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The application of an improved method for *trans*-resveratrol to determine the origin of Greek red wines

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Abstract

A rapid and sensitive method has been developed for the determination of *trans*-resveratrol in wine. This method consists of a solid phase extraction step followed by a rapid HPLC quantification step (30 min). The improvement of this method consists of the washing step of the solid phase extraction method carried out at pH 8.0 (12% ethanol in a phosphate buffer solution), thus permitting a more efficient removal of the interfering phenolic compounds in conjunction with the required low volume of tested sample. *Trans*-resveratrol content was determined for 29 red Greek wines of appellation of origin. The concentrations found varied between 0.550 and 2.534 mg/l. The wines produced by grape varieties grown in the Greek islands (Rhodes, Crete and Paros) were richer in *trans*-resveratrol. The grape variety Mandilaria, in particular, was the Greek variety with the highest *trans*-resveratrol content.

Keywords: *Trans*-resveratrol; Greek red wines; Solid phase extraction; HPLC

1. Introduction

trans-resveratrol (3,5,4′-trihydroxystilbene) has been identified as a constituent of various plant species (Hart, 1981), but grapes and related products are considered the most important dietary sources of this compound (Goldberg, 1995; Mattivi, Reniero, & Korhammer, 1995).

Resveratrol is synthesized in response to microbial infection or stress (Langcake & Pryce, 1976). However, it is also produced after chemical treatments, such as herbicide or fungicide application and by UV light exposure (Langcake & Pryce, 1976; Threlfall, Morris, & Mauromoustakos, 1999). Clarifying agents and filters can reduce resveratrol levels (Lamuela-Raventos, Romero-Perez, Waterhouse, & Torre-Boronat 1995; Soleas, Goldberg, Karumanchiri, Diamandis, & Ng, 1995).

In grape berries, resveratrol synthesis is primarily located at the skin cells and it is absent or low, in the fruit flesh (Jeandet, Bessis, & Gautheron, 1991). In red vinification, maceration with skins and seeds, during fermentation, is responsible for the higher resveratrol levels of red wines in comparison with the white ones (Goldberg, Karumanchiri, Ng, Yan, Diamandis, & Soleas, 1995; Lamuela-Raventos et al., 1995). The variety of grapes also plays an important role in resveratrol synthesis which may be genetically controlled (Lauela-Raventos et al., 1995; Threlfall et al., 1999). Resveratrol concentrations increased during fermentation on the skins but the amount extracted was dependent on the variety and enological conditions (Lamuela-Raventos, Romero-Perez, Waterhouse, Lloret, & Torre-Boronat, 1997; Okuda & Yokotosuka, 1996; Soleas et al., 1995).

The presence of *trans*-resveratrol in herbal Japanese folk medications has led to a number of animal experiments suggesting that it may have anti-inflammatory and anti-coagulatory properties that could protect against atherosclerosis and coronary heart disease (CHD; Arichi, Kimura, Okuda, Baba, Kozawa, & Arichi, 1982; Kimura, Ohminami, Okuda, Baba, Kozawa, & Arichi, 1983). This potential effect could be due to resveratrol's ability to inhibit the oxidation of human low-density lipoprotein (LDL; Frankel, Waterhouse, & Kinsella, 1993; Vinson, Jang, Dabbagh, Serry, & Cai, 1995), block platelet aggregation (Bertelli et al., 1995) and eicosanoid synthesis (Kimura, Okuda, & Arichi, 1985). In addition, resveratrol can also inhibit cellular events associated with tumor initiation, promotion and progression (Fontecave, Lepoivre, Elleingand, Gerez, &

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Guitter, 1998), reduce cell death from oxidative stress (Chantavitayapongs, Dracynska-Lusiak, & Sun, 1997) and act as an agonist for the estrogen receptor (Gehm, McAndrews, Chien, & Jameson, 1997). It has therefore been suggested that *trans*-resveratrol may be one of the most active compounds of red wines that have been shown in epidemiological (Hegsted & Ausman, 1988), clinical (Seigneur, Bonnet, & Dorian, 1990), animal studies (Klurfeld & Kritchevsky, 1981) and in vitro studies (Pace-Asiak, Hahn, Diamandis, Soleas, & Goldberg, 1995) to confer protection against atherosclerosis and CHD (McMurtey, 1997).

Resveratrol levels reported in red US wines are below 1 mg/l (Lamuela-Raventos & Waterhouse, 1993; Sieman & Creasy, 1992) and much higher in Italian, French and Spanish wines (Jeandet, Bessis, & Maume, 1993; Mattivi, 1993; Lamuela-Raventos et al., 1995). Revilla and Ryan (2000) have reported a very interesting HPLC method with photodiode array detection which allows the simultaneous separation of several phenolic compounds (15) with a single run. Zhu, Coury, Long, Duda, Kissinger, and Kissinger (2000) have reported the development of a sensitive and selective liquid chromatography/electrochemistry method with multi-channel detection of *trans*-resveratrol in natural product such as wine and grape juice. Gu, Chub, O'Dwyer, and Zeece (2000) have compared the implementation of capillary electrophoresis to the classical HPLC. The former was shown to differentiate the *cis-trans* resveratrol isomers and to be effective for the analysis of glycosides and aglycones.

As far as the authors are aware, the resveratrol content of Greek appellation of origin red wines has not been determined until present. There is only one recent study (Dourtoglou, Makris, Bois-Doumas, & Zonas, 1999) concerning the *trans*-resveratrol content of some Greek red wines but this was focused on fifteen commercial red wines not including all the appellation of origin areas and all local varieties. In addition the wines studied were from different vintage years. Hence, it was of great interest to perform a complete survey of *trans*-resveratrol content of all Greek appellation of origin red wines so that enological techniques that maximize its extraction during fermentation can be developed. In addition, it was interesting to know whether wines obtained from different varieties and appellations could be statistically differentiated and classified by the amount of *trans*-resveratrol they contain. In order to have comparable statistical results, all wines examined were from the 1997 vintage.

To carry out this initial survey of *trans*-resveratrol concentration in Greek wines, we have developed a modified fast assay that may have wide application. It incorporates a solid-phase extraction step, where selective extraction takes place by adjusting the pH at 8, followed by reversed phase HPLC with a UV-visible diode array detector.

2. Materials and methods

2.1. Wine samples

All red wines used in this study were produced according to the Greek appellation of origin system in order to ensure that they have been made in accordance with the specified standards. An appellation regulation, requires the use of specified grape varieties, delineates the areas having the appropriate soils for the production of quality wine from those varieties, specifies the system of cultivation and sets a maximum level of vine yields and a minimum level of sugar content in the grape must. The wine varieties and production areas are presented in Table 1. All samples were commercial wines from the 1997 vintage. For each variety, more than one sample was obtained from different wineries. However for the wines 10, 11 and 15 (Table 1) only one sample was purchased since there were not more than one producer available. For each wine, triplicate *trans*-resveratrol analysis took place immediately after bottle opening.

2.2. Sample preparation

Solid-phase extraction of *trans*-resveratrol was carried out on C-18 Sep-Pak bonded porous silica cartridges (500 mg) purchased from Waters Associates and preconditioned by washing with 3 ml of ethyl acetate, followed by 3 ml 96% (v/v) ethanol and with 5 ml of 12% (v/v) ethanol twice. After passing the sample (2 ml) through the cartridge, a subsequent washing step was performed by 10 ml deionized water followed by 10 ml of 12% (v/v) ethanol adjusted to pH 8.0 by a buffer solution of 94/6 (v/v) K_2HPO_4 (1 M)/KH_2PO_4 (1 M). The cartridge was then dried for 15 min by a constant flow of nitrogen. The absorbed resveratrol was eluted with 10 ml ethyl acetate, concentrated by rotary evaporation at 30 °C and redissolved in 1 ml methanol. In order to avoid any carryover effects, the cartridges were only once used. Recovery was determined for the overall assay by adding varying quantities of *trans*-resveratrol standard in the range of 0.6 to 6 mg/l into a certain wine.

2.3. HPLC analysis

A Hewlett-Packard 1090M Series II chromatograph with an auto injector (10-μl injection volume) and a diode array detector recording at 310 nm was used to detect the *trans*-resveratrol. A reversed-phase ODS Hypersil column (250×4 mm, particle size 5 μm) was used at 40 °C with a flow rate of 1 ml/min. Solvent A was acetonitrile and solvent B was water containing perchloric acid (0.6 ml/l). Elution employed a linear gradient from 5 to 50% solvent A in 5 min, then to 60% in 10 min, held for 15 min to wash the column and

followed by a return to the initial conditions (5% solvent A) for 5 min. Peaks were identified by comparison of retention times and ultraviolet spectra with a commercial standard of *trans*-resveratrol (Sigma).

2.4. Calibration

Seven standards of *trans*-resveratrol covering the range 0.72–18 mg/l were made up in methanol and analysed in duplicate. The constructed calibration curve showed excellent linearity (correlation coefficient: 0.9999).

Detection and quantification limits were calculated by measuring the magnitude of analytical background response by running six blanks (pure methanol), using the maximum sensitivity allowed by the system and calculating the standard deviation of this response (STD). The detection limit (DL) was estimated by multiplying the STD by a factor of 3, and the quantification limit (QL) was defined as 10 times the STD.

Precision was determined by performing six replicate analyses of the same sample on six different days, with each of the replicates passed independently through the cartridge prior to elution with ethyl acetate.

2.5. Statistical analysis

2.5.1. Cluster analysis

The hierarchical clustering method was employed based on average linkage by using SPSS. 9.0. Hierarchical clustering was opted because of its distinct advantages in terms of its simplicity and availability compared to other non-hierarchical methods (Jacobsen & Gunderson, 1986).

2.5.2. Principal Component Analysis (PCA)

A PCA usually occurs in three stages: (1) matrix of correlation (2) extraction of factors and (3) rotation aiming at maximization of relationships between variables and

Table 1
Wine codes, grape varieties, geographical origin, appellation name and *trans*-resveratrol content of Greek red wines

Code	Variety	Area of production	Appellation of origin	*Trans*-resveratrol (mg/l)[a]
1	Agiorgitiko	South Greece (Peloponnese)	Nemea	0.892±0.029
2	Agiorgitiko	South Greece (Peloponnese)	Nemea	0.811±0.010
3	Agiorgitiko	South Greece (Peloponnese)	Nemea	0.675±0.0 14
4	Agiorgitiko	South Greece (Peloponnese)	Nemea	0.683±0.002
5	Agiorgitiko	South Greece (Peloponnese)	Nemea	0.733±0.015
6	Xinomauro & Negoska	North Greece	Goumenissa	0.797±0.315
7	Xinomauro & Negoska	North Greece	Goumenissa	1.205±0.032
8	Xinomauro & Negoska	North Greece	Goumenissa	0.867±0.035
9	Xinomauro & Negoska	North Greece	Goumenissa	1.344±0.019
10	Xinomauro Krasato & Stavroto	Thessaly	Rapsafli	0.675±0.013
11	Xinomauro	North Greece	Aminteo	0.890±0.043
12	Xinomauro	North Greece	Naousa	0.874±0.052
13	Xinomauro	North Greece	Naousa	0.550±0.017
14	Xinomauro	North Greece	Naousa	0.912±0.008
15	Limnio, Cabernet Sauvignon	Chalkidiki	Cotes de Meliton	1.187±0.030
16	Liatiko	Crete	Sitia	0.983±0.016
17	Kotsifali & Mandilaria	Crete	Peza	1.148±0.034
18	Kotsifali & Mandilaria	Crete	Peza	0.992±0.026
19	Kotsifalj & Mandjlaria	Crete	Peza	2.534±0.050
20	Liatiko	Crete	Dafnes	0.823±0.012
21	Liatiko	Crete	Dafnes	1.405±0.010
22	Kostifali & Mantilaria	Crete	Archanes	1.378±0.021
23	Kostifali & Mantilaria	Crete	Archanes	1.812±0.013
24	Kostifali & Mantilaria	Crete	Archanes	0.508±0.018
25	Monemvasia & Mandilaria	Paros	Paros	0.986±0.037
26	Monemvasia & Mandilaria	Paros	Paros	1.125±0.044.
27	Monemvasia & Mandilaria	Paros	Paros	2.013±0.021
28	Mandilaria	Rodes	Rhodes	1.407±0.003
29	Mandilaria	Rodes	Rhodes	1.831±0.029

[a] Mean±standard deviation ($n=3$).

some of the factors. PCA provides the information regarding the number of factors (or axes) required for achieving a reconstruction of matrix that is sufficiently good to account satisfactorily for the correlation it contains. The PCA was carried out by employing the SPSS 9.0 program.

3. Results and discussion

3.1. Recovery

The mean recovery over the range 0.6–1.2 mg/l was 99.4% whereas at the concentration range 2.4–3.6 mg/l was 100.9%. At higher concentrations (6 mg/l), a modest reduction occurred to 92%.

3.2. Detection and quantification limits

At 306 nm the DL and the QL for *trans*-resveratrol was 0.002 mg/l and the QL was 0.007 mg/l from the current calibration curve. The QL was subsequently validated by the analysis of six standards prepared at a concentration of 0.007 mg/l.

3.3. Precision

The precision or the degree of reproducibility is expressed as the coefficient of variation (CV). The results in milligrams per liter as mean $\pm$ STD (CV%) were as follows: 1.356 ± 0.018 (1.33).

The method described above is rapid and sensitive and has good precision. It can be employed as a routine method for the quantification of *trans*-resveratrol at concentrations as low as 7 μg/l. Linearity was excellent up to concentrations of 18 mg/l and recovery was very satisfactory up to concentrations of 6 mg/l.

Since 1992, when Siemann and Creasy described the presence of *trans*-resveratrol in wines, many different methods have been described to determine this compound in wines, including these based on HPLC (Lamuela-Raventos & Waterhouse, 1993; Ribeiro de Lima et al., 1999; Roggero & Archie, 1994; Threlfall et al., 1999). However, because of the multiple steps employed in order to obtain an extract sufficiently enriched in *trans*-resveratrol, while being low in interfering compounds, large initial sample volumes have typically been used. This problem was overcome by the direct HPLC injection of the sample employed by McMurtey, Minn, Pobanz, and Schultz, (1994), Pexet, Pont, and Cuenat (1994) and Lamuela-Raventos et al., (1995) who employed electrochemical, fluorimetric and UV detectors respectively. Goldberg et al. (1994), managed to reduce the sample volume by employing a solid phase extraction step but this was followed by Gas Chromatographic/Mass Spectrometric (GC/MS) analysis. One milliliter of wine sample was enough to assess the *trans*-resveratrol content of the wine. Solid phase extraction was also employed by Mattivi (1993) followed by HPLC analysis but this method required 50 ml of wine. In addition, Okuda and Yokotsuka (1996) pre-purified their samples using solid phase extraction starting with 8 and 30 ml volumes of red and white wines, respectively. *trans*-resveratrol was then determined by HPLC but the method required connected columns in series and was long (60 min).

The method reported here consists of a modification of the Goldberg et al. (1994) solid phase extraction step followed by a rapid HPLC quantification step (30 min). The analysis was performed with only 2 ml of wine samples but the very low quantification limit, permits the use of even smaller volumes of 1 ml or less. The higher pH (8.0) of the washing buffer (12% ethanol in a phosphate buffer solution) permitted a more efficient removal of the interfering phenolic compounds that are also present in wine. Fig. 1 shows a typical HPLC chromatogram.

4. *Trans*-resveratrol concentration in Greek red wines

Hierarchical cluster analysis was applied in an attempt to identify relatively homogenous groups of wines based on resveratrol content, using an algorithm that starts with each wine in a separate cluster and combines them until only one is left. Distance and/or similarity measures were generated by the proximities procedure and displayment of statistics allows the best solution to be selected at each stage (SPSS 9.0, 1999). Fig. 2, shows a dendrogram for the analyzed wines where the cohesiveness of the clusters formed can be assessed.

According to Fig. 2, most of the wines coming from North Greece (Nos. 10, 11, 12, 14, 15, 6) but only one coming from Crete (No. 16) belong to the same group. Similarly, most of the wines from south Greece and the Aegean Sea islands fall in groups B, C, D and E. The Average Distance Method was employed as a compromise to the nearest and furthest neighbor methods thus providing a result midway between the extremes (Jacobsen & Gunderson, 1986).

Principal Component Analysis (PCA) is another technique aimed at the extraction of principal factors, after rotation (i.e. varimax) to maximize the relationships between the variables and some of the factors. In PCA analysis, *trans*-resveratrol values obtained for Portuguese, Spanish, French and Japanese wines (Lamuela-Raventos et al., 1995; Okuda & Yokotsuka, 1996; Ribeiro de Lima et al., 1999) were included in order to find out whether any clear differentiation between Greek and foreign wines is possible. Table 2, shows the principal components and the total variance

Table 2
Principal components and total variance explained[a]

Component	Extraction sums of squared loadings			Rotation sums of squared loadings		
	Total	% Variance	% Cumulative	Total	% Variance	% Cumulative
1	13.92	49.71	49.71	12.11	43.26	43.26
2	6.91	24.69	74.40	7.09	25.33	68.59
3	3.60	12.85	87.25	3.24	11.56	80.15
4	2.26	8.08	95.33			
5	1.14	4.06	99.39			

[a] Extraction method: principal component analysis. Rotation method: Varimax with Kaiser normalization.

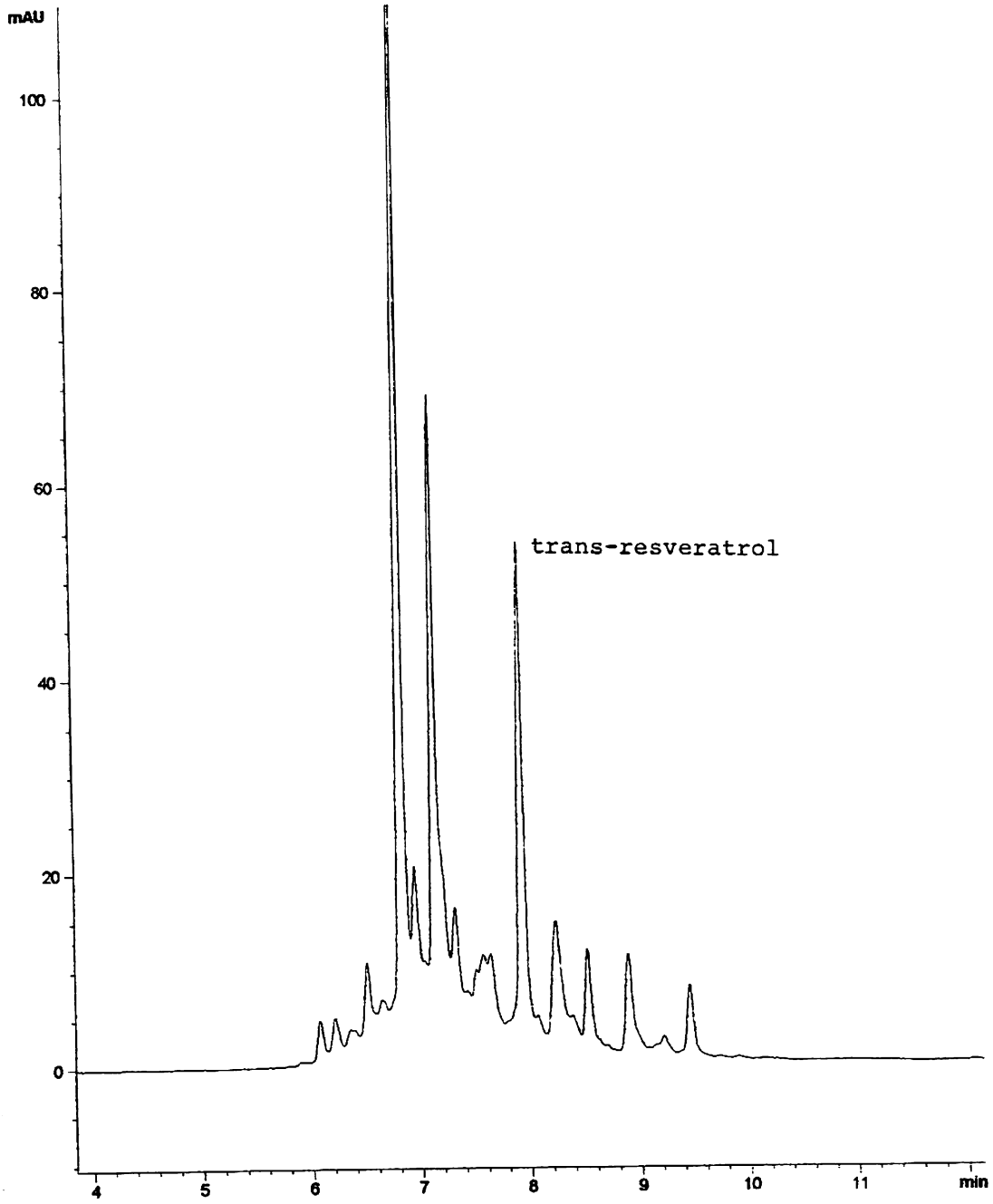

Fig. 1. Typical HPLC chromatogram recorded at 310 nm of the solid phase extracted wine samples.

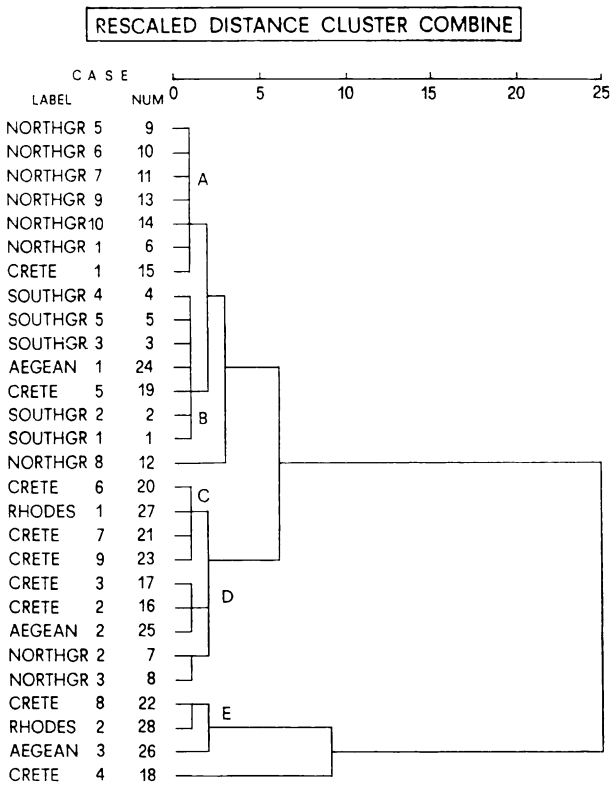

Fig. 2. Dendrogram using average linkage between groups.

obtained. In fact, Fig. 3 shows that most foreign wines are located almost at the centre of the figure (group A) whereas there are at least two more groups (indicated as B and C) which both stand for South Greece and Aegean Islands wines. Furthermore, one could possibly distinguish two groups (D and E, five wines altogether) representing North Greek wines. PC2 vs PC3 and PC1 vs PC3 figures resulted in similar classification patterns.

5. General discussion

Recently, the research related to resveratrol has been intensified in view of the great importance attributed to its therapeutic action. However, on several occasions, deviating values have been reported which are occasionally due to different methods employed. Apart from the employed method for resveratrol determination, there are several other factors which are responsible for resveratrol variation; grape variety, UV light exposure, enzyme addition, fining agent and skin contact time (Romero-Perez, Lamuela-Raventos, Waterhouse, & Torre-Boronat, 1999; Okuda & Yokotsuka, 1996; Threlfall et al., 1999).

Table 1 shows the *trans*-resveratrol values obtained after the analysis of the 29 wine samples as well as their geographical origin, their appellation and the variety used. The amount of *trans*-resveratrol in red wines varied considerably in the different types of wine, depending on the grape variety, environmental factors in the vineyard and wine-processing techniques. The levels of this compound were between 0.550 and 2.534 mg/l for all wines studied. These values are higher than the values reported by Dourtoglou et al. (1999; 0.367–1.569 mg/l) probably due to the older vintage years they used in their study. The wines with the highest levels were those made by Mandilaria grapes grown in Rhodes island (average 1.62 mg/l) followed by the wines made by a combination of Kotsifali and Mandilaria varieties grown at Crete island (average 1.56 mg/l). The combination of Monemvasia and Mandilaria grape varieties grown at Paros island (Aegean Sea) also gave wines with comparatively high amounts of *trans*-resveratrol (average 1.37 mg/l). Grapes of Liatiko variety grown at Crete gave an average *trans*-resveratrol level of 1.07 mg/l. The grape varieties grown at North Greece produced wines with lower average *trans*-resveratrol levels. For example, the combination of Xinomavro and Negoska varieties grown at Goumenissa gave wines with 0.95 mg/l and Ximomavro variety grown at Naousa gave 0.78 mg/l. The grape variety Agiorgitiko grown at Peloponnese, also gave wines with low *trans*-resveratrol average content (0.76 mg/l). Wines made by the varieties Xinomavro (grown at Aminteo), combination of Xinomavro, Krasato and Stavroto (grown at Rapsani), and combination of Limnio and Cabernet Sauvignon (grown at Chalkidiki) gave 0.89, 0.65 and 1.19 mg/l of *trans*-resveratrol, respectively.

It is known that *trans*-resveratrol is produced by grape berries in response to fungal infection and UV irradiation (Creasy & Coffee, 1988; Jeandet et al., 1991). Thus, it is possible that sun exposure of grapes grown at the Greek islands may be a factor in increased resveratrol levels observed in the wines coming from Rhodes, Crete and Paros. In addition, the grape varieties Mandilaria, Kotsifali and Monemvasia may be genetically richer in this compound than the other Greek varieties. In particular, Mandilaria may be the richer Greek variety since it is present in all the island wines, either alone or in combination with the other two varieties mentioned above. This is in agreement with the findings of Dourtoglou et al. (1999) who observed an increased concentration in *trans*-resveratrol in wines made from the cultivars Mandilaria and Kotsifali. However, since they studied wines from different vintage years, it is not possible to compare their results with the average results reported here.

However, in agreement with McMurtey et al. (1997), there was a considerable variability in resveratrol concentrations even in wines produced by the same grape variety. These results were not unexpected since a number of factors such as climate, geographical area of cultivation, growing conditions, wine-making techniques and storage conditions affect resveratrol content of wines (McMurtey et al. 1997).

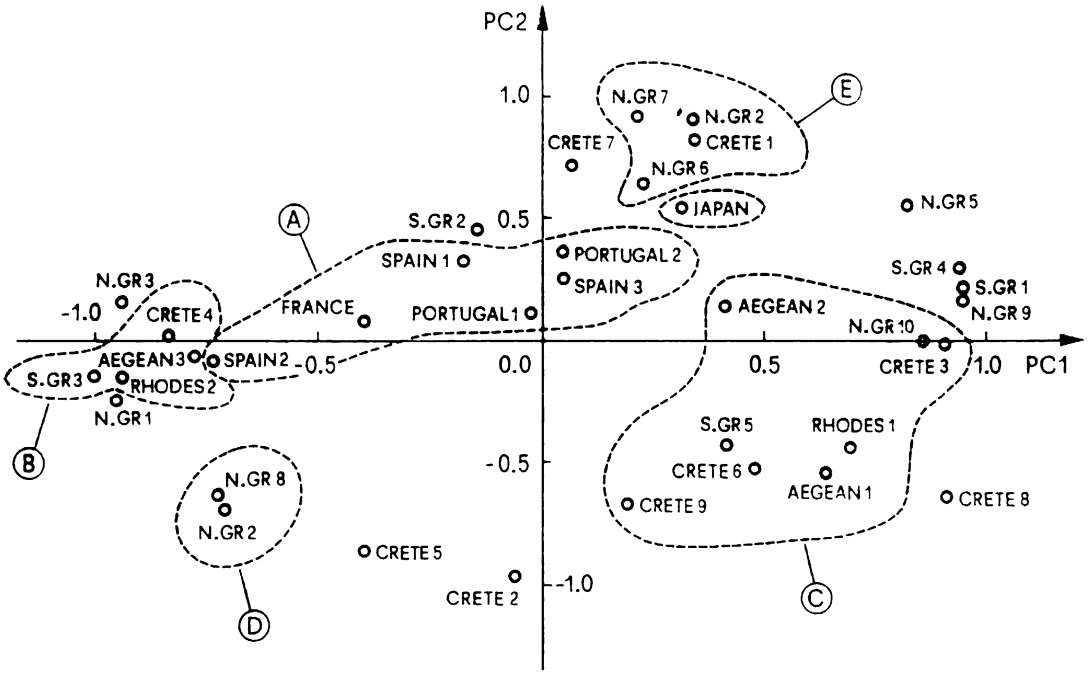

Fig. 3. PCA scores for some Greek and foreign wines.

In comparison with wines made from grapes grown in foreign countries, Greek wines had a much higher *trans*-resveratrol content than the Japanese wines (0.08–0.244 mg/l; Okuda & Yokotsuka, 1996). Californian wines made by Cabernet Sauvignon also were lower than the Greek wines in *trans*-resveratrol level 0.46–0.74 mg/l (McMurtey, 1997), 0.0022 mg/l (Sieman & Creasy, 1992); 0.05 to 0.09 mg/l (Lamuela-Raventos & Waterhouse, 1993). However Californian wines made by blended varieties showed a higher *trans*-resveratrol content (2.74–5.77 mg/l) (McMurtey, 1997). The richest California variety was Pinor Noir containing from 3.72 to 7.99 mg/l *trans*-resveratrol. Portuguese red wines had a similar *trans*-resveratrol content with the Greek wines (average values 1.0 mg/l for monovarietal and 1.5 mg/l for blended wines; Ribeiro de Lima et al., 1999) whereas French (3 mg/l) and Spanish wines (5.13 mg/l from Pinor Noir grape varieties, 3.99 mg/l from Merlot and 2.43 mg/l from Grenache) were higher in *trans*-resveratrol level (Lamuela-Raventos et al., 1995; Ribeiro de Lima et al., 1999). However, Spanish wines made by Cabernet Sauvignon (1.42 mg/l) and by Tempranillo (1.33 mg/l) grape varieties had a similar *trans*-resveratrol content with the Greek wines (Lamuela-Raventos et al., 1995). For Italial wines, it ranged between 0.5 and 10 mg/l depending on cultivar, area of cultivation, climate and wine-making technology (Celotti, Ferrarini, & Zironi, 1996). In another study (Mozzon, Frega, & Pallotta, 1996), 32 Tuscan red wines were found to contain between 0.3 and 2.1 mg/l *trans*-resveratrol.

All wine samples analyzed showed significant levels of *trans*-resveratrol which is known to be an active molecule against low-density lipoprotein oxidation. Frankel et al. (1993) suggests that a level near 2 mg/l would be required to obtain 80 per cent inhibition of LDL oxidation. In addition, Blond, Denis, and Bezard (1995) found that the same level of *trans*-resveratrol (2 mg/l) would inhibit 80% of lipid peroxidation in liposomes. Furthermore, Bertelli et al. (1995) found that *trans*-resveratrol at a concentration of 3.56 μg/l inhibited 50.3% platelet aggregation in vivo on human plasma.

Moderate consumers of Greek red wine (200 ml/day) would ingest between 0.11 and 0.51 mg/day *trans*-resveratrol. In addition, constant moderate consumption of red wine provides a significant amount of other phenolic compounds which may have beneficial effects and also act as synergists.

In conclusion, a new, rapid and sensitive method has been developed for the determination of *trans*-resveratrol in wine. This method consists of a solid phase extraction step followed by a rapid HPLC quantification step (30 min). This method is rapid and sensitive and has good precision. It can be employed as a routine method for quantification of *trans*-resveratrol to concentrations up to 7 μg/l. Linearity was excellent up to concentrations of 18 mg/l and recovery was very satisfactory up to concentrations of 6 mg/l. The method was applied in order to measure the concentration of *trans*-resveratrol in 29 red Greek wines of appellation of origin. The levels were between 0.550 and 2.534 mg/l. The wines produced by grape varieties grown at the Greek islands were richer in *trans*-resveratrol possibly due to the higher sun exposure or the genetically richer grape varieties used for the wine making.

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