Stability to Oxidation of Virgin Olive Oils as Related to Olive Conditions: Study of Polar Compounds by Chemometric Methods

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ABSTRACT: Polar compounds of virgin olive oils were analyzed. They influence oil flavor and aroma and improve the shelf-life of the oil. The orthodiphenolic fraction is particularly significant for oil stability because of its antioxidative activity. A relationship between the composition of the whole fraction of polar compounds and the state of health of the olives was established. For this purpose, oil samples were obtained from olives that had reached different degrees of ripeness and that had been affected by *Dacus oleae* infestation differently. The polar compounds were then analyzed by high-performance liquid chromatography. The data set was studied by means of chemometric methods. Partial least squares regression was used to obtain models that show a significant correlation between composition of the oil's polar compounds and conditions of the olives sampled. In particular, compounds with antioxidative activity were directly linked with the state of health of the olives. The models obtained allow tracing of the state of health of the olives sampled through analysis of the polar fraction of virgin olive oil with a high degree of accuracy, and thus prediction of the oil's expected shelf life.
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KEY WORDS: Chemometrics, HPLC, oil stability, olive conditions, OLS, orthodiphenols, PCA, PLS, polar compounds, virgin olive oils.

Polar compounds of virgin olive oils are extremely important; they include phenolic compounds that contribute significantly to oil flavor and aroma (1–4) and to its oxidation stability (5). Composition and amounts of phenolic compounds are influenced by olive cultivar, climatic conditions, cultivation methods, industrial procedures employed for oil extraction (1,2, 6–8), and in particular, by the degree of ripeness (1,6,9–12) and the state of health of the olives. Recent studies also showed that infestation by *Dacus oleae* and treatments used to fight it also influence phenolic compounds in virgin olive oils (13,14). These factors can reduce the orthodiphenolic fraction that ensures oxidation stability and is a major determinant for oil shelf life (1,3,15–18). The loss of orthodiphenolics is clearly visible when infested olives contain pupae and/or exit holes of adult insects (13).

In addition to phenolic compounds with well-known structures, high-performance liquid chromatography (HPLC) chromatograms of polar compounds in virgin olive oil samples show other compounds that have structures that are still being studied and appear to be correlated with the state of olive health. An extended analysis that includes these compounds thus provides a high number of variables that depend significantly on the state of olive health. The use of chemometric methods, already widely tested in the characterization of virgin olive oils (19–23), allows a simultaneous and optimized use of all available information.

This study analyzed polar compounds in virgin olive oil samples obtained from olives whose degree of ripeness and state of health were known. It aimed at establishing if the analysis of polar compounds, integrated with chemometric methods, allowed the definition of models to evaluate the quality of virgin olive oils.

EXPERIMENTAL PROCEDURES

Samples. Oil samples were obtained from an experimental grove located in Levanto (East Liguria, Italy). Two different 1-ha areas were marked: in the first area, olive trees did not undergo any treatment; in the second area, dimethoate was used for anti-*Dacus* treatment and applied to whole trees (treated = t).

Olives were picked directly from the tree. They were examined by stereoscopic microscope to select thirty-two 1-kg samples, homogeneous with regard to *Dacus oleae* infestation: 100% healthy olives [H]; 100% olives with third instar larvae [L]; 100% olives with *Dacus* pupae and/or exit holes [P]; and unselected olives, i.e., olives reflecting infestation conditions in the selected area [U].

Other 1-kg olive samples were obtained by collecting olives that had spontaneously fallen into nets spread under the trees [F]. Each sample was immediately crushed and pressed in the laboratory by simulating industrial processing conditions.

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Two further identical samples of unselected olives (1 kg each) were collected, then stored and processed after 7 d [U7; U7t] and 10 d [U10; U10t], respectively. This allowed evaluation of the consequences on polar compounds of the common farming practice of accumulating olives for a few days before processing.

This research was carried out on 1993 and 1994 crops. In November 1993, olives were picked when they reached a maximum in oil content (8 samples). Because of widespread infestation, in 1993 it was not possible to isolate a sample of 100% healthy olives. In 1994, three different ripening stages were considered: olives were picked when their oil content was still rising, after 20 d, when the maximum was reached, and the last batch was picked after another 20 d. These three different olive-ripening stages are indicated with 1, 2, and 3 as the prefix for 1994 oil samples (24 samples).

Chemicals. HPLC or analytical-grade reagents were supplied by Merck (Darmstadt, Germany). Hydroxytyrosol [(3,4-dihydroxyphenyl)ethanol] was synthesized according the method of Baraldi *et al*. (24). Oleoeuropeine glycoside was obtained from Roth Chemica (Karlsruhe, Germany). Tyrosol [(4-hydroxyphenyl)ethanol], vanillic acid [4-hydroxy-3-methoxy-benzoic acid], *p*-coumaric acid [3-(4-hydroxyphenyl)propenoic acid] and syringic acid [4-hydroxy-3,5-dimethoxy-benzoic acid] were supplied by Fluka (Buchs, Switzerland). The latter was used as internal standard (i.s.).

Extraction and purification of polar compounds. Olive oil (20 g) was dissolved in 10 mL hexane. A solution (1 mL) of the internal standard in methanol (10 mg/100 mL) was added. The obtained solution was extracted three times with 12.5 mL of methanol/water (60:40, vol/vol); each time it was stirred over a magnetic plate for 15 min and then separated into the two phases by centrifugation at $3000 \times g$ for 10 min. Hydroalcoholic extracts were then combined and washed twice with 15 mL hexane. The hydroalcoholic extracts (10 mL) were evaporated under vacuum at a temperature below 30°C and then dissolved in 1 mL methanol immediately before HPLC analysis.

HPLC analysis. HPLC was performed with a Hewlett-Packard Series 1050 liquid chromatographic system (Waldbronn, Germany) (loop 20 µL) equipped with a diode array detector. A Lichrosorb RP18 column (4.0 mm i.d. × 250 mm; particle size 5 µm) (Merck, Darmstadt) was used. Elution was performed at a flow rate of 1.0 mL/min with a mobile phase of water/acetic acid (98:2, vol/vol) (solvent A) and methanol/acetonitrile (50:50, vol/vol) (solvent B), starting with 5% B and increasing B to levels of 30% at 25 min, 40% at 35 min, 52% at 40 min, 70% at 50 min, 100% at 55 min, and continuing for 5 min. A re-equilibration time of 15 min was then required.

Quantitation was achieved at 280 nm by internal standard method. Tyrosol response factor was detected by multiple level calibration ($R^2 = 0.99999$), and applied to all extracted polar compounds because pure standards were not available for several compounds. The results are expressed as mg/kg oil (as tyrosol).

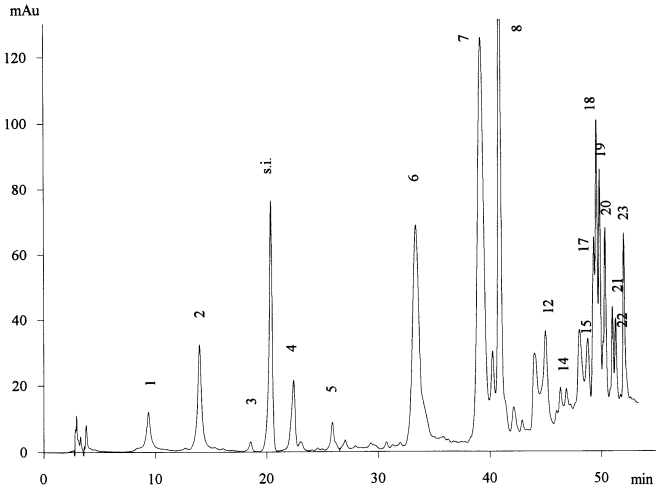

FIG. 1. High-performance liquid chromatographic plot of polar compounds of virgin olive oil. 1, hydroxytyrosol (λ_{max} = 280 nm); 2, tyrosol (λ_{max} = 275 nm); 3, vanillic acid (λ_{max} = 259 nm); 4, n.e. (chemical structure not elucidated) (λ_{max} = 310 nm); 5, *p*-coumaric acid (λ_{max} = 310 nm); 6, dialdehydic form of elenolic acid bonded with hydroxytyrosol (λ_{max} = 280 nm) (28); 7, dialdehydic form of elenolic acid bonded with tyrosol (λ_{max} = 275 nm) (28); 8, n.e. (λ_{max} = 280 nm); 12, oleoeuropeine aglycone (λ_{max} = 278 nm) (28); 15, n.e. (λ_{max} = 300 nm); 17, n.e. (λ_{max} = 298 nm); 18, n.e. (λ_{max} = 284 nm); 19, n.e. (the spectrum shows a decreasing absorbance); 20, n.e. (λ_{max} = 272 nm); 21, n.e. (λ_{max} = 314 nm); 22, n.e. (λ_{max} = 274 nm); 23, n.e. (λ_{max} = 280 nm).

Of the polar compounds identifiable on the chromatogram, only 18 had identical ultraviolet spectra ($\lambda \geq 250$ nm) in all samples. They are indicated on the chromatogram in Figure 1 and were used for the chemometric study.

Packages. Multivariate data evaluation was done by QPARVUS, running under DOS (25).

Chemometric methods. Principal component analysis (PCA) (26) is a well-known technique to visualize data and to find the real dimension of a data set. The *p* parameters, measured for each sample, describe each sample (object) in a *p*-dimensional space (*p* variables). PCA generates a set of new orthogonal variables (axes), linear combinations of the original variables, so that the maximal amount of variance contained in the data set (information) is concentrated in the first principal components. The significant principal components, that is, the new variables, can be used in place of the original variables for successive treatment or to visualize the information contained in the data set.

Loadings are the coefficients of the original variables that define each principal component. Scores are the projections of the objects on the new axes. Because PCA concentrates the variance of the data set in a smaller number of variables than the original one, it is suitable to reduce the dimensionality of large data matrices by eliminating the nonsignificant principal components so that the successive treatments on reduced data sets are easier. Data were autoscaled (subtraction of the mean and division by standard deviation for each variable) before principal-component computation to assign the same numerical weight to each variable.

Partial least squares (PLS) regression (27) is a multivariate linear regression technique. Regression methods build the model to describe relations between one or more response variables and predictor variables. For example, responses can be sensorial scores and biological activity, while predictors are usually measured chemical, physical, and chemical-physical parameters. Ordinary least-squares regression cannot be used when the object number is smaller than the predictor number and when predictors are correlated. In these cases, biased methods must be used. PLS is a biased regression method that is often used in chemistry when predictors are correlated. After computing latent variables, linear combinations of predictors that are similar to principal components, PLS finds the maximum correlation direction between response and latent variables. PLS simultaneously maximizes both the variance of latent variables and the correlation with response. Complexity of the model depends on the number of latent variables that are significant, according to the maximum percentage of cross-validated explained variance. After a regression equation is obtained, it is possible to measure the importance of predictors by considering the regression coefficients divided by standard deviation of each predictor.

Although it is important to know the model that describes the relationship between the response and predictors, the final aim is to use the model to predict an unknown response from measured predictors. For this reason the model must be validated. A data set has to be divided into training and evaluation sets. Models are built with the objects of the training set and successively tested on the objects of the evaluation set, the responses of which are considered unknown. In this study, 10 random different evaluation groups were used to evaluate the quality of model prediction (leave-more-out cross validation method).

Feature selection is performed to find the relevant variables that describe the relationship between response and predictors. Even by using biased methods, it is possible to obtain better models after the deletion of nonrelevant predictors. In this study, PLS regression was used, performing a number of cycles equal to the number of predictors. Each time, the predictor with the lowest importance was deleted**.** The models computed with the remaining predictors were compared, and predictor selection was performed on the basis of maximum prediction power. The procedure used, that is, PLS on selected features, was validated with three independent test sets obtained by random selection of 10 objects out of 32 samples.

RESULTS AND DISCUSSION

First, the original data set (Table 1) was examined univariately to test skewness of variables: variables 1, 2, 5, 12, and 14 showed an asymmetric distribution; therefore, a logarithmic transform was performed.

The 18 predictors (amount of the single polar compounds or their logarithmic transforms) were used to compute principal components. Figure 2 shows the loading and score plot of the first two principal components, which explain 67.7% of the total variance. The first principal component clearly shows the direction of the state of olive health, which im-

TABLE 1
Data Set

32 objects:	Code name rxyyzye
18 variables:	Amounts of the polar compounds (mg/kg oil) or their logarithmic transforms in order of their chromatographic elution.
1 response variable:	Health score

Object code name

r = 1, 2, 3, null; x = H, L, P, U, F; yy = 7, 10, null; z = t, null; ye = 93, 94 (year)

Variables

Peak	Variable	Peak	Variable	Peak	Variable	Peak	Variable
1	log('1')	6	6	15	15	21	21
2	log('2')	7	7	17	17	22	22
3	3	8	8	18	18	23	23
4	4	12	log('12')	19	19		
5	log('5')	14	log('14')	20	20		

Response variable

Health score	Object	Health score	Object
1	3P94	8	3Ut94
2	P93, 2P94, 2Pt94, F93, Ft93, 2F94, 2Ft94	9	Ut93, 2Ut94
3	1P94, 1Pt94	10	1Ut94
4	U793, U7t93, 2U794, 2U7t94, U1093, U10t93, 2U1094, 2U10t94	11	3H94, 3Ht94
5	3U94	12	2H94, 2Ht94
6	2U94, 2L94	13	1H94, 1Ht94
7	1U94		

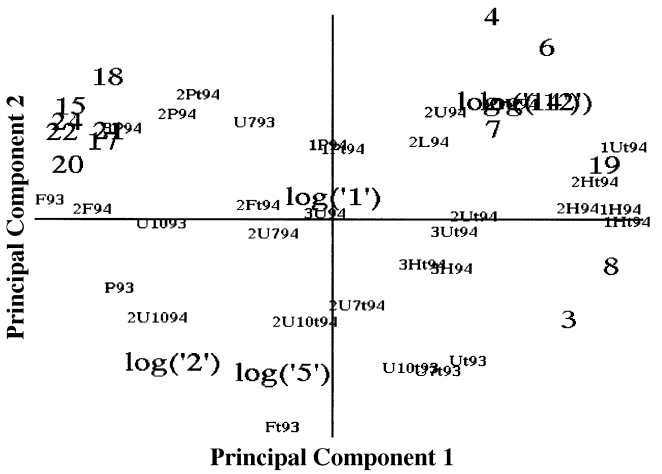

FIG. 2. Loading and score plot of the first two principal components computed with 18 variables.

proves from left to right. The second principal component shows the direction of anti-*Dacus* treatment: scores for samples obtained from treated olives are generally lower than those for samples obtained from olives that had the same state of health but had not been treated.

By observing loadings, that is, the coefficients of original variables, it is possible to obtain information about the composition of principal components: the "health" direction (first principal component) depends on high levels of the chromatographic peaks 3, 6, 8, 12, 14, 19, and low levels of most of the peaks at the end of the chromatographic plot, 15–18 and 20–23. An analysis of loadings confirms the results of previous studies (12–14). In fact, loadings 6 and 12 correspond to the peaks of two hydroxytyrosol [(3,4-dihydroxyphenyl)-ethanol] derivatives and, in particular, to the dialdehydic form of elenolic acid linked to hydroxytyrosol (peak 6) and to an isomer of oleoeuropeine aglycone (peak 12), recently identified by Montedoro *et al*. (28). Because of their orthodiphenolic structure, these two compounds are responsible for most of the antioxidative activity of the total polar fraction.

By observing the plot in greater detail, it can be seen that the scores from healthier olives [healthy (H) and unselected (U)] are located on the right side. Moving from right to left, the first samples were obtained with the best olives (1Ht94, 2Ht94, 1H94, and 2H94). The samples obtained with unselected, not too-ripe olives (1U94 and 2U94) are immediately after them and near samples obtained with unselected olives that were protected by anti-*Dacus* treatments (Ut93, 2Ut94, and 3Ut94). Since this treatment improves olive conditions, scores 1Ut94, 2Ut94, and 3Ut94 obviously precede 1U94, 2U94, and 3U94, respectively. In particular, sample 1Ut94 is comparable to those obtained with the best healthy olives, both because olive ripening was not advanced and because anti-*Dacus* treatments contributed to the preservation of the polar compounds. Samples 3H94 and 3Ht94 are located a distance from the other samples obtained with healthy olives because their advanced ripeness damages polar compounds, as has already been discussed in a previous study (12). Sample 3U94 is further separated from the best samples because it was obtained with very ripe olives that had not been protected with anti-*Dacus* treatments. There are only four samples obtained from highly infested or otherwise damaged olives that can be located on the right side of the diagram: 2L94, U7t93, U10t93, and 2U7t94. This can be easily explained. 2L94 was obtained from olives in which parasites had not yet reached the last degree of larval development and thus had not entirely compromised olive integrity. U7t93, U10t93, and 2U7t94, on the other hand, were obtained from unselected olives that were certainly damaged by the storage period, but the state of health of which was still acceptable as a result of anti-*Dacus* treatments.

The following samples from the left side of the diagram were obtained from the worst olives. First were samples obtained from heavily damaged olives, treated but not very ripe (1P94 and 1Pt94), then those obtained from untreated olives at a more advanced degree of ripeness (2P94, P93, 3P94) or from untreated and stored (U1093 and 2U1094) olives, and, finally, were those obtained from olives that had fallen into the nets, both treated and untreated (F93, Ft93 and 2F94). The second principal component shows the effect of the anti-*Dacus* treatment that was particularly marked in samples of unselected olives. The unselected olives were positively affected by the treatment.

Chromatographic separation of peaks 15–23 is not easy, particularly without a high-resolution chromatographic system. Therefore, peaks 15–23 were summed, even if peak 19 increased with improved olive health, and behaved inversely with the other peaks. Summing the peaks means that they lose their individuality, and thus it is not necessary to know the identity of the substances represented by individual peaks, according to the blind assay method proposed by Saxberg *et al*. (29). The principal components were thus calculated with 11 variables. The first two principal components explain 66.6% of the total variance. The plot is similar to that of Figure 2, meaning that the variable sum brings the same information of the original variables. Because many "routine" HPLC units are not able to separate the nine peaks (15–23), the use of their sum allows the same information to be obtained by means of less complex and expensive apparatus.

Instead of the absolute value of the variable sum, its ratios with variables that seemed to depend more directly on the state of olive health were considered. Logarithmic transforms were used to avoid skewness. Thus 15 variables were obtained, and Figure 3 shows the loading and score plot of the first two principal components that explain 71.8% of total variance. The first principal component is confirmed as the direction of olive health.

Thus, principal components are useful for visualizing the information of the data set. The aim of this study, however, was mainly to obtain a quantitative measure of the state of olive health. Then, oils were classified according to degree of ripeness and health of the olives, with a score ranging from 1

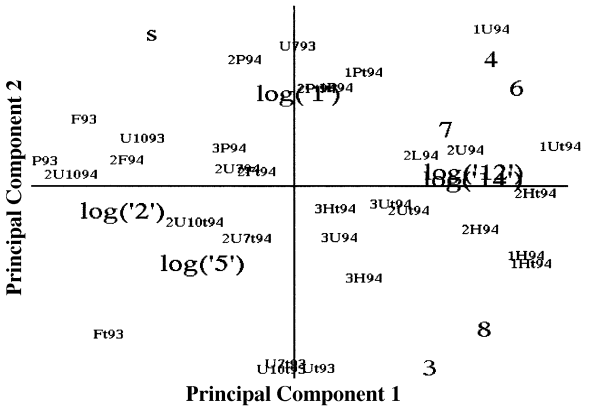

FIG. 3. Loading and score plot of the first two principal components computed with 15 variables.

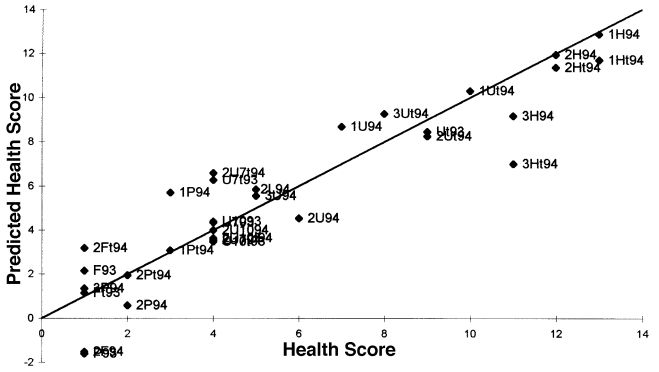

FIG. 4. Predicted health score vs. health score plot. Partial least squares model obtained by 15 predictors.

to 13 (health score), in the direction of increasing quality (Table 1).

The health score can be considered a response variable to be correlated with the 15 previously used variables (predictor variables). A model that describes the relationship between response and predictors was obtained by the PLS method. Table 2 shows interesting results. The best linear model in prediction is obtained by five latent variables. This model explains 75.2% of the cross-validated variance, in other words, the health score of an unknown sample can be predicted with a predictive ability of 75.2% and corresponds to a mean error of prediction of 1.7. This means that the 15 predictors are useful to describe olive health. Figure 4 shows the plot of predicted health scores vs. health scores: considering the large variability of the response, a good linear relation is evident ($R^2_a = 0.864$ and cross-validated $R^2_a = 0.752$). In Table 2, the importance of variables is also listed; variables 4, log9s/‘6’) and log(s/‘8’) are the most important to build the linear model.

TABLE 2
Partial Least Squares Model Computed by 15 Predictors

Latent variable	Explained variance (%) (fitting)	Cross-validated explained variance (%) (prediction)	Mean error (prediction)
1	71.78	69.29	1.84
2	75.50	63.74	1.93
3	84.90	62.63	1.93
4	85.40	72.49	1.68
5[a]	**86.40**	**75.20**	**1.66**
6	86.25	73.12	1.62
7	86.50	71.49	1.70
8	86.31	72.94	1.68
	Importance of variables		
4	0.1605	log(s/″12′)	0.0686
log(s/″6′)	0.1325	log(‘14′)	0.0470
log(s/″8′)	0.1113	6	0.0374
s	0.0925	log(‘1′)	0.0268
log(‘2′)	0.0725	7	0.0182
log(‘12′)	0.0715	log(s/″7′)	0.0166
8	0.0714	log(‘5′)	0.0039
3	0.0694		

[a]Boldface: best linear model in prediction.

An attempt to find a better model was made by selecting the relevant predictors by using PLS regression; the criterion of selection was the importance of predictors. The results of feature selection are shown in Figure 5. The maximum percentage of cross-validated variance (85.3%) was obtained after elimination of eight predictors. Further elimination of variables did not improve the predictive ability of the model, and thus, the best model was built with seven predictors, log(‘2’), 4, 8, s, log(s/‘6’), log(s/‘12’), and log(s/‘8’). Figure 6 shows the plot of predicted health scores vs. health scores. It displays a linear relation similar to that obtained with 15 predictors. The model obtained with seven predictors has a good prediction ability, with a mean error of 1.1, in other words, the health score is predicted plus or minus one score value. An analysis of predicted scores for the various sample groups shows only slight errors in comparison with health scores for samples obtained from healthy olives, when the latter had not reached an advanced degree of ripeness. Instead, samples 3H94 and 3Ht94 show higher errors. In particular, the error of the latter sample (off by 4 score values) was the highest in the entire model. Also, in samples obtained from unselected olives, there were slight differences between predicted health scores and health scores, and the same can be said for samples obtained from olives in the worst conditions. For only two samples (2F94 and P93), higher errors were probably due to the fact that olives were particularly damaged, thus making it difficult to assign them any score.

The mean errors in prediction with the three validation test sets were 2.3, 1.6, and 2.4. This shows that the predicted scores can be used to represent the state of olive health with an independent test set. Of course, these results could be much better if the model were built on a larger data set.

The principal-component analysis allowed the visualization of a relationship between the composition of the polar fraction of virgin olive oils and the state of health of employed olives. Orthodiphenolic compounds were found to be a particularly useful tool to differentiate analyzed oils accord-

FIG. 5. Feature selection by partial least squares regression.

ing to the state of health of the olives. Because these compounds also play a significant role in protecting olive oils from oxidation, the direction of the state of health identified by the first principal component can also be considered as a direction of prolonged shelf life. Building a PLS model by including the results of this study can be useful because it allows the evaluation, with a good degree of accuracy, of both the state of health of the employed olives and the shelf life of the oil itself through analysis of the polar fraction of any olive oil sample.

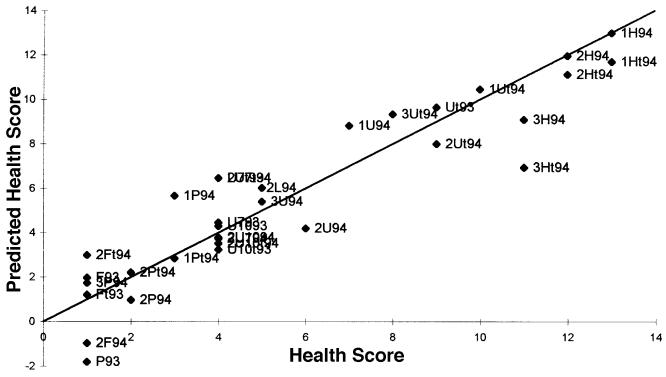

FIG. 6. Predicted health score vs. health score plot. Partial least squares model obtained by seven predictors.

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