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Determination of 2,5-diketopiperazines in Greek Processed Olives by Liquid Chromatography/Mass Spectrometry Analysis

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ABSTRACT

Diketopiperazines (DKPs) are cyclic dipeptides which have been detected in a variety of natural products, especially in thermally treated or fermented foods and beverages, providing a metallic bitter taste. DKPs, mainly due to their characteristic heterocyclic system, have been reported to exhibit a broad spectrum of biological activities including antimicrobial, antiviral, antitumor, antihyperglycaemic and antimutagenic. In the present study, several DKPs were identified in seven different Greek varieties of processed olives using HR-LC-MS^n. The identification of DKPs in olive samples was achieved by comparison of their retention time and fragmentation pattern with reference DKP standards. The MS^n spectra were identical to confirm the presence of specific compounds because their results associate both fragmentation pattern and fragments' intensity. Nine compounds were found out of a total of 19 standard DKPs. The most prominent diketopiperazine was the cyclo(Phe-Phe) followed by and cyclo(Phe-Pro). Varieties where most DKPs were identified were Kothreiki, Kalamon, Throumpoelies and Helidoni.

Keywords: 2,5-diketopiperazines; HR-LC-MS^n; olives; fragmentation pattern.

INTRODUCTION

Olives have been cultivated in the Mediterranean area for at least 5,000 years and Greece is the one of the largest producers with approximately 2.5 million tons per year. Olives are significant source of antiradical, antioxidant and anti-inflammatory phytonutrients as simple phenols, flavonoids and terpenes and have gained an important place in the Mediterranean diet[1]). Green fermented (Spanish-type), black ripe or Californian-style, and naturally black or Greek-style olives in brine are the main types of brine-cured olives[2]. Brine-curing process, involves salt treatment, washing, and brine fermentation, sizing, and packaging[3]. Many changes in flavour and phytonutrient composition of olives can take place during the brine-curing process.

Diketopiperazines (DKPs), which are the smallest possible cyclic peptides composed of two-amino acids, are abundant natural compounds produced by various bacteria like *Bacillus subtilis*[4], *Pseudomonas aeruginosa*[5], or *Lactobacillus* *plantarum*[6] and fungi, e.g., *Aspergillus flavus*[7] or *Alternaria alternata*[8] as well as marine sponges[9]. Recently, the interest in this substantial class has increased due to their immense bioactivities, including antitumor, antiviral, antibacterial, antifungal, anthelmintic and anticancer[10,11,12,13,14]. Diketopiperazines have the ability to bind to a wide range of enzymes and receptors making them attractive scaffolds for drug discovery[15].

Considering the presence of diketopiperazines in numerous natural products, even in very low concentrations which makes them hardly traceable and their potential in medicine through their biological effects, it is of great interest to detect them in commonly consumed food commodities. To this extent, we present here the identification of 2,5-diketopiperazines in commercial fermented olives via the application of liquid chromatography/tandem mass spectrometry (LC/MS^n), using an electrospray ionization (ESI) Orbitrap mass spectrometer. To the best of our knowledge, this is the first report on the investigation of these biologically active compounds from fermented olives.

MATERIALS AND METHODS

Reagents and Standards

The cyclic dipeptides standards were: cyclo(Ala-Gly), cyclo-D-(Ala-Pro), cyclo-D-(Ala-Val), cyclo(Pro-Val), cyclo(Ala-His), cyclo(Leu-Pro), cyclo(Phe-Pro), cyclo(Leu-Phe), cyclo(Phe-Phe), cyclo(His-Phe), cyclo(Leu-Trp), cyclo(Asp-Gly), cyclo(Asp-Asp), cyclo(Trp-Tyr), cyclo(Val-Val), cyclo(Gly-Leu), cyclo(Ser-Tyr), cyclo(Phe-Ser) and cyclo(Gly-Gly). All standards were purchased from Sigma Chemical Co (Sigma-Aldrich Company, St. Louis, MO, USA). All solvents used for sample preparation were of analytical (LC-MS) grade from Merck (Darmstadt. Germany).

Sampling and sample preparation

Commercial fermented olives of seven olive tree cultivars (*Olea europaea* L.) from different regions of Greece were purchased and studied: ‘Amphissis’, ‘Hondroelia’, ‘Kalamon’, ‘Throumbolia’, ‘Koroneiki’,‘Megaritiki’ and ‘Kothreiki’. Concerning Kalamon variety, fruit size varies from small to giant and mammoth. The complete sample-set is presented in Table 2.

Fermentation process for selected cultivars includes packaging of the fruits into airtight and food-grade containers covered with brine. The lids of the containers were closed loosely and the filled containers were stored at 25 ^{o}C.

Isolation of 2,5-diketopiperazines from olive pastes

Olive’s pastes were prepared by removing the stone and homogenised the rest of the fruit using a blender. Pastes (30 g) were extracted by solid-liquid extraction according to Ryan et al.[16] with the following modifications. For pigments and lipids removal n-hexane extraction was applied four times (4 x 50 mL) at room temperature for 30 min. After centrifugation, the residual material was extracted 4 times with acetone/water (70/30, v/v) for 45 min at room temperature under stirring. The procedure was repeated thrice (3 x 50 mL), the samples were centrifuged and the combined supernatants were dried over anhydrous sodium sulphate, filtered and acetone was removed using a rotary evaporator at 30 ^{o}C.. The 2,5-diketopiperazines were extracted from the aqueous solution obtained with dichloromethane (DCM) by liquid/liquid extraction. The procedure was repeated thrice (3 x 30 mL) and the combined extracts dried over anhydrous sodium sulphate, filtered and concentrated in pre-weighed vials in a rotary evaporator to constant weight, to determine the extractable content. Afterwards, the extracted 2,5-diketopiperazines were redissolved in DCM, filtered through a 0.45 ìm filter and stored at -20 ºC until used for LC-MS^n analysis.

Liquid Chromatography – Mass Spectrometry (LC-MS)

Chromatographic conditions

LC analyses were performed using an a Thermo Accela U-HPLC injecting 1 µL sample from a cooled tray (10°C) directly onto an Kromasil column (2.1 mm × 100 mm, 1.8 µm particles, equilibrated in 90% solvent A (0.1% aqueous solution of acetic acid) and 10% solvent B (acetonitrile containing 0.1% acetic acid). The compounds were eluted using flow rate 250 µL/min by linearly increasing solvent B concentration from 10% to final 60% over 7 min . The column was then washed increasing solvent B up to 100% (3 min) and re-equilibrated in 90% solvent A, 10% solvent B. The total run time, including column wash and equilibration, was 15 min.

Table 1: ESI-MS^n data of protonated 2,5-diketopiperazines standards

2,5-diketopiperazines	Rt	ms^1 (MH$^+$) experimental m/z	ms^1 (MH$^+$) theoretical m/z	Delta (ppm)	ms^2	ms^3	ms^4
Cyclo D-Ala-Pro $C_8H_{12}N_2O_2$	1.61	169.0972	169.09705	-0.9	152.0715 (19), 141.0882 (57), 124.0725 (24), 113.1010 (1), 70.0447 (100)	-	-
Cyclo Pro-Val $C_{10}H_{16}N_2O_2$	4.02	197.1285	197.12848	-0.1	180.0976 (11), 169.1190 (100), 155.9611 (6), 141.1104 (10), 124.1116 (4), 72.0719 (6), 70.0441 (14)	141.1113 (100), 124.1116 (92), 72.0719 (4), 70.0441 (4)	124.1116 (100), 112.2508 (4), 70.0441 (5)
Cyclo Val-Val $C_{10}H_{18}N_2O_2$	4.95	199.1441	199.14415	0.2	171.1223 (100), 154.1027 (32), 126.1556 (7), 72.0649 (12)	126.1556 (100), 72.0649 (91)	109.1594 (100), 83.1121 (7), 70.0998 (85), 96.1712 (22), 67.1334 (27), 58.1351 (24)
Cyclo Leu-Pro $C_{11}H_{18}N_2O_2$	5.26	211.1441	211.1442	0.5	194.0910 (23), 183.1413 (100), 166.0809 (5), 155.0913 (11), 138.1223 (7), 127.0902 (4), 86.0968 (61), 70.0447 (35)	165.1234 (4), 156.0789 (3), 155.0913 (94), 138.1223 (100), 127.0902 (41), 110.2324 (1), 98.2212 (2), 86.0968 (11), 70.0447 (2)	123.0332 (74), 110.2324 (1), 108.0232 (25), 96.1112 (42), 95.1807 (10), 82.1414 (74), 70.0447 (100)

Cyclo Ala-His $C_9H_{12}N_4O_2$	0.76	209.1033	209.10339	0.4	192.0459, 181.1180 (100), 166.0686, 164.0912, 138.1237 , 136.0954, 110.1667	164.0912, 138.1237 , 136.0954, 110.1667	136.0954
Cyclo Gly-Gly $C_4H_6N_2O_2$	0.89	115.0502	115.05006	-1.2	98. 0334 (8), 97.0712 (60), 94.3567 (16), 91.0116 (8), 87.0457 (100), 80.1424 (6), 73.0671 (9), 70.9500 (10), 70.0013 (10), 69.0569 (13), 59.1112 (8)	59.0034 (100)	
Cyclo Asp-Gly $C_6H_8N_2O_4$	0.90	173.0557	173.05566	-0.2	155.0346 (100), 150.0706 (1), 131.9429 (16)	127.0326 (95), 113.0309 (100), 99.1152 (15), 85.0943 (89)	85.0157 (100)
Cyclo Asp-Asp $C_8H_{10}N_2O_6$	0.94	231.0612	231.06087	-0.3	213.1090 (100), 195.1009 (1), 188.9608 (2), 171.9804 (1), 170.9812 (7)	195.0423 (100), 171.1012 (1)	167.0123 (39), 153.0367 (19), 125.0552 (100), 124.1182 (41), 98.1082 (23)
Cyclo Ala-Gly $C_5H_8N_2O_2$	0.94	129.0659	129.06572	-1.4	112.0046 (10), 111.0544 (3), 101.0724 (100), 100.1613 (1), 84.0611 (24), 83.1568 (1).	83.9935 (100)	

Cyclo-D-Ala-Val $C_8H_{12}N_2O_2$	2.07	171.1128	169.11272	-0.5	143.0985 (100), 126.0907 (89), 98.1011 (2), 72.0634 (41)	115.0520 (11), 98.1096 (39), 72.1565 (100)	54.9810 (100)
Cyclo Gly-Leu $C_8H_{14}N_2O_2$	3.62	171.1128	171.11269	-0.7	154.0612 (9), 153.1334 (4), 143.1320 (62), 126.0928 (100), 115.0976 (45), 114.1421 (11), 87.0809 (19), 86.1315 (47)	108.0710 (33), 98.00989 (100), 95.0734 (28), 81.1217 (25), 69.1115 (8)	83.1423 (43), 81.0718 (36), 70.1710 (31), 69.0567 (65), 67.1443 (42), 55.9808 (100)
Cyclo Trp-Tyr $C_{20}H_{19}N_3O_3$	5.55	350.1499	350.14929	-1.7	333.1894 (21), 322.2266 (3), 305.2446 (6), 219.1049 (5), 191.0855 (5), 181.0931 (5), 170.0350 (14) , 136.0539 (13), 132.0477 (38), 130.0844 (100), 117.1609 (2)	-	-
Cyclo Phe-Pro $C_{14}H_{16}N_2O_2$	5.59	245.1285	245.12833	-0.7	217.1558 (100), 228.0948 (2), 200.0919 (4), 189.1433 (2), 120.0862 (61), 98.1401 (*),70.1247 (10)	172.10 (100)	157.1198 (10), 155.0533 (9), 144.0462 (100), 143.0296 (15), 130.0653 (5), 129.0496 (20), 105.0946 (10), 91.0810 (2), 68.0275 (2)

Cyclo Leu-Trp $C_{17}H_{21}N_3O_2$	6.30	300.1707	300.17056	-0.5	283.2324 (6), 272.2537 (2), 255.2095 (1), 170.0391 (7), 169.1170(1), 132.0329 (28), 130.0727 (100)	-	-
Cyclo Leu-Phe $C_{15}H_{20}N_2O_2$	6.54	261.1598	261.15955	-1.0	244.1782 (1), 233.1786 (100), 216.1437 (31), 205.0758 (1), 188.1208 (1), 148.1770 (*), 120.0578 (46), 86.0968 (9)	216.1272 (2), 205.1228 (2), 188.1725 (100), 177.1394 (5), 148.1770 (1) , 132.1192 (8), 120.2131 (98), 105.2924 (1), 86.2793 (46)	171.0853 (26), 159.0783 (18), 146.0469 (11), 145.0730 (4), 143.0459 (19), 132.1192 (56), 129.0533 (18), 120.0578 (17), 117.0749 (17), 105.1032 (100), 96.1908 (3), 91.1076 (4), 84.1504(1)
Cyclo Phe-Phe $C_{18}H_{18}N_2O_2$	6.85	295.1441	295.14395	-0.5	278.2380 (1), 267.2574 (100), 250.2240 (6), 222.1727 (1), 120.1214 (48)	250.2240 (2), 222.1727 (2), 130.1475 (17), 120.2656 (100)	103.0847 (100)
Cyclo His-Phe $C_{15}H_{16}N_4O_2$	1.94	285.1346	285.13434	-0.9	268.2142 (54), 257.2142 (100), 240.1753 (10), 224.1747 (2), 212.1779 (30), 166.0500 (27),	240.1753 (78), 239.3858 (9), 212.1779 (100), 175.1889 (10), 138.0925 (17), 120.1379 (6),	195.0721 (94), 185.1238 (14), 183.0926 (11), 130.0482 (63),

					138.0925 (3), 120.1379 (2), 110.1199 (18)	110.1199 (62), 83.2689 (2).	120.1379 (100), 83.1260 (48).
Cyclo Phe-Ser $C_{12}H_{14}N_2O_3$	3.59	235.1077	235.10767	-0.1	218.0464 (*), 217.1461 (12) , 207.1324 (100) , 190.1201 (1) , 189.1138 (17) , 167.1102 (3) , 162.1047 (3) , 120.1004 (3)	207.1105 (12), 190.0646 (7), 189.1050 (11), 177.1141 (9), 162.1708 (86), 160.3000 (3), 144.2778 (22), 121.0335 (13), 120.3363 (100), 88.0757 (24), 87.4081 (56).	-
Cyclo Ser-Tyr $C_{12}H_{14}N_2O_4$	1.48	251.1026	251.10252	-0.3	234.2054 (*) , 233.1349 (10), 223.1624 (100), 206.1135 (1), 205.1278 (13), 178.1202(2), 145.0716 (12), 136.1216 (3), 107.1013 (5)	206.0938 (6), 205.1278 (11), 193.1215 (9), 188.0735 (1), 178.1202 (80), 160.2467 (20), 137.0477 (6), 136.1216 (100), 88.0399 (14), 87.3757 (29), 70.6184 (5).	119.0214 (100), 91.0941 (10).

Table 2: Sample set of Greek olive varieties

Sample No	Cultivar
1	'Throumbolia' large sized fruit from Megara
2	'Kothreiki' medium sized fruit from Amphissa
3	'Kalamon' medium sized fruit from Korinthos
4	'Kalamon' medium sized fruit from Ilia
5	'Amphissis' medium sized fruit from Fthiotida
6	'Hondroelia' large sized fruit from Amphissa
7	'Megaritiki' medium sized fruit from Megara
8	'Kalamon' sweet giant sized fruit from Kalamata
9	'Kalamon' sweet medium sized fruit from Kalamata
10	'Kalamon' giant sized fruit from Kalamata
11	'Kalamon' mammoth sized fruit from Kalamata
12	'Amphissis' medium sized fruit from Fokida
13	'Kalamon' medium sized fruit from Megara
14	'Koroneiki' medium sized fruit from Ilia

Mass spectrometry analysis

For LC/ESI-MS experiments, a Thermo Scientific LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was connected to the UHPLC instrument via an HESI interface. Centroided mass spectra were acquired with AGC target value of 1E6, resolution of 30,000 (FWHM as defined at m/z 400), and maximum ion injection time (IT) of 100 ms, for full scan analysis in the mass range of m/z 80 to 380 Da followed by data dependent MS/MS on the linear ion trap on the most intense ion from parent list. The normalized collision energy for the high efficiency collision-induced dissociation (CID) was adjusted to 35%, and the isolation width of precursor ions was m/z 2.0

The mass spectrometer was operated in the positive ion mode using a source voltage of 3.50 kV. The capillary temperature was 300 ^{o}C and the source heater temperature was 200 ^{o}C. The auxillary gas and sheath gas flow rates were set at 35 Arb and 15 Arb, respectively. The instrument was calibrated daily using the manufacturer's calibration mixture ProteoMass LTQ/FT-Hybrid ESI Pos. Mode Cal Mix (SUPELCO, Bellefonte, PA, USA).

Mass Frontier 6.0 (High Chem Ltd.) was implemented to produce possible fragments and mechanisms using standard databases (HighChem ESI Pos 2008 and HighChem Fragmentation Library). Liquid Chromatography – Mass Spectrometry (LC-MS)

Table 3: DKPs identified in the 14 samples

DKP	Times found	Samples 1	2	3	4	5	6	7	8	9	10	11	12	13	14
Phe_Phe	14	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Leu_Phe	7		√	√	√	√	√	√							√
Pro_Val	6		√	√	√	√	√								√
Phe_Pro	6		√	√	√	√	√								√
Leu_Pro	6		√	√	√	√	√								√
D_Ala_Pro	5		√	√	√	√	√								√
Leu_Trp	2		√				√								
Val-Val	1				√										

Table 4: DKPs detected in Greek olive varieties

S.	Category	DKPs	Expected m/z	Detected m/z	Expected RT	Actual RT	Delta mDa	Delta ppm	Ion Intensity
1	A	Phe_Phe	295.1441	295.14340	6.85	6.84	-0.7	-2.4	61875
2	NA	D_Ala_Pro	169.0972	169.097	1.61	1.64	-0.2	-1.2	21567
	NA	Pro_Val	197.1285	197.12849	4.02	4.03	0.0	0.0	193204
	NA	Leu_Pro	211.1441	211.1442	5.26	5.27	0.1	0.5	647370
	A	Phe_Pro	245.1285	245.12814	5.59	5.62	-0.4	-1.5	563572
	A	Leu_Trp	300.1707	300.16977	6.3	6.42	-0.9	-3.1	81708
	A	Leu_Phe	261.1598	261.15961	6.54	6.56	-0.2	-0.7	228411
	A	Phe_Phe	295.1441	295.14377	6.85	6.85	-0.3	-1.1	193890
3	NA	D_Ala_Pro	169.0972	169.09682	1.61	1.68	-0.4	-2.3	26174
	NA	Pro_Val	197.1285	197.12852	4.02	4.1	0.0	0.1	175677
	NA	Leu_Pro	211.1441	211.1441	5.26	5.34	0.0	0.0	441283
	A	Phe_Pro	245.1285	245.12831	5.59	5.67	-0.2	-0.8	312225
	A	Leu_Phe	261.1598	261.15961	6.54	6.63	-0.2	-0.7	65427
	A	Phe_Phe	295.1441	295.14352	6.85	6.94	-0.6	-2.0	87246
4	NA	D_Ala_Pro	169.0972	169.09705	1.61	1.68	-0.2	-0.9	39764
	NA	Pro_Val	197.1285	197.12849	4.02	4.05	-0.0	-0.0	286386
	NA	Val_Val	199.1441	199.1441	4.95	5.01	0.0	0.0	24917
	NA	Leu_Pro	211.1441	211.1441	5.26	5.3	0.0	0.0	1715003
	A	Phe_Pro	245.1285	245.12825	5.59	5.63	-0.2	-1.0	1043307
	A	Leu_Phe	261.1598	261.15961	6.54	6.58	-0.2	-0.7	1024470
	A	Phe_Phe	295.1444	295.14383	6.85	6.89	-0.3	-0.9	265837
5	NA	D_Ala_Pro	169.0972	169.09686	1.61	1.65	-0.3	-2.0	22418
	NA	Pro_Val	197.1285	197.12863	4.02	4.02	0.1	0.7	118060
	NA	Leu_Pro	211.1441	211.14413	5.26	5.26	0.0	0.2	648795
	A	Phe_Pro	245.1285	245.12831	5.59	5.61	-0.2	-0.8	331521
	A	Leu_Phe	261.1598	261.15964	6.54	6.55	-0.2	-0.6	641009
	A	Phe_Phe	295.1441	295.14392	6.85	6.86	-0.2	-0.6	296654
6	NA	D_Ala_Pro	169.0972	169.09715	1.61	1.73	-0.0	-0.3	23377
	NA	Pro_Val	197.1285	197.12852	4.02	4.1	0.0	0.1	99863
	NA	Leu_Pro	211.1441	211.14417	5.26	5.28	0.1	0.3	662646
	A	Phe_Pro	245.1285	245.12828	5.59	5.62	-0.2	-0.9	671771
	A	Leu_Trp	300.1707	300.17212	6.3	6.31	1.4	4.7	15331
	A	Leu_Phe	261.1598	261.1598	6.54	6.55	-0.2	-0.7	981456
	A	Phe_Phe	295.1441	295.14398	6.85	6.85	-0.1	-0.4	486008
7	A	Leu_Phe	261.1598	261.15961	6.54	6.54	-0.2	-0.7	17954
	A	Phe_Phe	295.1441	295.14365	6.85	6.84	-0.5	-1.5	73411
8	A	Phe_Phe	295.1441	295.14349	6.85	6.85	-0.6	-2.1	44665
9	A	Phe_Phe	295.1441	295.1438	6.85	6.86	-0.3	-1.0	77874
10	A	Phe_Phe	295.1441	295.1438	6.85	6.87	-0.3	-1.0	45481
11	A	Phe_Phe	295.1441	295.1434	6.85	6.84	-0.7	-2.4	77116
12	A	Phe_Phe	295.1441	295.14374	6.85	6.89	-0.4	-1.2	96764
13	A	Phe_Phe	295.1441	295.14359	6.85	6.89	-0.5	-1.7	101429
14	NA	Pro_Val	197.1285	197.12849	4.02	4.12	-0.0	-0.0	27507
	NA	Leu_Pro	211.1441	211.14406	5.26	5.32	-0.0	-0.2	177882
	A	Phe_Pro	245.1285	245.12827	5.59	5.66	-0.2	-1.0	176189
	A	Leu_Phe	261.1598	261.1597	6.54	6.59	-0.1	-0.4	217984
	A	Phe_Phe	295.1441	295.14377	6.85	6.91	-0.3	-1.1	72453

A: Aromatic DKP; NA: Non aromatic DKP

RESULTS AND DISCUSSION

The presence of DKPs in the studied olives is the result of fermentation from microorganisms. Their presence increases the olives' nutritional value and contributes to their organoleptic characteristics, giving bitter and metallic taste. The identification of the 2,5-diketopiperazines(2,5-DKPs) in the 14 different samples was performed by comparison of their retention times and mass spectra with those of reference compounds. The retention times, the molecular masses of the protonated DKP standards as identified by ESI-MS^1 and expressed in mono-isotopic basis as well as their mass spectral characteristics are specified in Table 1. The high-resolution mass data of protonated 2,5-DKPs confirmed their elemental composition, given a mass error of below 1.7 ppm with their theoretical molecular mass. The complete chromatogram of the 19 standard DKPs is provided in Figure 1 and the total ion chromatograms of olive samples are presented in Figure 2.

Fragment ions with intensities <5% of the base peak were mentioned only in the case they were needed for identification or comparison. The 2,5-diketopiperazines showed similar fragmentation patterns and revealed the characteristic mass spectrometric behavior in ESI($^+$) experiments, producing M^+ ions in their MS^1 spectra and the product ions $[MH^+ - 28]$, $[MH^+ - 17]$ and $[MH^+ - 45]$ in their MS^2 spectra[17], with different intensities of the fragments.

Results showed that only 8 out of 19 standard DKPs were identified in the 14 samples (Tables 3 and 4). Figure 3 presents the chromatograms of the 8 standard DKPs found and their corresponding peaks in the olive samples. One common identified DKP in all 14 samples is cyclo(Phe-Phe) followed by cyclo(Leu-Phe) found in 7 samples and cyclo(Pro-Val), cyclo(Phe-Pro) together with cyclo(Leu-Pro) were found in 6 samples (Tables 2 and 3).

Cyclo(Phe-Phe) has also been detected in a wide variety of beverages and foods such as beef, cheddar cheese, cocoa, white wine, yeast extract, etc. It is a naturally occurring 2,5-diketopiperazine and is dual inhibitor of the serotonin transporter (SERT) and acetylcholinesterase (AChE) *in vitro*[18].

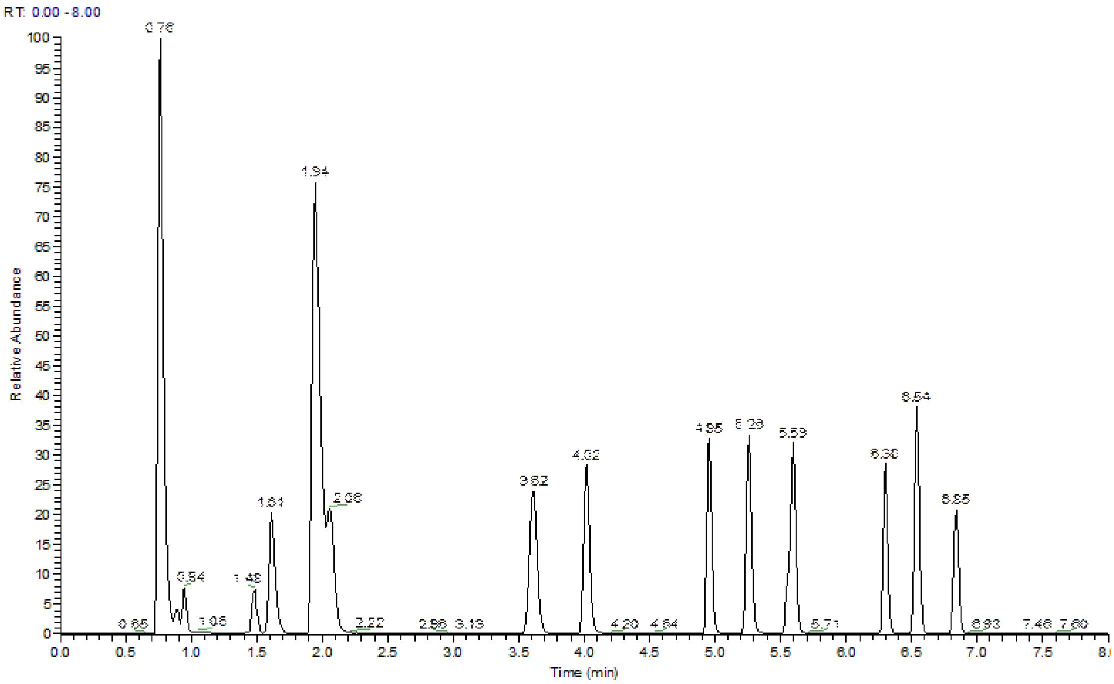

Fig. 1: Chromatogram of standard DKPs

The asymmetric and aromatic DKPs cyclo(Leu-Phe) and cyclo(Phe-Pro) have also been detected in cheddar cheese, chicken, coffee, cocoa, roasted pork, red wine, white wine and balsamic vinegars. Cyclo(Leu-Phe) has exhibited an important antiradical activity against the free hydroxyl radicals[10,18].

Identification of aromatic 2,5-diketopiperazines

The symmetric aromatic cyclo(Phe-Phe) was detected in all olive samples irrespective variety and region (Tables 3 and 4). Cyclo(Phe-Phe) revealed the parent ion (MH^+) at m/z 295.1441 and produced fragment ions at m/z 267.2574, 278.2380, 250.2240 and 222.1727 in its MS^2 spectra (Table 1), indicating the loss of a (C=O), (NH_3), ($HCONH_2$) and (C=O + $HCONH_2$) moieties, respectively. The ion at m/z 120.1214 represented the Phe amino acid after CO_2H elimination (Ph-CH_2-CH(NH_2)-). Furthermore, the unique fragment at m/z 103.0847 in the MS^4 spectra was obtained by a NH_3 elimination from ion (Ph-CH_2-CH(NH_2)-).

The asymmetric and aromatic cyclo(Leu-Phe) was detected in most of the studied varieties (Table 3) and more specifically in samples 2-7 and 14. Cyclo(Leu-Phe) its (MH^+) ion at m/z 261.1598 (Table 1 and 4) released fragments at m/z 233.1786, 244.1782, 216.1437 and 188.1208, resulting from the loss of (C=O), (NH_3), ($HCONH_2$) and (C=O + $HCONH_2$) moieties, respectively. The ions at m/z 148.1770 and 120.0578 represented the Phe amino acid after OH and CO_2H elimination, respectively, whereas the ion at m/z 86.0968 the Leu amino acid after CO_2H moiety loss. Regarding cyclo(Phe-Pro), the presence of an ion at m/z 172.1002 after the loss of 73 amu only in its MS^3 spectrum, pointed that this compound is a proline-based DKPs analogue[17].

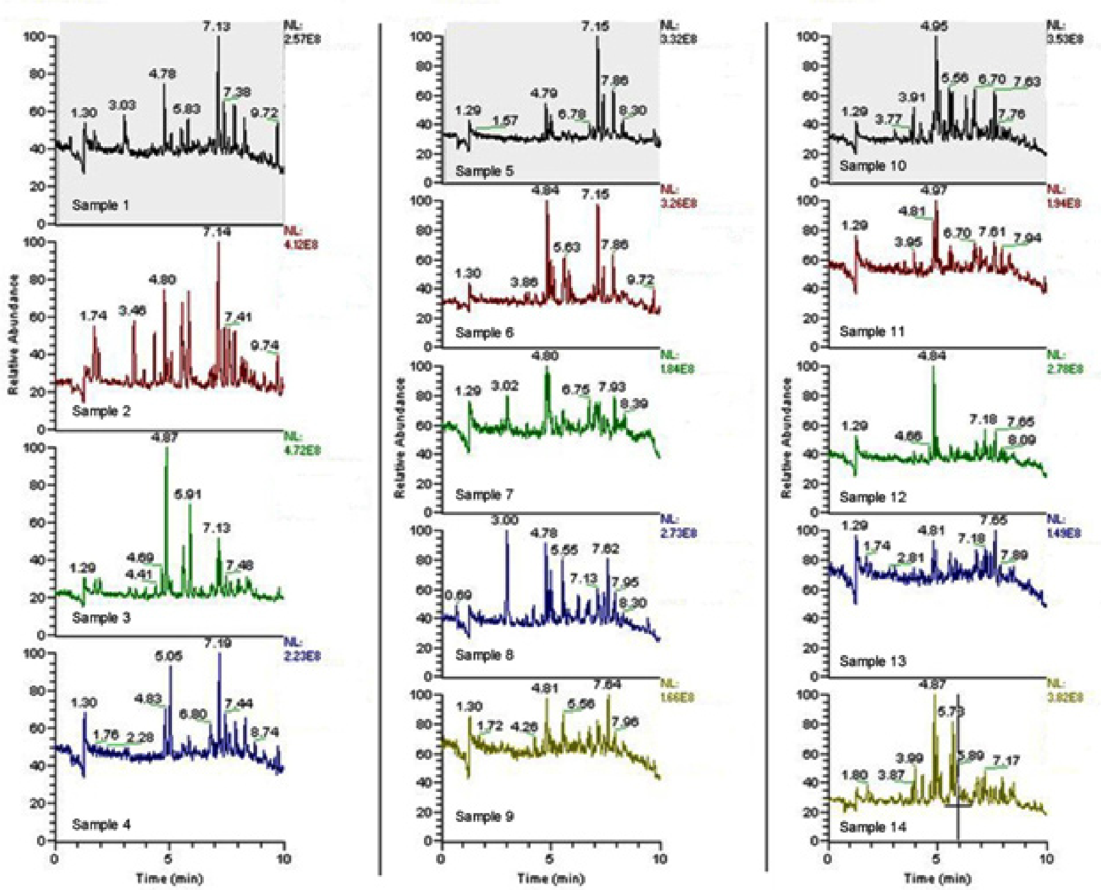

Fig. 2: Total ion chromatograms of olive extracts' samples

Cyclo(Leu-Trp) exhibited the parent ion (MH^+) at m/z 300.1707 and cyclo(Trp-Tyr) at m/z 350.1499 (Table 1 and 4). Both compounds produced fragment at m/z 130 in relative abundance 100% which is the typical fragment pointing to the tryptophan side chain (3-methylene-indole positive ion) in the MS^2 spectra[17]. Furthermore, the characteristic fragments for Trp amino acid at m/z 132 and 170 were also observed, corresponding to 3-methyl-indole protonated and 3-(3-indol)-propenal protonated ions, respectively[17].

Cyclo (Leu-Trp) has also been isolated from marine fungus *Acremonium strictum* and sponge *Callyspongia* sp. cyclo (Leu-Trp) displays important antiradical activity against the free hydroxyl radical and higher antioxidant activity than vitamin E[10,18].

Identification of non-aromatic 2,5-diketopiperazines

The diketopiperazines, cyclo(Leu-Pro), cyclo(Pro-Val) and cyclo(D-Ala-Pro) presented their parent ions (MH^+) at m/z 211.1441, 197.1285 and 169.0972, respectively (Table 1 and 4). They also exhibited similar fragmentation pattern via the loss of 56 amu, which is resulted by the consequent elimination of C_2H_2 and C=O moieties from the parent ion[17], yielding peaks at m/z 155.0913, 141.1104 and 113.1010, respectively. This fragmentation pathway allowed assigning them as proline-based DKPs analogues. Furthermore, the ions at m/z 98 and 70 (Table 1) represented the Pro amino acid after OH and CO_2H elimination, respectively. These proline containing DKPs are also found in a wide variety of food and beverages as well as in cultures of bacteria

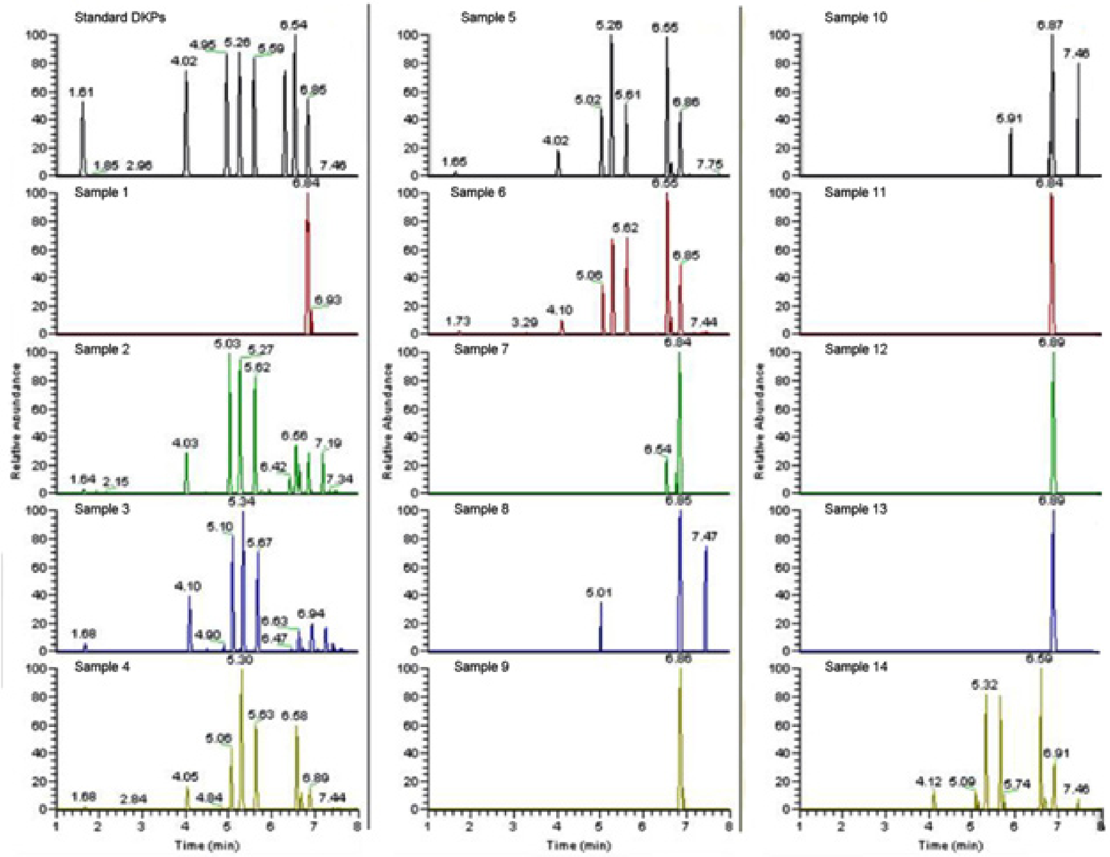

Fig. 3: Chromatograms of the 8 standard DKPs found and their corresponding peaks in the olive samples

and fungi. Cyclo(Pro-Val) was identified as the most important 2,5-diketopiperazine contributing to the bitter taste of several foods[10,16,18,19].

To a further extent, the symmetric aliphatic DKP cyclo(Val-Val) was detected only in one olive variety (Table 3). Its (MH^+) ion at m/z 199.1441 (Tables 1 and 4) released in the MS^2 spectrum, fragments at m/z 171.1223, 154.1027 and 126.1556, resulting from the loss of (C=O), ($HCONH_2$) and (C=O + $HCONH_2$) moieties, respectively. The ion at m/z 72.0649 represented the Val amino acid after CO_2H elimination. Cyclo(Val-Val) has also been detected in beef, bread, chicken, cocoa and yeast extract. It has also been isolated from marine microorganism *Bacillus subtilis* and has been shown to have antimalarial activity[18,20].

CONCLUSIONS

LC/ESI-MS analysis identified 8 aromatic and aliphatic 2,5-diketopeperazines firstly reported for Greek processed olives. Two of the richest varieties were 'Kothreiki' medium sized fruit (Sample 2) and 'Hondroelia' large sized fruit (Sample 6) both from Amphissa, with 7 common DKPs. This fact may indicate the importance of the geographical origin and cultivation practices to the metabolic profile of the fruit. Seven DKPs were also found in 'Kalamon' medium sized fruit from Ilia (Sample 4) which is the only sample where cyclo(Val-Val) was identified. Regarding DKPs, the most abundant were those based on phenylalanine-and proline- aminoacids with cyclo(Phe-Phe) being the only diketopiperazine found in all 14 samples. Overall, the different profile of 2,5-diketopiperazines, obtained for the studied varieties is probably related to genetic and geographical factors, cultivation conditions, as well as the handling and storage methods before and during fermentation process.

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