 nm for 3-MPA/OPA derivates of primary AA and 262 nm for FMOC-Cl derivates of secondary AA. We used a Zorbax Eclipse AAA (4.6 × 150 mm) column with 5 μm particle size with a guard column Zorbax Eclipse AAA (4.6 × 12.5 mm) with the same particle size (Agilent, Palo Alto, USA). Mobile phase flow rate was 2 mL/min. The separation was carried out using a gradient of four eluents. Eluent A was ultra pure water, eluent B acetonitrile, C methanol and eluent D 40 mM Na_2HPO_4 adjusted to pH 7.8 with 10 M NaOH. The initial mobile phase composition was 100% D, which changed linearly from 1.9 min to 18.1 min to the composition of 5.7% A, 25.7% B, 25.7% C and 42.9% D. At 18.1 min the second linear gradient starts until 18.6 min, where composition was 10% A, 45% B and 45% C and this composition remained

constant until 22.3 min. Then, the composition changed to the initial one linearly in 1 min. The column was then equilibrated for 3 min with the initial mobile phase composition.

3. 2. Sugars Determination

Analyses were performed using HPLC system with following modules: degasser X-Act (Jour Research, VICI AG International), autosampler Marathon-XT (Spark Holland, Emmen, The Netherlands), isocratic pump Maxi star K-1000 and RI detector K-2301 (Knauer, Berlin, Germany). Aminex HPX-87H (7.8 × 300 mm) column was used (Bio-Rad, Hercules, USA). Mobile phase (2.5 mM H_2SO_4) flow rate was 0.5 ml/min. Injection volume was 20 μL.

4. Results and Discussion

Free amino acid content was monitored during fermentation of grape must of four different white grape varieties: Chardonnay, Malvasia, Sauvignon and Welsh Riesling. In the described samples, AA were determined at different stages of fermentation (Figure 3). Therefore, samples considerably differed in their sugar content (Table 1), as concentrations of glucose and fructose in the starting musts were approximately around 100 g/L of each, while the concentration of both sugars after fermentation to young wine was less than 2 g/L.

It was observed, however, that the extent of derivatization of AA IS norvaline and sarcosine in different samples differed considerably. A correlation between sugar content and peak areas of AA IS derivatives was sought for. In grape musts, where the initial concentrations of sugars were between 175 and 195 g/L, peak areas for norvaline derivative were approximately 65% smaller comparing to peak areas in young wines. The same was observed for sarcosine derivative, where peak areas were even by 75% smaller. In the partly fermented must, where approximately 30% of sugars were fermented (concentration 115–160 g/L), peak areas of both internal standards derivatives were 45–55% smaller compared to peak areas in young wines. After the amount of sugars dropped to approximately 50% of the initial sugar content (92–105 g/L), peak areas for norvaline derivative were smaller by 27% and for sarcosine derivative by 37% compared to young wines. Differences in peaks areas of IS derivatives in young wines and in musts after more than 60% of initial sugar fermented (concentration < 60 g/L) are not significantly different to those in young wines anymore. In relative terms, the observed results are independent of the volume of added IS solution (e.g. 10 or 20 μL, Table 1).

The observed influence of the presence of sugars on derivatization of AA with 3-MPA/OPA/FMOC-Cl was also studied with model sugar solutions. Peak areas of norvaline and sarcosine derivatives in solutions with different glucose and fructose concentrations were monitored (Figure 4). In the concentration range of 0.5–10 g/L, the relative standard deviations (RSD) of peak areas were less than 1.5% for norvaline and less than 6% for sarcosine derivative. Peak areas of sarcosine derivative at a concentration of sugars 50 g/L were significantly smaller (by 22% compared to the peak area in the absence of sugars), while peak areas of norvaline derivatives were still not significantly affected. At concentrations of sugars above

Table 1: Sugar contents and peak areas for derivatives of AA internal standards.

	Chardonnay (10 μL of IS added)			Malvasia (10 μL of IS added)			Sauvignon (20 μL of IS added)			Welsh Riesling (20 μL of IS added)		
	sugar content (g/L)	peak area (mAU* s) norvaline	sarcosine	sugar content (g/L)	peak area (mAU* s) norvaline	sarcosine	sugar content (g/L)	peak area (mAU* s) norvaline	sarcosine	sugar content (g/L)	peak area (mAU* s) norvaline	sarcosine
must	193.1	60	36	187.9	56	32	174.9	119	52	192.9	125	68
party fermented musts	162.6	59	36	150.3	75	49	149.2	138	77	192.9	101	59
	145.8	74	42	118.9	86	52	60.6	373	251	121.1	184	129
	145.8	77	41	118.9	77	55	58.3	–	225	103.2	234	145
	143.5	88	55	116.6	84	51	56.1	399	227	98.7	255	156
	143.5	84	42	116.6	75	60	56.1	402	226	96.4	253	168
	141.3	87	55	114.4	94	62	–	–	–	94.2	265	169
	141.3	83	55	–	–	–	–	–	–	92	263	189
young wines	4.6	147	97	7.3	190	139	2.4	345	256	1.8	328	235
	1.6	172	104	1.6	167	131	1.9	367	213	1.8	367	265
	1.4	167	93	1.5	181	125	1.8	347	224	1.7	342	297
	1.0	180	112	1.5	165	122	1.2	352	258	1.1	343	269
	1.0	173	84	1.0	182	127	0.8	328	198	0.6	345	267
	0.9	165	94	0.9	138	119	0.7	355	211	0.6	361	272
	0.8	188	102	0.9	171	128	0.7	341	214	0.5	367	276
	0.8	177	114	0.7	183	130	0.6	345	194	0.5	348	249

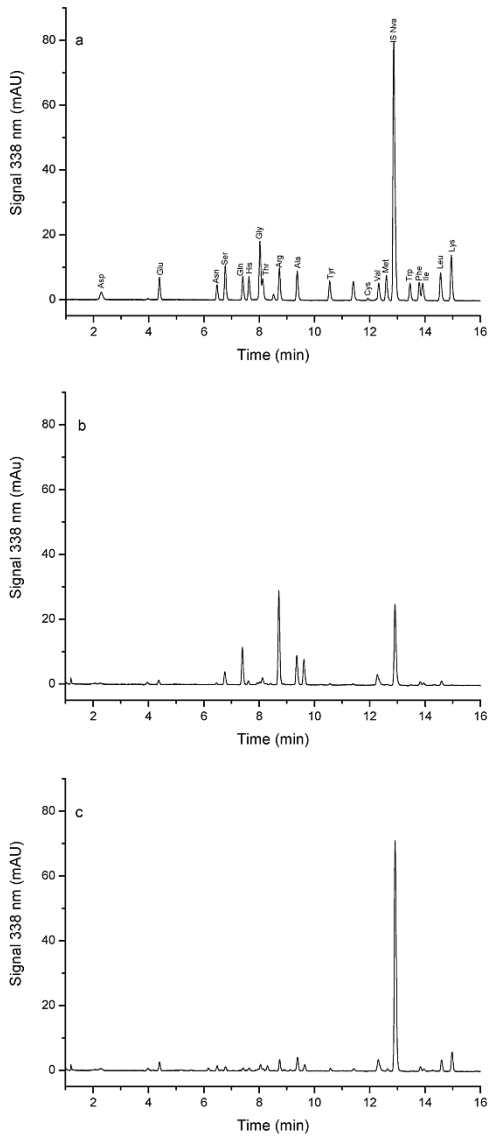


Figure 3: HPLC chromatograms of primary AA: (a) standard solution, (b) must and (c) young wine. In all instances 20 μL of IS norvaline was added.

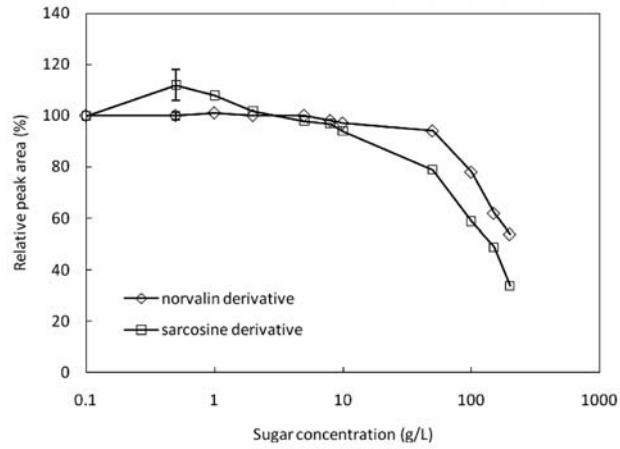


Figure 4: Peak areas for AA internal standard derivatives relative to peak areas in solutions without sugar. The error bar represents standard deviation.

100 g/L, the peak area for sarcosine derivative decreased down to 65% at 200 g/L. The change of peak area of the norvaline derivative was less pronounced and decreased by 45% at 200 g/L of sugars, compared to the peak area in the absence of sugars.

These results confirm that the presence of sugars at concentrations above 50 g/L in must and wine samples, considerably influences the derivatization of primary AA with 3-MPA/OPA and at concentrations above 10 g/L the effect is statistically significant for secondary AA derivatized with FMOC-Cl.

According to the results, we propose that quantitative determination of individual AA in must and wine samples is performed using calibration curves taking into account the response factors for AA standards relative to response factors of IS. The evaluation of AA content in musts ought to be corrected according to the reduction of IS peak areas relative to IS peaks areas in wines, where no effect of the presence of sugars was observed.

This improved procedure will be use in our forthcoming study of fermentation of musts using a variety of different procedures.

5. Conclusions

To avoid a systematic error in determination of AA in the presence of elevated concentration of sugars, the use of external calibration in combination with internal standards is suggested. This is especially important when AA content is monitored in samples with very variable sugar content, such as during fermentation of grape juice to wine.

6. Acknowledgement

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7. References

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Povzetek

Določevali smo proste aminokisline (AK) v moštih in mladih vinih s pred-kolonsko derivatizacijo primarnih AK s 3-MPA/OPA in sekundarnih AK s FMOC-Cl. Ker imajo ti vzorci zelo različno vsebnost sladkorjev, smo preučevali vpliv sladkorjev na derivatizacijo z uporabo dveh internih standardov: (i) primarno AK norvalinom in (ii) sekundarno AK sarkozinom in sicer v realnih vzorcih ter v modelnih raztopinah fruktoze in glukoze. Opazili smo velik vpliv matrice, še posebno, če je bila koncentracija sladkorjev večja kot 50 g/L. Vpliv sladkorjev je bil večji pri derivatizaciji sekundarne AK sarkozina kot pri derivatizaciji primarne AK norvalina. Za kvantitativno vrednotenje AK v vzorcih s spremenljivo in visoko vsebnostjo sladkorjev predlagamo uporabo umeritvene premice v kombinaciji z metodo internega standarda, kjer se odzivni faktor za posamezno AK korigira glede na odziv internih standardov.