Effect of Storage under CO_2 Atmosphere on the Volatile, Amino Acid, and Pigment Constituents in Red Grape (*Vitis vinifera* L. Var. Agiorgitiko)

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Volatiles of whole red grapes (*Vitis vinifera* L. var. Agiorgitiko) were isolated by using head-space technique. The grape prior to analysis were exposed for a period of 10 days under carbon dioxide atmosphere. This technique is currently used by wine-makers before the grapes are crushed and is called "carbonic maceration". The chemical composition of total volatiles was compared to this of control sample obtained from the same variety of grapes and stored for the same time period under normal atmosphere conditions. The composition of the amino acids and anthocyans, before and after carbon dioxide treatment, was also investigated. Significant differences in the constituents between the two groups were observed regarding the composition of the amino acids and anthocyans, as well as the volatile constituents, where 114 compounds were identified in the carbon dioxide treated sample, compared to 60 of the control sample.

INTRODUCTION

The exposure of whole grapes for 10 or more days under carbon dioxide atmosphere before their crushing is the technique known as "carbonic maceration" (Flanzy, 1935; Amerine and Ough, 1968, 1969). What results is an intracellular modification of aroma precursors and especially of the amino acids such as phenylalanine and leucine. This leads to a typical aroma of the grape must which persists after the fermentation of the treated grapes. The wine produced possesses a more intense "fruity-floral" aroma with "vanilla" and "rose" notes (Amerine and Fong, 1974), and it is characterized as "young wine".

Vitis vinifera L. var. Agiorgitiko is a *Vitis* variety from the region of Nemea in Greece, probably one of the oldest varieties, which gives a red wine rich in tannins and with a neutral olfactory profile. When experimental vinifications of *V. vinifera* L. var. Agiorgitiko grapes were carried out by using carbonic maceration, a fruity and floral wine was produced. The typical enriched volatiles of this wine giving the fruity-floral aroma were formed during the step of storage under carbon dioxide atmosphere.

Similar results were reported by Ducruet et al. (1983) when they treated red grapes of Carignan variety, before their vinification, with carbon dioxide. They concluded that among the typical volatiles were phenols, ethyl and methyl vanillate, and ethyl cinnamate and that they were formed during the first phase of vinification when whole grapes were kept under carbon dioxide atmosphere. Dubois et al. (1977) studied also the volatiles of wines obtained through carbonic maceration and they concluded that the typical aroma was due to compounds such as vinyl-4-guaiacol, vinyl-4-phenol, eugenol, methyl and ethyl vanillate, and ethyl cinnamate.

On the basis of these observations in Carignan we undertook this project with *V. vinifera* L. var. Agiorgitiko to investigate whether the volatiles obtained by carbonic maceration are independent of the grape variety and if they are formed exclusively in the whole grapes during the storage under carbon dioxide. In this study 114 compounds were identified in the grapes exposed to carbon dioxide prior to fermentation, against 60 of the control sample. In previous studies 50 compounds were reported in Carignan variety (Ducruet et al., 1983).

EXPERIMENTAL PROCEDURES

Preparation of the Plant Material. Technologically mature Agiorgitiko grapes (total sugar content 218 g/L, titrable acidity 6.45 g/L expressed as tartaric acid) were collected from the vineyard A. Schinochoritis in Archaia Nemea. The plants belong to *V. vinifera* var. Agiorgitiko, and they were not grafted to subject resistance to phylloxera. The grapes (1000 kg) were transported overnight to Athens and divided in two parts (MC and AIR).

Seven hundred kilograms of grapes, the first part (MC), was placed in seven heavy duty plastic vessels (110-L volume each) in the dark; the vessels were closed hermetically and were connected to a carbon dioxide cylinder though PVC tubes. The vessels were equipped with a stop air system which assured the evacuation of excess of carbon dioxide and prevented the entry of air. During the storage period, twice in a day, a purge with 99.99% carbon dioxide was carried out to maintain absolutely anaerobic conditions.

Three hundred kilograms of grapes, the second part (AIR), was placed in three heavy duty plastic vessels (110-L volume each) in the dark, under air. In both cases, care was taken to avoid damage or crushing of the grapes during transportation and treatment. During this period the temperature was held between 23 and 27 °C. After 10 days of storage, the vessels were opened and 10 kg of grapes was selected from each part (MC and AIR). The sample of 10 kg was placed in a cold room at 0 °C for 20 h and then crushed by hand pressing. The two blends derived from parts MC and AIR called, respectively, MC-1 and AIR-1, were subjected to analysis.

Isolation of Volatiles. Four hundred grams of each sample MC-1 and AIR-1 was separately placed in a head-space apparatus like the one described by Joulain (1986). A vacuum of 500 mTorr was applied, and a cold trap at −70 °C was used to collect the water and the volatiles codistilled due to the high vacuum. The whole process lasted for 4 h. Then the trap was disconnected from the system and was joined with a 500-mL round-bottom flask, where the trap content was left to thaw. The liquid volume collected was ~250 mL, and it was extracted three times with 50 mL of distilled Freon 11. The combined freon extracts were evaporated with a Vigreux column at room temperature, and the

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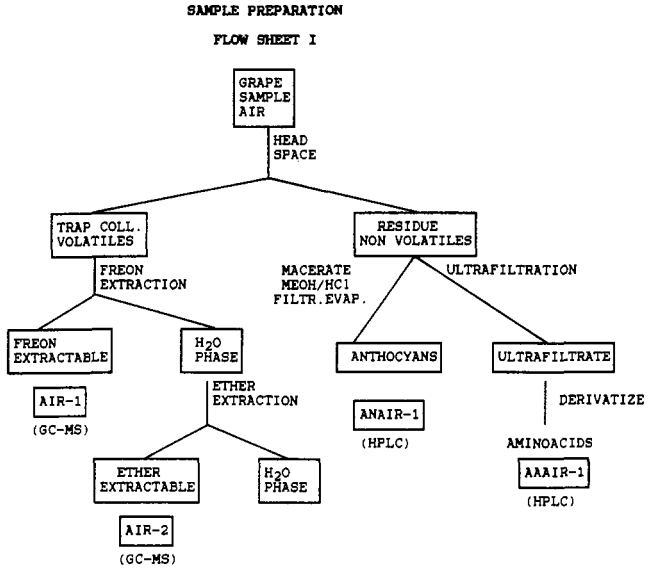
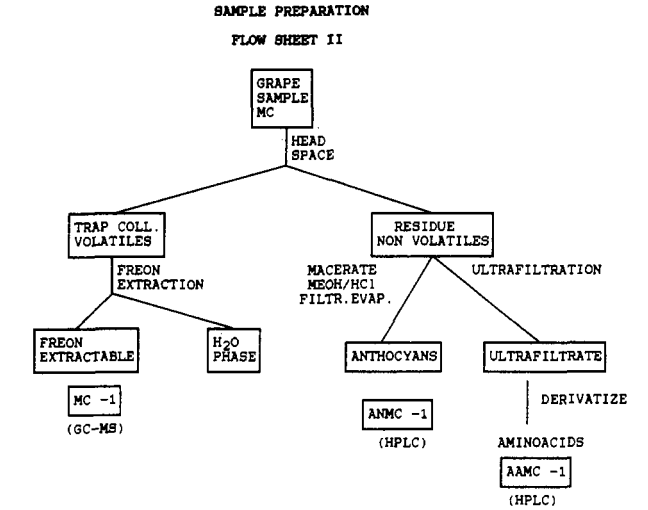
residues (~15 mg) were analyzed by GC–MS (see flow sheet I, samples MC-1 and AIR-1).



The water phase was subsequently extracted three times with 100 mL of diethyl ether. Ether extracts were dried over sodium sulfate, and the solvent was removed by evaporation at room temperature under water pressure vacuum (see flow sheet I, sample AIR-2). The residue (~20 mg) was analyzed by GC–MS. After the above treatment of the isolation of volatiles, the residual sample was used for amino acid and anthocyan analysis.

Isolation of the Anthocyans. Twenty grams of whole grapes (skin plus flesh) after the removal of the volatiles was macerated with 100 mL of methanol/HCl (99:1 v/v) in a cold room at 0 °C for 24 h. Then they were filtered through filter paper, and the filtrate was evaporated under vacuum to a standard weight. To this residue was added methanol to a volume of 11 mL, and this solution was the one used for the HPLC analysis (see flow sheets I and II).



Isolation of the Amino Acids. Twenty grams of whole grapes (skin plus flesh) after the removal of the volatiles was placed in cheesecloth and squeezed to obtain the grape juice. The samples were named AAMC-1 and AAAIR-1, respectively. Subsequently, the juice was ultrafiltrated by using a membrane with a MW cutoff of 1000 greater to eliminate all compounds with molecular weight greater than 1000. This technique has been used extensively in our laboratory and tested against standard amino acid mixtures in fruit and juice analysis. Of the ultrafiltrate, 4000 μL was adjusted to pH 8.90 with 1 N NaOH and then derivatized according to the following procedure, a modification of the method described by Kuneman et al. (1988).

Derivatization Procedure. One milliliter of the pH-adjusted ultrafiltrate was placed in a screw-top reaction vial; then the following were added and well mixed: 20 μL of D-isoleucine as internal standard. 100 μL of 2 N sodium carbonate solution, and 500 μL of Marfey's reagent. The mixture was placed in a oven at 75 °C for 1 h. Then 100 μL of 2 N HCl was added, and the mixture was analyzed by HPLC. The response factors of all amino acids have already been determined by using standard amino acids and were used for the quantitative determination of the amino acids of the grape samples AAMC-1 and AAAIR-1 (see flow sheets I and II).

GC–MS Analysis. A Hewlett-Packard 5070 chromatograph was coupled with a VG12-250 mass spectrometer system equipped with EI source. Gas chromatographic conditions were as follows: column, Carbowax 20 fused silica capillary; length, 25 m; i.d., 0.3 mm; film thickness, 0.2 μm; injector temperature, 200 °C; transfer line temperature, 230 °C; carrier gas, He; flow rate, 0.0166 cm^3/s; sample volume, 0.1 μL; split ratio, 1:100. For the analysis the following multistep temperature program was used:

initial temp, °C	initial time, min	rate, °C/min	final temp, °C	final time, min
70	3.0	2.00	150	1.00
		5.00	200	0.00
		10.00	230	20.00

The compounds were identified by comparison of their mass spectra with these of authentic samples either from the Vioryl S.A. private collection mass spectral library or from the NBS library. The compounds were confirmed by coinjection with authentic samples.

HPLC Conditions for Amino Acid and Anthocyan Analysis. A Perkin-Elmer Series 4 system equipped with a UV–vis variable-wavelength LC 85/autocontrol detector, and an LC-100 integrator were used.

Amino Acid Analysis. The chromatographic conditions were modified from the conditions of Kuneman et al. (1988). The column used and mainly the accurate pH control play a significant role in the resolution of amino acids such as aspartic acid, arginine, and glytamic acid. Column: Spherisorb ODS-1 5 μm RPC18; size 4.6 × 250 mm. Detector: UV–vis, λ = 340 nm.

The solvent system was a mixture of three solvents (A + B + C): (A) acetonitrile/water (10/90 v/v), triethylamine 3.36 g/L, and phosphoric acid to pH 3.00; (B) acetonitrile/water (40/60 v/v), triethylamine 3.36 g/L, and phosphoric acid to pH 3.00; (C) methanol 100%. For the analysis of amino acids a multistep solvent gradient was used:

time, min	flow, mL/min	A	B	C	gradient curve
0.5	1.5	100	0	0	
60	1.5	25	75	0	linear
5	1.5	0	100	0	linear
5	1.5	0	0	100	
20	1.5	0	0	100	

Anthocyan Analysis. Column: Spherisorb ODS-2 5 μm RPC_{18}; size 4.6 × 250 mm. Detector: UV–vis λ = 520, 280 nm.

Solvent system: (A) water/formic acid/acetonitrile (85/10/9 v/v); (B) methanol 100%. For the analysis of anthocyans according to the literature (Nagel and Wulf, 1979; Hong and Wrolstad, 1990a,b) the following multistep gradient program of solvents was used:

time, min	flow, mL/min	A	B	gradient curve
0	1	85	15	
10	1	85	15	
5	1	60	40	linear
5	1	60	40	
5	1	40	60	linear
15	1	0	100	
5	1	0	100	linear
15	1.5	85	15	

Materials. All of the solvents used were of HPLC grade supplied from Lab Scan. Marfey's reagent is 1-fluoro-2,4-dinitro-

Table 1. GC–MS Analysis of the Volatiles of Red Grape Samples: MC-1 (Stored under CO_2) and AIR-1 and AIR-2 (Stored under Air)

compound						compound					
no.	name	MC-1	AIR-1	AIR-2	pathway	no.	name	MC-1	AIR-1	AIR-2	pathway
1	hexanal	+	+		LXG	68	phenyl ethyl acetate	+			SHK
2	butanol			+		69	damascenone	+			CAR
3	3-methylbutyl acetate	+			LEU	70	calamene	+			TERP
4	pyridine			+		71	*trans*-anethole		+		SHK
5	ethyl but-2-enoate	+				72	methyl laurate	+			B-OXD
6	heptan-2-one	+				73	$C_{18}H_{38}$	+			
7	limonene	+	+		TERP	74	phenyl ethyl propionate	+			SHK
8	3-methylbutyl alcohol	+	+	+	LEU	75	2,6-dimethylundeca-2,6-dien-10-one (geranyl acetone)	+		+	TERP
9	*trans*-hex-2-enal	+	+		LXG						
10	diethyl disulfide			+		76	geraniol	+			TERP
11	ethyl hexanoate	+			LXG	77	benzyl alcohol	+			SHK
12	methylthiophene			+		78	ethyl dodecanoate	+			B-OXD
13	vinylbenzene	+			SHK	79	phenyl ethyl butyrate	+		+	SHK
14	hexyl acetate	+			LXG	80	butyl salicylate	+	+		SHK
15	3-hydroxy-2-butanone (acetoine)	+		+		81	phenylethyl alcohol	+	+		SHK
						82	benzothiazole	+			
16	2-methyl-2-hepten-6-one	+				83	γ-ionone	+		+	CAR
17	1-hexanol	+	+	+	LXG	84	phenyl propyl acetate	+			SHK
18	*cis*-hex-3-enol	+	+	+	LXG	85	$C_{19}H_{40}$	+		+	
19	nonanal	+			LXG	86	phenyl ethyl hexanoate	+			LXG/SHK
20	*trans*-hex-2-enol	+	+	+	LXG	87	heptanoic acid				B-OXD
21	diethylformamide			+		88	anisaldehyde	+	+		SHK
22	$C_{14}H_{30}$	+				89	dodecanol	+	+	+	B-OXD
23	acetic acid			+		90	phenol				SHK
24	ethyl octanoate	+			B-OXD	91	3-methylbutyl salicylate	+	+	+	SHK/LEU
25	α-copaene	+			TERP	92	phenyl propyl alcohol	+			SHK
26	butyrolactone			+		93	$C_{20}H_{42}$	+			
27	octyl acetate	+			B-OXD	94	hexyl benzoate	+			SHK/LXG
28	2-ethylpyridine			+		95	methyl branched acids			+	LEU
29	camphor	+			TERP	96	octanoic acid	+			B-OXD
30	methyl octyl ketone	+			B-OXD	97	pentyl salicylate	+			SHK
31	decanal	+			B-OXD	98	glyceryl triacetate (triacetine)	+			
32	3-methylbutyl hexanoate	+			LXG/LEU	99	decatrianol	+			
33	benzaldehyde	+			SHK	100	ethyl cinnamate	+			SHK
34	$C_{15}H_{32}$	+	+			101	*cis*-hexen-3-en-1-yl benzoate	+			SHK/LXG
35	nonanal diethyl acetal	+			LXG	102	γ-decalactone	+			CAR
36	linalol		+		TERP	103	$C_{21}H_{44}$	+			
37	octanol	+			B-OXO	104	eugenol	+			SHK
38	linalyl acetate		+		TERP	105	hexyl salicylate	+	+		SHK/LXG
39	dimethyl succinate	+				106	γ-undecalactone	+		+	
40	bornyl acetate		+		TERP	107	tetradecol		+	+	
41	methyl nonyl ketone	+				108	acetyleugenol	+			SHK
42	undecanal	+				109	$C_{22}H_{44}$	+			
43	silicon bcg	+			SHK	110	dihydroactiniolide	+			CAR
44	phenylacetaldehyde	+			SHK	111	veratraldehyde	+	+		SHK
45	$C_{16}H_{34}$	+			SHK	112	coumarine		+		SHK
46	acetophenone	+				113	benzyl hexanoate	+	+		SHK/LXG
47	diethyl succinate				0	114	ethyl palmitate	+		+	
48	decanal diethyl acetal	+			B-OXD	115	decanoic acid	+		+	B-OXD
49	ethyl decanoate	+			B-OXD	116	perinaphthindenone	+	+		
50	estragole	+	+			117	$C_{23}H_{48}$	+		+	
51	3-methylbutyl octanoate	+			B-OXD/LEU	118	diphenyl ketone	+	+		SHK
52	decyl acetate	+			B-OXD	119	$C_{24}H_{50}$	+	+		
53	naphthalene	+			TERP	120	dodecanoic acid	+			B-OXD
54	6,8-*p*-menthadien-2-one (carvone)	+			TERP	121	vanillin	+		+	SHK
						122	phthalate	+			
55	benzyl acetate	+	+		SHK	123	$C_{25}H_{52}$	+		+	
56	geranial	+			TERP	124	benzyl benzoate	+		+	SHK
57	*m*-dimethoxybenzene	+		+	SHK	125	$C_{26}H_{54}$	+		+	
58	$C_{17}H_{36}$	+				126	$C_{27}H_{56}$	+		+	
59	*cis*-anethole	+				127	$C_{28}H_{58}$	+		+	
60	methyl salicylate	+			SHK	128	palmitic acid	+		+	
61	hexanoic acid			+	LXG	129	$C_{29}H_{60}$	+		+	
62	methyl phenyl acetate	+			SHK	130	$C_{30}H_{62}$	+			
63	phenyl isopropyl alcohol	+			SHK	131	squalene	+			
64	geranyl acetate	+			TERP	132	oleic acid			+	
65	ethyl phenyl acetate	+			SHK	133	*p*-(1,3-dimethylbutylamino)-diphenylamine			+	
66	aliphatic hydrocarbon	+									
67	ethyl salicylate	+			SHK						

5-phenyl-L-alanine amide from Sigma. D-Isoleucine that was used as internal standard and pure amino acids standards were supplied from Janssen. The ultrafiltration system and the MW1000 membrane was supplied from Amicon.

RESULTS AND DISCUSSION

In the first part of this work we compare the composition of volatiles formed in whole grapes stored under carbon

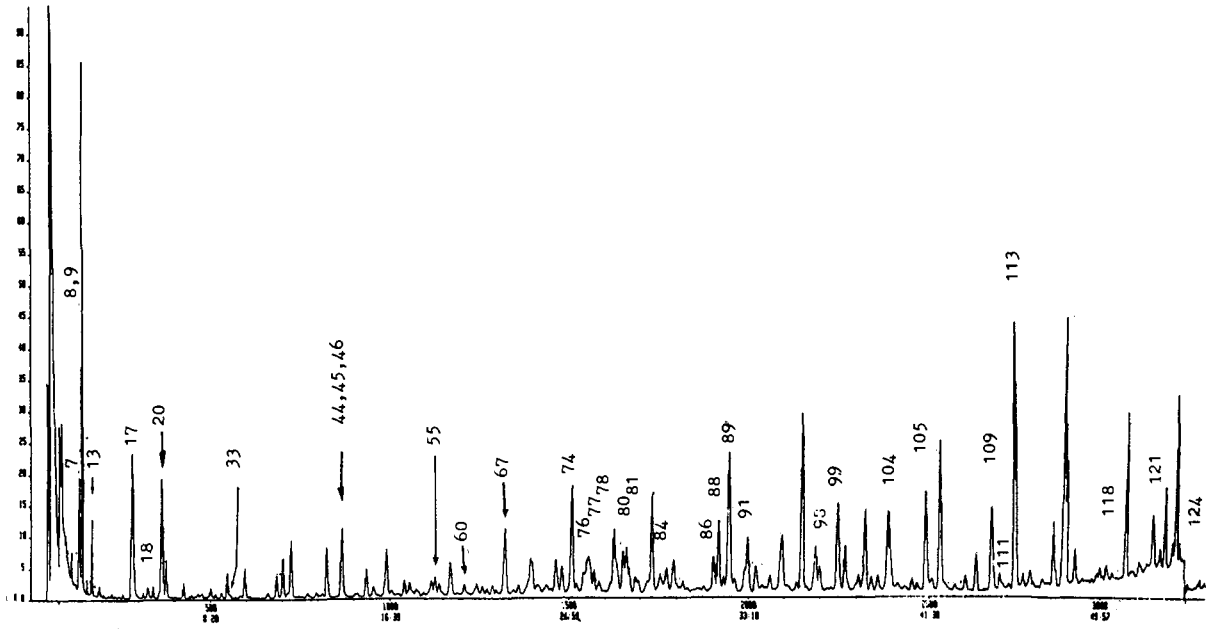


Figure 1. GC–MS analysis of the volatiles of sample MC-1.

Table 2. Amino Acid Composition of Grapes Stored under CO_2 (AAMC-1) and Air (AAAIR-1)

amino acid	retention time, min	mg/L in AAMC-1	mg/L in AAAIR-1	ratio
L-histidine	24.3	64.33	41.75	1.54
L-serine		137.6	102.6	1.34
L-aspartic	27.2	970.6	655.0	1.48
L-arginine	29.5	1827.5	1710.0	1.06
L-glutamic	31.0	140.0	224.0	0.62
L-glycine	34.3	55.3	12.0	4.60
L-alanine	36.1	283.5	132.5	2.15
proline	38.5	2851.5	1035.0	2.75
L-tyrosine	46.9	26.7	26.0	1.0
GABA	51.9	830.0	158.0	5.25
L-phenylalanine	65.02	45.2	22.6	2.0
L-leucine	65.8	23.3	12.6	1.85

Table 3. Anthocyan Composition of Grapes Stored under CO_2 (ANMC-1) and Air (ANAIR-1)

retention time, min	CO_2 (ANMC-1)	air (ANAIR-1)	peak area ratio CO_2/air
2.65	1051	3680	0.28
4.50	221	902	2.50
5.78	728		
6.50	3103	2314	1.30
8.80	2361	2372	1.00
9.50	27906	14330	1.95
20.77	2323	918	2.50
21.82	2588	1087	2.40
23.18	1370	60	2.30
25.50	11600	1855	6.20

dioxide atmosphere (MC-1) against that of the control sample (AIR-1) by using a head-space isolation technique in both samples. The head-space method with a "cold finger apparatus" was chosen to eliminate any interferences deriving from the extraction techniques. Another reason for the choice of this technique was the high efficiency of the method to give a real profile as it is perceived as aroma during flavor evaluation.

In previous work linear aliphatic alcohols and aldehydes such as (*Z*)-3-hexenol, 1-hexanol, and hexanal were reported in the same type of wines giving a more "vegetable" aroma. Aromatic aldehydes and ketones such as benzaldehyde and general secondary metabolites deriving from shikimic acid metabolism, characteristic of the carbonic maceration wines, were also reported (Flanzy et al., 1987). Vinylbenzene, ethyl cinnamate, eugenol, and methyl vanillate are typical volatiles produced after the anaerobic maceration of wine grapes, and they contribute to the fruity and spicy aroma of these wines (Flanzy et al., 1987).

The composition of the volatiles was determined in three samples. The first sample corresponds to carbonic maceration treated grapes (MC-1), and the others correspond to the grapes stored under air (AIR-1 and AIR-2). The volatiles identified are listed in Table 1. It is evident from the comparison of the chromatograms that the profile of volatiles after carbonic maceration (Figure 1, MC-1 GC–MS) was richer in volatile components than the control after storage under air (Figure 2, AIR-1 GC–MS). It is easily to explain why the wines derived from this technique are by far richer in aroma. The volatiles identified are formed through six different biogenetic pathways, which are listed below. In Table 1 there is a correlation between the compounds identified by GC–MS and the biogenetic pathways to which they can be attributed according to the literature. The differences observed between samples AIR-1 and AIR-2 are due to the different solvents employed (freon in AIR-1, ether in AIR-2).

1. *The shikimic acid pathway* (SHK in Table 1), passing through a phenylalanine step, is the most important, giving 34 volatile compounds.

2. *The* L-*leucine degradation pathway* (LEU in Table 1) leads to the formation of components which contribute to the overall aroma created after the storage of the grape under carbon dioxide. According to Schreier (1982), 3-methylbutyl compounds originate from this amino acid. Biogenesis of 3-methyl-1-butanol is catalyzed by L-leucine aminotransferase in a coupled reaction with 2-ketoglutamic acid, which acts as an amino group acceptor (Straus et al., 1986). The product of this reaction is glutamic acid, which by the action of L-glutamate α-decarboxylase gives γ-aminobutyric acid (GABA). In support of the above, our results showed that the GABA content was significantly increased 5.25 times higher after storage under carbon dioxide (Table 2), and this was also observed by Flanzy et al. (1987).

3. *Another minor group* of identified compounds originates from octanoic, decanoic, and dodecanoic acid (B-OXD in Table 1).

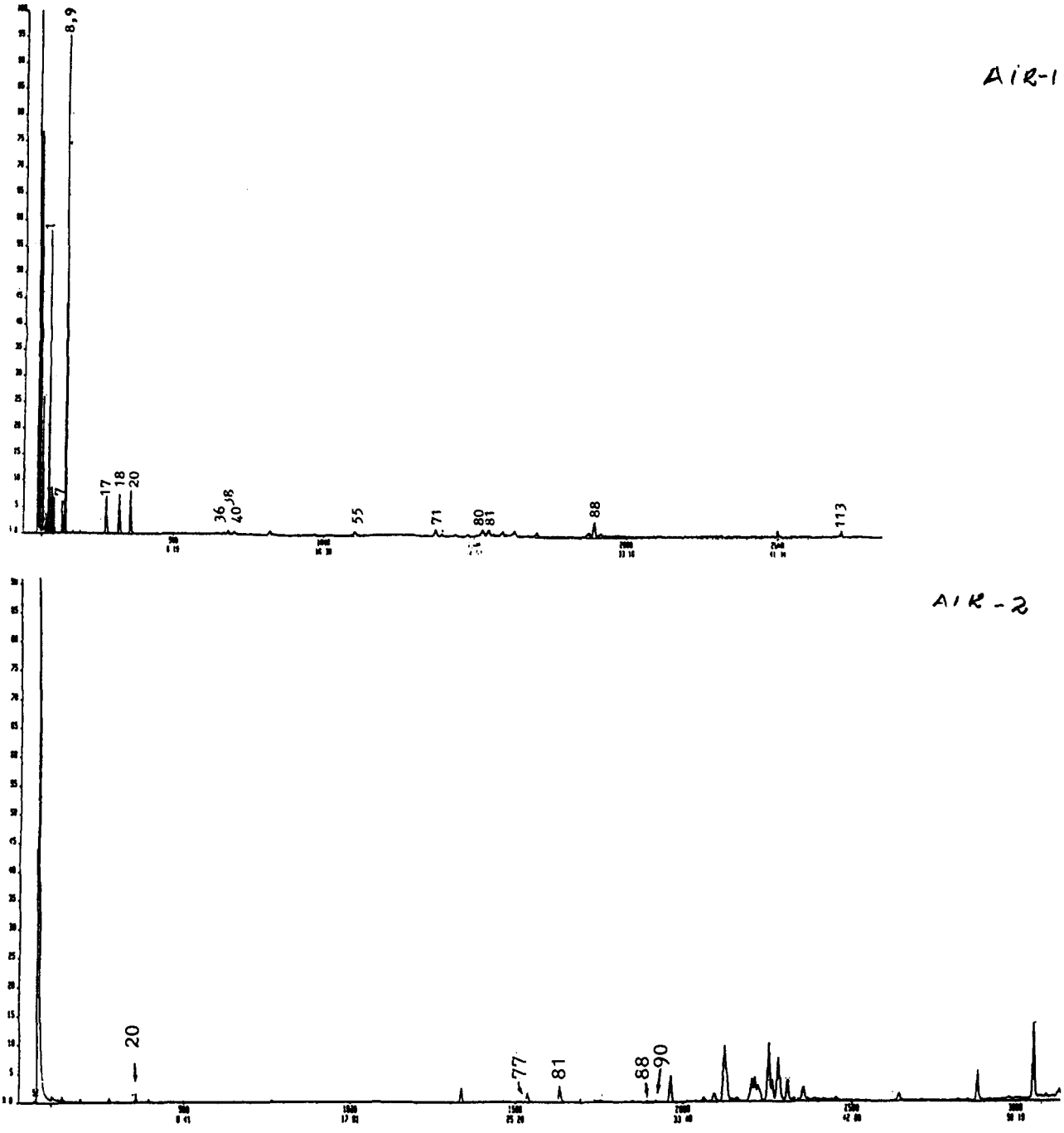


Figure 2. GC-MS analysis of the volatiles of samples AIR-1 and AIR-2.

4. In both experiments (MC and AIR) a mixture of hexanal, *trans*-hex-2-enal, 1-hexanol, *cis*-hex-3-enol, and *trans*-hex-2-enol was detected, contributing to the vegetable flavor. However, in the case of MC these are rather minor contituents among 100 other volatile components, and their off-flavor is not so evident and detectable. In contrast, in AIR experiments their presence is evident and significantly contributes to the whole aroma profile. These "stress metabolites" are the result of the physical disruption of the cell wall of the grape, and they are originate from unsaturated C_{18} fatty acids (Schreier et al., 1976) (LxG in Table 1).

5. Another group of flavor compounds are derivatives or degradation products of carotenoids such as damascenones, 8-ionone, γ-decalactone, and dihydroactiniolide (CAR in Table 1).

6. Finally some terpenes were detected only in trace amounts (Figures 1 and 2). We do not believe they affect the final aroma of this variety. Therefore, it is possible to classify Agiorgitiko as a rather neutral variety having flavor largely independent of monoterpenes (Straus et al., 1986) (TERP in Table 1).

The GC–MS identified volatile components and the biogenetic pathways through whoch they probably derive are listed in Table 1.

In Table 2 is presented a quantitative amino acid composition of grapes stored under CO_2 (sample AAMC-1) in comparison with that obtained from grapes stored in air (sample AAAIR-1), according to a modified method of Kuneman et al. (1988). The quantitative determination of each sample was based on calculation from two repeated derivatization procedures and three chromatographic runs

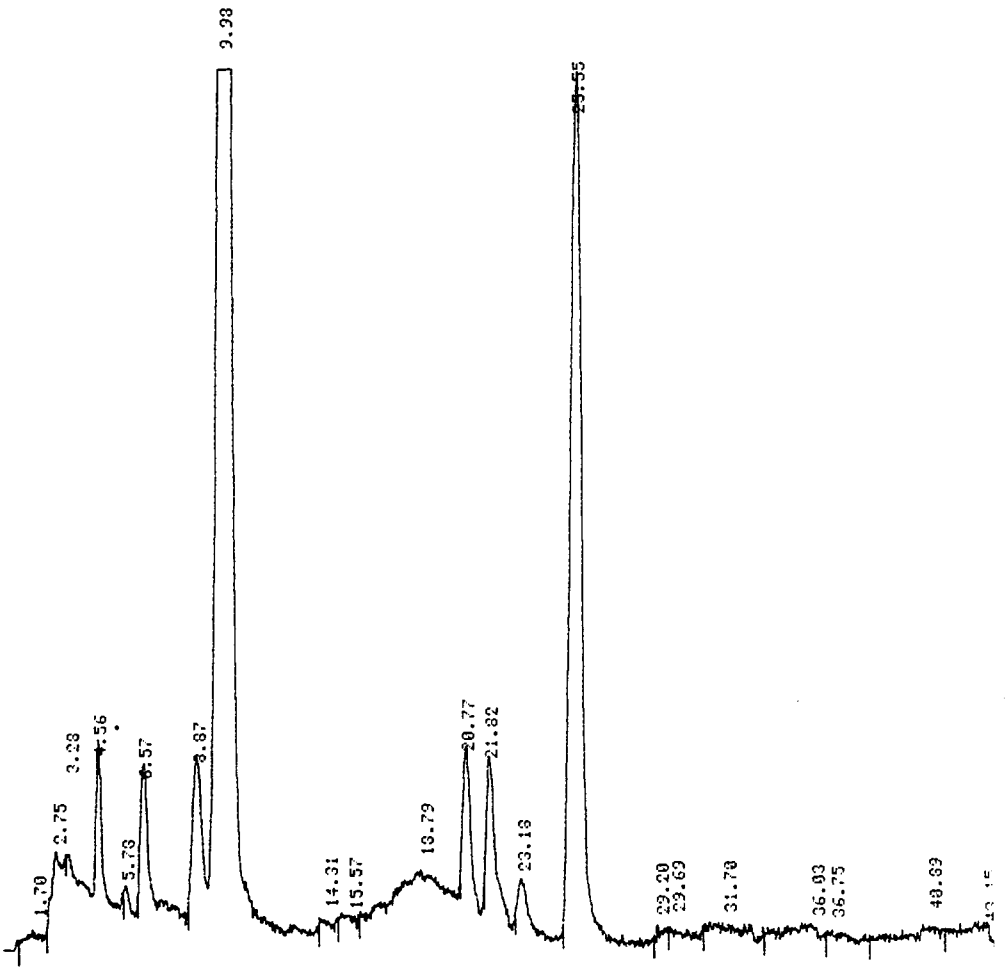


Figure 3. HPLC analysis of the anthocyans of sample ANMC-1.

(six chromatograms in total for each sample). Furthermore, the response factors using internal standard for each amino acid have been determined and were used for the quantitative results.

The efficiency of the ultrafiltration treatment of the juice has been tested, with standard mixtures of known quantities of amino acids, and was found to be excellent. An increase of the amounts of amino acids when grapes were stored under CO_2 was observed. According to the work of Flanzy et al. (1987) in the Carignan variety, a similar result has been obtained. The amounts of amino acids are expressed in milligrams per liter, and they were determined in extracts which had been previously concentrated during the evaporation of the volatiles and thus were higher, as revealed by the amino acid analysis of an untreated sample. Therefore, the true quantity of amino acids is about the half of the observed values. In this work and as previously reported in the work of Flanzy et al. (1987), GABA and L-glycine are the two amino acids for which storage under carbon dioxide atmosphere increased their amounts. In general, an increase was also observed in the quantities of most acids with the exception of glutamic acid.

In Table 3 and in Figures 3 (ANMC-1) and 4 (ANAIR-1) the anthocyan results are presented (samples ANMC-1 and ANAIR-1). As we can see from the peak area ratios CO_2/air in the third column, there are significant differences of almost all peaks detected at λ = 520 nm. The more pronounced difference was observed for the peak with RT 25.50, where a 6-fold increase appeared. The peak with RT 9.50 (probably malvidin glycoside, comparison with authentic sample), which was the major peak of the chromatograms, shows a 2-fold increase, while the peaks with RT 4.50, 20.77, 21.82, and 23.18 show a 2.5 fold increase.

Peaks unaffected were the RT 6.5 and 8.8, while a peak reduced in the CO_2 was the RT 2.65. Further work on the isolation and structure determination of the anthocyans is required.

CONCLUSION

As we can observe from GC–MS and HPLC compiled results, in connection with the previous works presented in the discussion of our results, the volatile compounds contributing to the flavor of carbon dioxide treated grapes are largely independent of the grape variety and they can be attributed to specific biochemical pathway regarding specific amino acids. Experiments made with other grape varieties by different authors conclude that these particular aroma compounds, found also in this grape variety when it is treated with carbon dioxide, persist after the alcoholic fermentation and characterize the wine. Anthocyan composition is also affected.

Aroma is essentially due to the phenyl-propanoid metabolism products, which, as we postulate, are reactivated postharvest and upon exposure of the intact red grapes to CO_2 atmosphere.

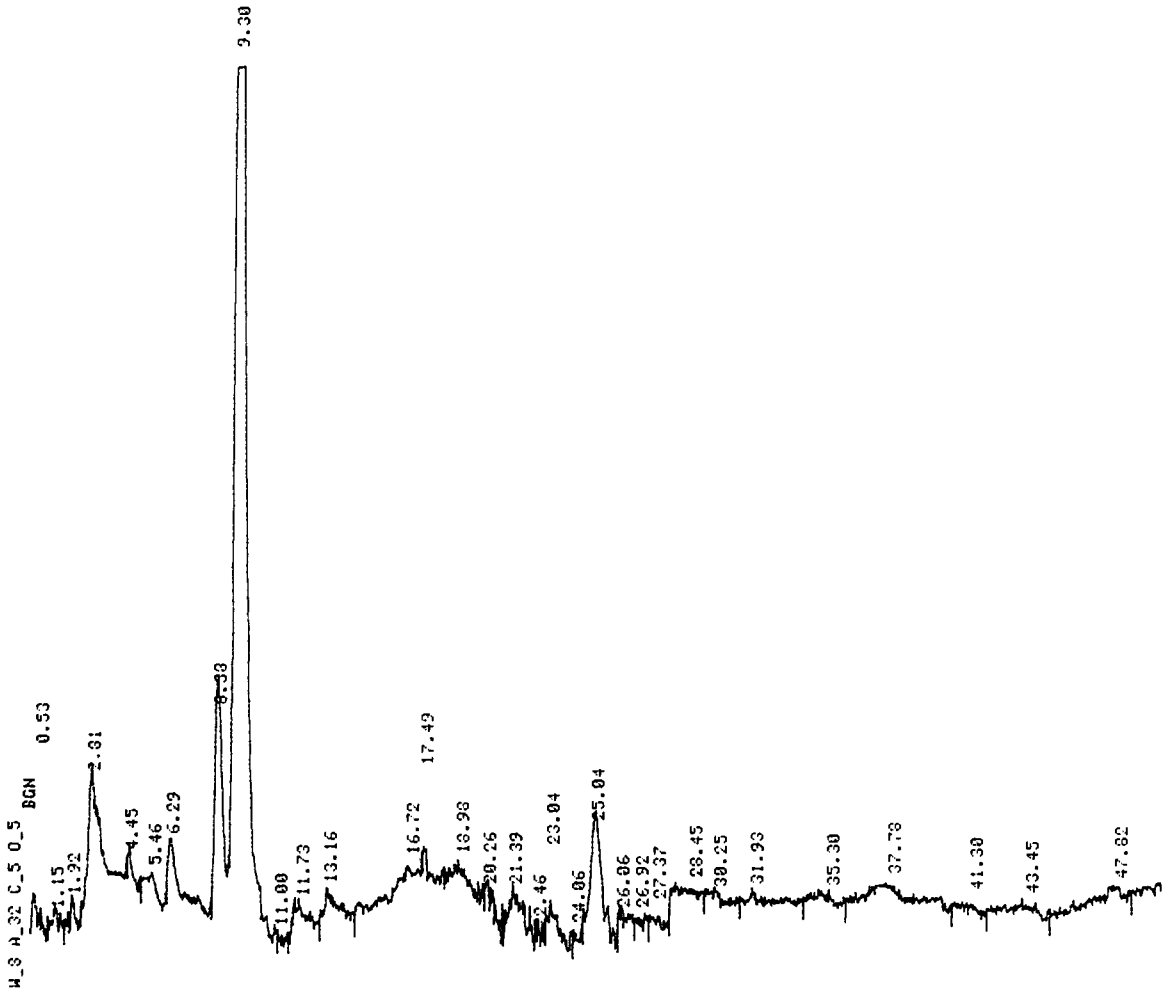


Figure 4. HPLC analysis of the anthocyans of sample ANAIR-1.

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