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molecular pharmaceutics

^{1}H NMR Monitoring of the Canine Metabolic Profile after Oral Administration of Xenobiotics Using Multivariate Statistics

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Received June 16, 2006; Revised Manuscript Received November 30, 2006; Accepted December 4, 2006

Abstract: This study describes a chemometric analysis of ^{1}H NMR spectra of canine plasma following oral single dose administrations of two food components (lycopene and oleuropein) and of two drug products, Fungoral (ketoconazole) and Adalat (nifedipine). Due to the high interday physiological variation, ^{1}H NMR plasma data were first filtered (by applying orthogonal signal correction) and then subjected to principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). A distinct discrimination was achieved between samples obtained with and without administration of xenobiotics using both techniques. According to distance to model criterion and as shown by the Cooman's plots there was no overlap between the four models which proved to be specific for each xenobiotic. Moreover, it was shown that this approach has the potential to recognize subtle variations in the metabolic profile even if the administered xenobiotic itself could not be detected in the canine plasma by conventional HPLC methods.

Keywords: ^{1}H NMR spectroscopy; canine plasma; lycopene; oleuropein; ketoconazole; nifedipine; principal component analysis (PCA); partial least squares discriminant analysis (PLS-DA); orthogonal signal correction (OSC); metabolic profile

Introduction

^{1}H NMR spectroscopy facilitates the detection of a wide range of low molecular weight metabolites found in tissues and biofluids including urine and plasma.[1] In combination with multivariate statistics including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), neural networks, etc., it is a powerful technique for investigating changes in the metabolic profile, as a result of pathological disorders or exogenous stimuli in a living organism.[2−6] NMR coupled with chemometrics has already been applied as a tool in disease diagnosis[7,8] or in the investigation of the biological response to drugs[9,10] and other xenobiotics.[11] Recently, relevant NMR based investigations

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10.1021/mp060069z CCC: $37.00
Published on Web 01/31/2007

have been used to characterize the biological fingerprint of anabolic treatment in cattle, indicating possible application of these techniques in doping control as well.[12,13] On the other hand, NMR profiles of biofluids may also contain latent information on the more subtle consequences of diet[14−18] or physiological variation related, i.e., to the response to exposure to a maternal separation stress[6] or to diurnal cycles.[19] These studies indicate that global metabolic response suffices to provide discrimination between treated and nontreated subjects and may offer further insight into the mechanisms of biochemical pathways. In many of the above studies,[14−17] data filtering methods have been used, in order to avoid variations in the NMR signals that are not relevant to the response under investigation like intersubject or interday interference. Orthogonal signal correction (OSC) is one of the most frequently used data-filtering methods. This approach has been already applied for the diagnosis of coronary heart disease,[20] or to remove variations due to instrument and physiological variations.[21]

Absorption of xenobiotics and their influence in human metabolism is considered of major importance in pharmaceutical research. During the course of our research on the absorption behavior of orally administered compounds we were confronted with the investigation of two food components, lycopene and oleuropein. A considerable body of evidence exists concerning the beneficial activity of these natural products in human health.[22−28] The absorption studies were conducted on dog, which is one of the most frequently

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used animal models for such studies.[29−31] Both compounds showed very poor absorption producing detection difficulties in plasma.[31] Initial trials to detect those components or their metabolites by NMR spectroscopy proved unsuccessful. However, in the light of the considerations described above, it was challenging to exploit the large amount of information included in the NMR spectra by using multivariate statistics in order to recognize the biological signature of these components in the canine plasma. To our knowledge, although NMR spectra of canine plasma have been published,[32] no canine plasma mapping has been reported based on NMR in conjunction with multivariate statistics. The same procedure was further applied to two other xenobiotics, the widely used therapeutic agents ketoconazole and nifedipine, which also present bioavailability problems, and quantification in plasma is difficult.[33,34] The aim of the study was to generate models for classification of samples obtained with or without administration of the above xenobiotics and evaluate the specificity of these models for each substance administered. The classification results were further compared with plasma levels at different time points in order to evaluate the long acting effect of the xenobiotics on the metabolic profile.

Materials and Methods

Substances and Formulations Administered to the Dogs. *all-trans*-Lycopene was extracted and isolated from tomato paste (purity 93%, Laboratory of Pharmacognosy and Natural Products (National and Kapodistrian University of Athens, Athens, Greece).[31] Lycopene, a carotenoid with antioxidant activities, is responsible for tomato red color and correlates inversely with prostate cancer risk.[35,36]

Oleuropein was isolated from the leaves of the olive tree (*Olea europaea*) with purity greater than 99% as it was assessed by NMR analysis (Laboratory of Pharmacognosy and Natural Products, National and Kapodistrian University of Athens, Athens, Greece).[37] Oleuropein, a glycon-glucoside, is the ester of elenolic acid with hydroxytyrosol, and it is known to possess antioxidant activity.[38,39]

Ketoconazole, a synthetic imidazole antifungal agent[40] was provided in the form of tablets (Fungoral 200 mg/tablet) by Janssen-Cilag (Athens, Greece).

Nifedipine, a dihydropyridine calcium antagonist,[41] was provided in the form of capsules (Adalat 5 mg/capsule) by Bayer AG (Athens, Greece).

Canine Studies. Single dose studies were performed in two female mongrel dogs 3 and 4 years old that weighed 24 and 31 kg, respectively. The animal facility operates in the Laboratory of Biopharmaceutics and Pharmacokinetics according to European Union regulations for the maintenance and experimentation on animals and has been approved by the Veterinary Directorate of the Municipality of Athens. Dogs were fasted for 16 h from food, but not water, before each administration and were offered a standard meal (150 g of pellets and 200 mL of tap water) 6 h after administration. All administrations were performed using an orogastric tube. Blood samples were drawn by means of an indwelling

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catheter positioned and remaining in a suitable foreleg vein up to 12 h after dosing. At 12 h the catheter was removed and the dog returned to her cage, where she was allowed to eat and drink ad libitum. If required, samples after 12, 24, and 48 h were collected by individual venipuncture. Blood samples were collected in glass tubes containing EDTA (BD Vacutainer Systems, K3E 15% 0.054 mL, Plymouth, U.K.) and centrifuged. Plasma samples were kept −20 °C until analyzed.

On four different occasions dog #1 was administered orally 75 mg of lycopene suspended in 250 mL of water, 300 mg of lycopene suspended in 250 mL of water, 750 mg of lycopene suspended in 500 mL of water, and 750 mg of lycopene suspended in 500 mL of milk (3.5% fat). Blood samples were drawn at 0, 2, 4, 6, and 8 h after administration.

Oleuropein was administered in dog #1 and dog #2 as a suspension in milk (3.5% fat) (750 mg in 500 mL of milk). Blood samples were drawn at 0, 2, 4, 6, 8, 12, 24, and 48 h after administration.

One Fungoral tablet was administered in dog #1 and in dog #2 with 500 mL of milk (3.5% fat). In both cases, blood samples were drawn at 0, 0.25, 0.5, 1, 1.5, 3, 4, 6, 8, 10, and 12 h postdosing.

Two Adalat capsules were administered in dog #1 and in dog #2 with 500 mL of milk (3.5% fat). In both cases, blood samples were drawn at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, and 10 h postdosing.

Although, according elimination kinetics of all compounds, washout periods could be as short as 2 weeks, in order to minimize the possibility of carryover effects on the metabolic profile, in all cases the washout periods were at least 3 weeks.

Finally, dog #1 was administered 500 mL of milk (3.5% fat) and dog #2 was administered 250 mL of water. In both cases blood samples were drawn to create the blank samples at 0, 2, 4, 6, 8, 12, 24, and 48 h.

The above protocols were approved by the General Secretariat for Research and Development (Ministry of Development) that sponsored the in vivo studies (EPET II, CSF II, and PABET 2000).

Analysis of Plasma Samples. The chromatographic system consisted of a Spectra System P1000 pump, a Spectra System UV 2000 absorbance detector extended to the visible region, and an autosampler AS 3000. The above system was controlled by a Spectra System Controller SN 4000 and a software package Chromquest (Thermoquest Inc., San Jose).

Concentrations of lycopene, ketoconazole, and nifedipine in plasma were measured with HPLC−UV methods that have been recently published.[33,34,42]

For measuring oleuropein in plasma, a reversed-phase Symmetry C_8 column (250 × 4.6 mm, 5 μm particle size) equipped with a precolumn Hypersil BDS-C_{18} (10 × 4mm, 5 μm particle size) was used. The mobile phase was composed of methanol and water 40:60 v/v, and its flow rate was 1 mL/min. Injection volume was 50 μL. Experiments were performed at ambient temperature. Absorption was measured at 240 nm. Two hundred microliters of sample was transferred to a microcentrifuge tube, and 200 μL of perchloric acid 10% w/v was added for protein precipitation. After vortexing for 30 s, the sample was centrifuged for 10 min at 12000 rpm and 20 °C. The clear supernatant was immediately injected into the HPLC system. Calibration curves of oleuropein in plasma were linear in the concentration range of 0.2−10 μg/mL. Limit quantification in plasma was 200 ng/mL.

^{1}H NMR Spectroscopy. Forty microliters of deuterated water containing 0.01% 4,4-dimethyl-4-silapentane sodium sulfonate (DSS) for a magnetic field lock was added to each plasma sample (600 μL). ^{1}H NMR measurements were performed on a Bruker DRX-400 Avance spectrometer operating at 400 MHz and at 300 K. 1D spectra (64K FID data points) were collected using Carr−Purcell−Meiboom−Gill (CPMG) spin−echo pulse sequence which gave the clearest signature of changes concerning low molecular weight metabolites, with little extra information contained in the higher molecular weight components. Water suppression was included in the CPMG sequence.

The CPMG pulse sequence was D−[−90°−(τ−180°−τ)$_n$−FID], where D = 5 s (to allow T_1 relaxation); τ = 343 μs [a fixed delay to allow spectral editing via T_2 relaxation (attenuation of broad signals) and refocusing of spin-coupled multiplets]; and n = a fixed loop of 128 cycles, giving a total spin−spin relaxation delay 2$n\tau$ of 87.8 ms. Typically, 128 free induction decays (FIDs) were collected into 64K data points using a 10 μs pulse width (90° pulse angle) and an acquisition time of 5.86 s.[43,44] Prior to Fourier transformation (FT) the FIDs were zero-filled to 128K and multiplied by an exponential function corresponding to a line broadening of 1 Hz.

The acquired NMR spectra were phase corrected and referenced to the chemical shift of DSS at δ 0.0. A baseline correction factor was applied to each spectrum using a simple polynomial curve fitting.

Data Reduction and Chemometric Analysis of the NMR Spectra. In order to maximize the chances of using all the metabolic information in the spectra and to provide a reproducible procedure, the spectra were reduced to a series of descriptors.[7,11,45] This was achieved by segmenting the spectra into consecutive non-overlapped regions of 0.04 ppm

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(buckets) and integrating the signal intensity in each region using the AMIX program (Analysis of Mixtures, software package version 2.7, Bruker Analytische Messtechnik, Karlsruhe, Germany). The spectra were normalized to the total sum of the spectral integral covering the δ range 10−0.5 excluding the δ region 5.2−4.46 containing the residual peak from the suppressed water resonance. Since plasma samples were also prepared for HPLC analysis, therefore containing EDTA, the δ regions 2.52−2.70, 2.98−3.38, and 3.5−3.74 corresponding to resonances from free nonendogenous EDTA and EDTA metal complexes were also excluded. The resulting normalized integrals composed the data matrix that was submitted to multivariate analysis using SIMCA-P (version 8.0, Umetrics, Umeå, Sweden) software package.

Application of Mathematical Spectral Filters to the ^{1}H NMR Plasma Data. To reduce the effect of interday and intraday variability, orthogonal signal correction (OSC) as a spectral filter was applied in the training set. OSC is a data filtration method using information based on class (**Y** matrix), where **Y** matrix corresponds to a response vector and consists of dummy variables related to substance administration. OSC removes the systematic variation from the matrix **X** not related to the response vector **Y**,[46,47] thus highlighting the key variations related to the biochemical response of interest:[14−16,20,21] in our case, spectral alterations due to xenobiotic administration. Two components were removed, as recommended in SIMCA, and the resulted filtered data were used for generating PCA models.

For lycopene, the plasma concentration data were used to define the response vector **Y** and regardless of the milk coadministration three classes were identified: (a) class 1, blank samples (no administration of lycopene); (b) class 2, lycopene levels of about 40 ng/mL in plasma, corresponding to doses 75 and 300 mg; and (c) class 3, lycopene levels >40 ng/mL corresponding to 750 mg with or without milk.[31] Alternatively, two dummy variables were used corresponding to class 0 (before lycopene administration) and class 1 (after lycopene administration). The latter approach was also used for oleuropein, ketoconazole, and nifedipine.

Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA). PCA involves the calculation of linear combinations of the original descriptors, the PCs. Each PC is a linear combination of the original variables, whereby each successive PC explains the maximum amount of variance possible in the data set and each PC is orthogonal to every other PC.[14−16] Data were visualized by plotting the PC scores where each point in the score plot represents an individual sample and the PC loadings where each point represents one spectral region. Similarities and differences between samples can be detected in the score plots and can be used for sample discrimination. Data were divided into training and test sets. The separation was considered randomly. PCA was performed in the training set without or after OSC filtering. The test set was not submitted to filtering. Normalized prediction distances (DmodX) provided distances to the models whose critical values (Dcrit) were computed with 0.95 confidence intervals. By using Cooman's plot, the distance to each of the PC models was computed and plotted along with the critical distances. This approach was used in order to assess the classification performance of samples by predicting class membership in terms of distance to the model and to evaluate the specificity of the models.[47]

A supervised alternative of PCA is partial least squares discriminant analysis (PLS-DA) whereby the multivariate variables corresponding to the observations (spectral descriptors) are related to the class membership of each sample.[7,8] The method allows predictions assigning probabilities to new observations for class membership. A PLS-DA model was applied splitting the samples into classes according to the origin of samples: blank samples formed class 0, while samples obtained after treatment formed class 1. The validity of the PLS-DA model was assessed by statistical parameters: the correlation coefficient R^2 and the cross validation correlation coefficient Q^2. Q^2 was derived using the default option of SIMCA-P.[47] The same training and test data sets were used as in PCA. The training set was submitted to OSC filtering prior to PLS-DA. The predictive ability for correct class membership classification was evaluated according to the prediction option of SIMCA, using a cutoff value of 0.5.

Statistical Analysis. One-way ANOVA was used for the extraction of the statistically significant spectral variables between blank samples and samples treated with a xenobiotic. A calculated P value of less than 0.05 was considered statistically significant (SPSS, Inc. Chicago, IL, version 10.0).

Results and Discussion

^{1}H NMR Spectral Analysis of Blood Spectra. In the ^{1}H NMR spectra of plasma samples the signals from low molecular weight metabolites like amino acids (e.g., alanine, glutamine, glutamate), glucose, ketone bodies (e.g., acetoacetate), the signals of lipids, LDL, VLDL, etc. were detected.[44,48] As an example, a plasma CPMG spectrum before any administration of xenobiotic (blank) compared with a spectrum after the administration of oleuropein is depicted in Figure 1 (dog #1, 4 h postdose). The presence of lipoprotein residuals in the spectrum could be attributed to the relatively short spin−echo time (87 ms) of the CPMG experiment. No signals arising from the four xenobiotics or their metabolites were detected in the plasma NMR spectra even from those collected after the administration of high doses of lycopene and oleuropein, confirming the low plasma levels of these compounds due to their poor absorption characterization.

No statistically significant signal intensity variation was observed concerning different metabolites during time points. Consequently, all time points were considered together. In

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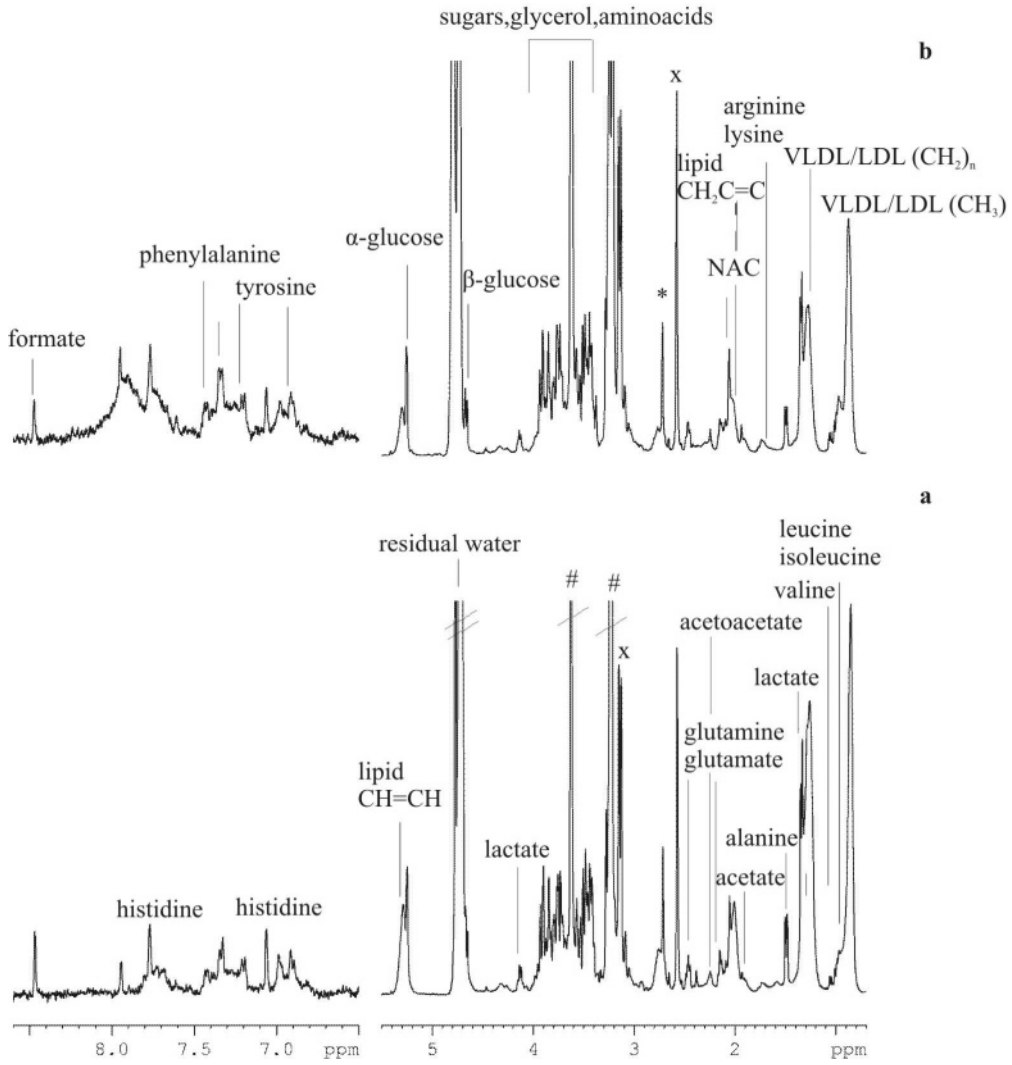

Figure 1. ^{1}H NMR 400 MHz CPMG plasma spectra (δ 0.7−5.5 ppm, δ 6.5−8.5 ppm): (a) prior to and (b) 4 h after the administration of 750 mg of oleuropein powder in 500 mL of milk to dog #1. Key: VLDL, very low density lipoprotein; LDL, low density lipoprotein; NAC, acetyl signal from α_1-acid glycoprotein; x, Ca-EDTA^{2-}; *, Mg-EDTA^{2-}; #, EDTA.

case of different doses (lycopene), they were considered separately. After this grouping, one way ANOVA showed statistically significant differences from blank samples for some endogenous metabolite signals depending on the xenobiotic as presented in Table 1. They include the signals of VLDL/LDL (δ 0.82−0.90, 1.22−1.30) and lipids (δ 2.0, 5.30), α-glucose (δ 5.26), the amino acids phenylalanine (δ 7.30−7.38), tyrosine (δ 6.90, 7.20), histidine (δ 7.06, 7.76), glutamine and glutamate (δ 2.10−2.18, 2.42−2.50), leucine, isoleucine, valine (δ 0.94−1.02, 1.06), alanine (δ 1.54), *N*-acetyl glycoproteins (δ 2.05), acetoacetate (δ 2.24), and formate (δ 8.5).

These results were compared with analogous findings reported in the literature using conventional techniques. According to these reports, supplementation of lycopene, as well as oleuropein, reduces plasma LDL and VLDL levels,[22−26] while they are also involved in glucose metabolism with a beneficial effect on inhibiting hyperglycemia.[27,28] In agreement with these previous reports, NMR signals showed reduction of signals of LDL and VLDL as well as unsaturated lipids and α-glucose. Other studies concerning treatment with oleuropein after ischemic reperfusion in rabbits showed that oleuropein restored aerobic metabolism.[26] The reduction in glucose levels, produced by both lycopene and oleuropein, with no significant or ambiguous increase in lactate, suggests that there is no influence in aerobic metabolism after administration of these food components, as it was reported in the case of other nutrients.[16] In the case of ketoconazole administration, a reduction in serum cholesterol has been reported in the literature.[49] Our findings revealed a decrease in levels of LDL and lipid signals (δ 1.3−1.22 and 2.0, respectively). Glucose as well as acetoacetate signals also declined while no alterations in acetate signals were observed. Further, literature data suggest that nifedipine influences glucose and lactate levels when administered in pathological

(49) Stalenhoef, A. F. H.; Stuyt, P. M. J.; de Graag, J. Effects of ketoconazole on plasma lipids and lipoprotein(a) in familial hypercholesterolaemia, compared with simvastatin. *Neth. J. Med.* **1997**, *51*, 10−15.

Table 1. Statistically Significant Changes ($P < 0.05$) in Plasma Metabolite Levels after the Administration of Lycopene (According to Different Doses), Oleuropein, Ketoconazole, and Nifedipine Compared to Blank Samples[a]

		lycopene[b] (mg)						
chemical shift	metabolite	75	300	750m	750w	oleuropein[c]	ketoconazole[c]	nifedipine[c]
0.82−0.9 (m)	LDL, C**H**$_3$(CH$_2$)$_n$; VLDL, C**H**$_3$CH$_2$CH$_2$CH═	↓	↓	↓	↓	↓	–	–
1.06 (d), 1.02−0.94 (m)	valine, leucine, isoleusine (γ, δ CH$_3$)	–	↓	↑	↑	↑	–	–
1.22−1.3 (m)	VLDL, LDL (C**H**$_2$)$_n$	↓	↓	↓	↓	↓	↓	↓
1.34 (d), 4.14 (q)	lactate (CH$_3$, CH)	–	–	–	–	–	–	↓
1.54 (d)	alanine (CH$_3$)	–	–	–	–	–	–	–
2.0 (m)	lipid C**H**$_2$C═C	↓	↓	↓	↓	–	–	↓
2.05 (s)	NAC (CH$_3$)	↓	↓	–	–	–	–	↓
2.22 (s)	acetoacetate (CH$_3$)	–	–	–	–	–	↓	↓
2.1−2.18(m), 2.42−2.5 (m)	glutamine, glutamate (β,γCH$_2$)	↓	↓	↓	↓	–	–	↓
5.26 (d)	α-glucose (CH)	↓	↓	↓	↓	↓	↓	↓
5.3 (m)	unsaturated lipids CH═CH	↓	↓	↓	↓	–	–	–
6.90 (d), 7.20 (d)	tyrosine (CH)	↑	–	–	↑	–	–	–
7.06 (s), 7.76 (s)	histidine (CH)	↓	–	–	–	↓	↓	↓
7.30−7.38 (m)	phenylalanine (CH)	–	–	–	↑	↑	↓	↓
8.5 (s)	formate (CH)	↑	↑	–	–	–	–	–

[a] Variations compared to blank samples: ↑, indicates relative increase in signal; ↓, relative decrease in signal; −, no change. Key: 750w, administration of 750 mg of lycopene powder in water; 750m, administration of 750 mg of lycopene powder in milk; s, singlet; d, doublet; q, quartet; m, multiplet; NAC, *N*-acetylglycoproteins. [b] Data from one dog. [c] Data from two dogs.

cases such as cerebral ischemia/reperfusion in rats.[50] It renormalizes the increased glucose and lactate plasma levels related to stimulation of gluconeogenesis in brain or its vasodilating action. Nifedipine administration may also influence ketone bodies by reducing their increased levels after ischemia. The Ca^{2+} influx after ischemia induces mitochondrial failure, decreases acetoacetate, and triggers the lipolysis event. In our study, glucose, lactate, and acetoacetate plasma levels declined, even if no pathological event had taken place before nifedipine administration. Nifedipine also produced a decrease in the residual signals of the lipoproteins as it was also stated in the literature.[51]

Chemometric Analysis of ^{1}H NMR Data Set

Lycopene Data. Figure 2 shows the plasma levels of lycopene after single oral administrations of 750 mg of lycopene powder to dog #1. Lower doses of *all-trans*-lycopene powder resulted in nonquantifiable plasma concentrations, i.e., <40 ng/mL.

Initial PCA of the reduced NMR spectra did not lead to any distinct discrimination according to the administered dose and time intervals (data not shown). OSC was then applied as a filtering method using plasma level concentrations as **Y** variable. After this procedure PCA was repeated and an adequate discrimination was achieved (Figure 3); blank

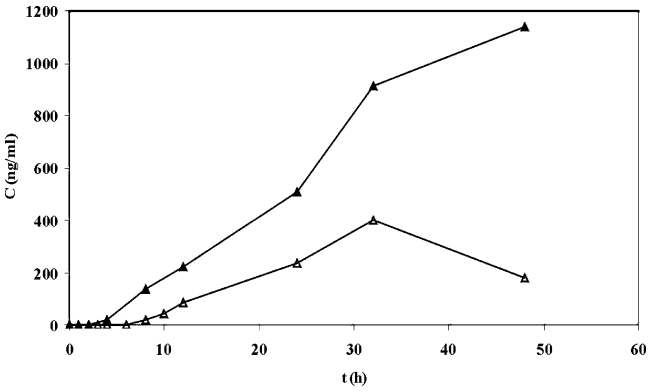

Figure 2. Plasma levels of lycopene after single oral administrations to dog #1 of 750 mg of lycopene powder suspended in 250 mL of water (Δ) and 500 mL of milk (▲).

samples were clustered in the right side of the score plot, samples with low concentrations of lycopene (after administration of 75 and 300 mg) were situated in the center of the score plot, and samples with higher lycopene levels (after administration of 750 mg with water or milk) were discriminated in the left side of the score plot (Figure 3, upper graph). In order to validate the robustness of the discrimination, the data set was divided into training and test sets. The test set was constructed by five samples randomly selected from those collected after the administration of various doses of lycopene. OSC was performed only for the training set. After PCA the same discrimination was obtained and the test samples were correctly classified (Figure 3, lower graph). Since in plasma CPMG NMR spectra lycopene signals were not detected and this technique aims to show lycopene effect on the metabolic profile, we considered it less biased to proceed in an alternative OSC filtering of the training set using as response vector **Y** two dummy variables corresponding to treatment (after lycopene administration) or no

(50) El-Abhar, H. S.; Shaalan, M.; Bakarat, M.; El-Denshary, E. S. Effect of melatonin and nifedipine on some antioxidant enzymes and different energy fuels in the blood and brain of global ischemic rats. *J. Pineal Res.* **2002**, *33*, 87−94.

(51) Garcia-Perez, B.; Ayala, I.; Castells, M. T.; Domenech, G.; Sanchez-Polo, M. T.; Garcia-Partida, P.; Valdes, M. Effects of nifedipine, verapamil and diltiazem on serum biochemical parameters and aortic composition of atherosclerotic chickens. *Biomed. Pharmacother.* **2005**, *59*, 1−7.

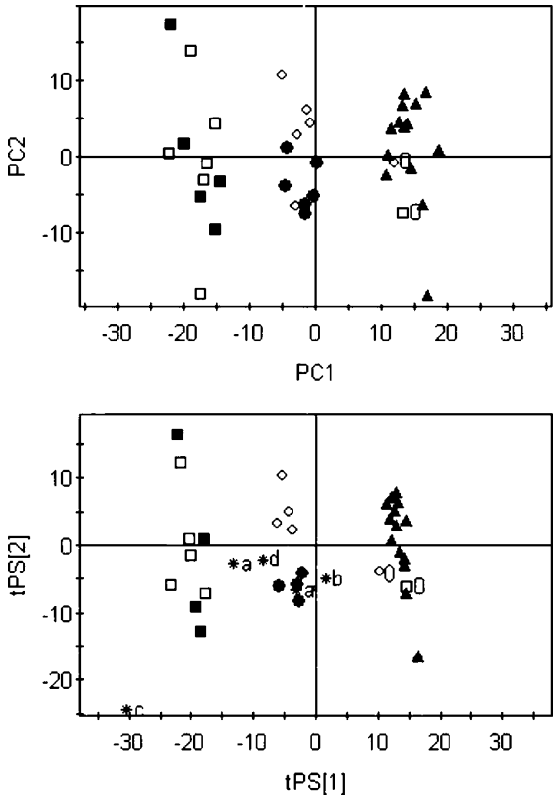

Figure 3. PC score plots of CPMG ^{1}H NMR spectra of canine plasma after application of OSC for samples collected prior to lycopene administration (▲) and after administration of 75 mg of lycopene powder in water (●), 300 mg in water (○), 750 mg in water (□), and 750 mg in milk (■). The upper graph contains the training set. In the lower graph the extra symbols (*) correspond to test set. Key: ○0, sample prior to the administration of 300 mg in water; □0, sample prior to the administration of 750 mg in water; *a, sample after administration of 75 mg in water; *b, sample after administration of 300 mg in water; *c, sample after administration of 750 mg in water; *d, sample after administration of 750 mg in milk.

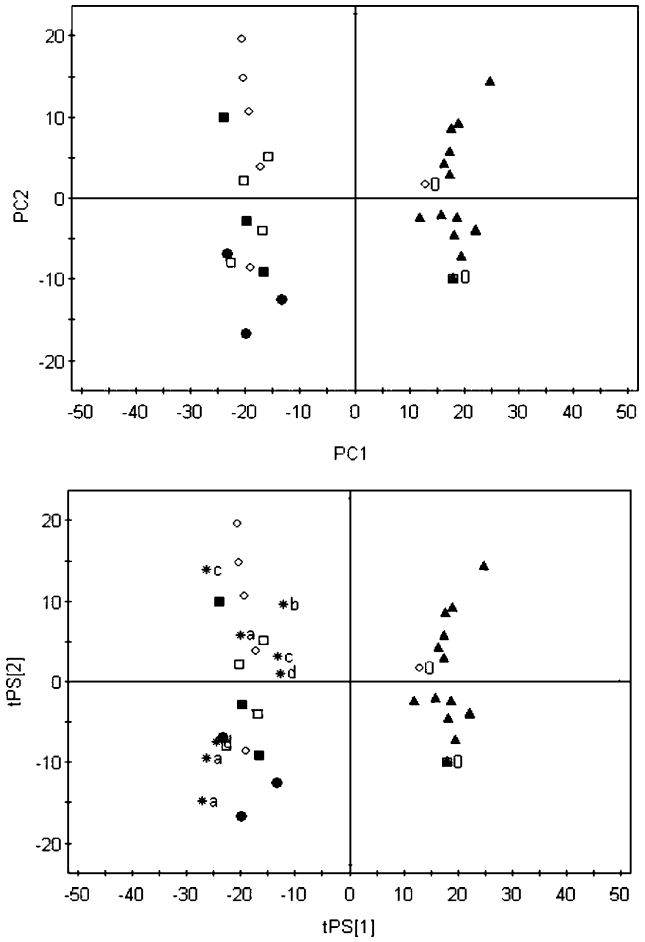

Figure 4. PC score plots of CPMG ^{1}H NMR spectra of canine plasma mapping samples collected prior to lycopene administration (▲) and after administration of 75 mg of lycopene powder in water (●), 300 mg in water (○), 750 mg in water (□), and 750 mg in milk (■). Plots resulted after the application of OSC using as response vector **Y** two dummy variables (0 and 1, prior to and after administration, respectively). The upper graph contains the training set. In the lower graph the extra symbols (*) correspond to the test set. The key is the same as in Figure 3.

treatment (no lycopene administration). This procedure would allow discrimination without prior knowledge of plasma concentrations. PCA was applied, and discrimination is shown in Figure 4. Blank samples were all located in the right side of the score plot, while samples collected after administration of various doses of lycopene were situated in the left side of the score plot (Figure 4, upper graph). All eight test samples were correctly classified in the plot as shown in Figure 4, lower graph.

Oleuropein Data. Oleuropein plasma levels at all sampling times were lower than 200 ng/mL and could not be quantified in plasma. The OSC approach using two dummy variables (treatment, no treatment) was applied. After PCA, oleuropein samples were discriminated in the left side of the score plot, away from the blank samples (Figure 5, upper graph). PCA was repeated after dividing the samples into training and test sets. The same OSC procedure was applied only to the training set. The test set was randomly selected and consisted of two blank samples and four samples collected after treatment. The blank samples and two of the oleuropein samples were correctly discriminated in the proper clusters, while two of the oleuropein samples were discriminated in the same direction of other oleuropein samples but not within the cluster (Figure 5, lower graph). It is interesting to note that discrimination was successful despite the fact that, after oral administration of oleuropein, no oleuropein could be actually quantified in plasma as a result of its poor absorption characteristics.

Fungoral and Adalat Data. Plasma concentration data showed maximum plasma ketoconazole concentrations 4−6 h postdosing, with the onset of plasma levels occurring 3 h after administration, whereas plasma concentrations were lower than the limit of quantification at times longer than 10 h.[33] Nifedipine plasma levels were <1.5 ng/mL (limit of quantification of the method) during the entire postadministration period.[34] Six randomly selected samples after

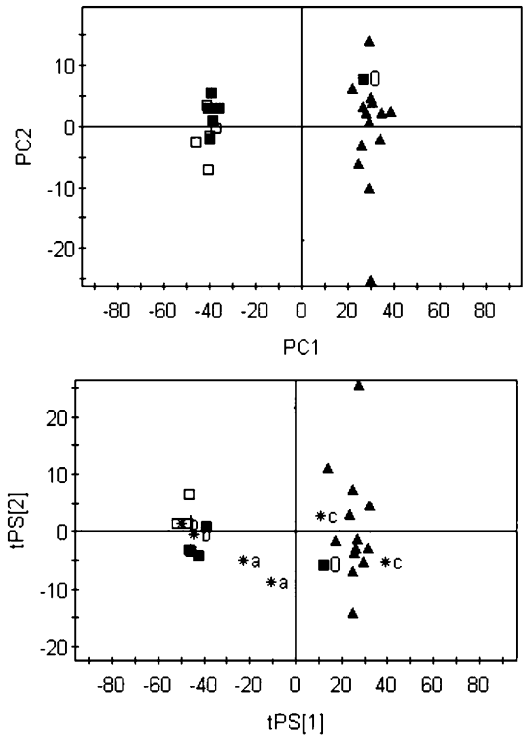

Figure 5. PC score plots of CPMG ^{1}H NMR spectra of canine plasma mapping samples collected prior to oleuropein administration (▲) and after administration of 750 mg of oleuropein powder in milk to dog #1 (■) and dog #2 (□). Plots resulted after the application of OSC using as response vector **Y** two dummy variables (0 and 1, prior to and after administration, respectively). The upper graph contains the training set. In the lower graph the extra symbols (*) correspond to the test set. Key: ■0, sample prior to the administration of 750 mg to dog #1; □0, sample prior to the administration of 750 mg to dog #2; *a, sample after administration of 750 mg to dog #1; *b, sample after administration of 750 mg to dog #2; *c, sample prior to the administration of oleuropein.

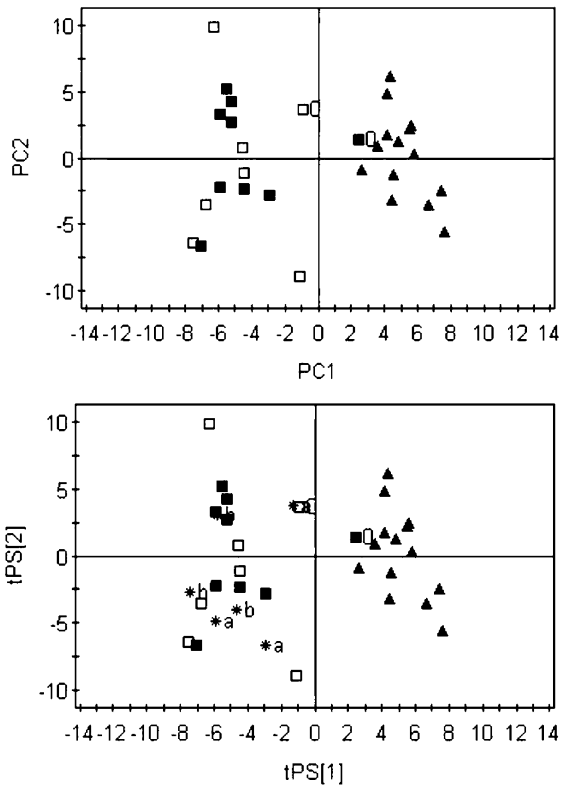

Figure 6. PC score plots of CPMG ^{1}H NMR spectra of canine plasma mapping samples collected prior to the administration of one Fungoral tablet (▲) and after administration of one Fungoral tablet in milk to dog #1 (■) and dog #2 (□). Plots resulted after the application of OSC using as response vector **Y** two dummy variables (0 and 1, prior to and after administration, respectively). The upper graph contains the training set. In the lower graph the extra symbols (*) correspond to the test set. Key: ■0, sample prior to the administration of one Fungoral tablet to dog #1; □0, sample prior to the administration of one Fungoral tablet to dog #2; *a, sample after administration of one Fungoral tablet to dog #1; *b, sample after administration of one Fungoral tablet to dog #2.

treatment with ketoconazole and nifedipine were considered as test sets. OSC was performed only for the training set using two dummy variables (treatment, no treatment). PCA led to successful discriminations as shown in Figures 6 and 7. According to the PC score plot, blank samples were discriminated in the right side of the score plot, while the samples after administration of drug products were situated in the left side in both cases (Figures 6 and 7). Interestingly, this discrimination included all samples obtained from treated dogs also at time points at which plasma concentrations could not be detected. This finding is in agreement with the analogous finding described above for oleuropein.

Specificity of the PCA Models. In order to test the specificity of the resulting models a further chemometric analysis was performed concerning the combined data sets, i.e., the two food components, the two drug products, and blank samples. OSC was applied using two dummy variables corresponding to treatment (food components or drug products) and no treatment. The analysis resulted in no discrimination (data not shown). Thus, the generation of a global model for all substances was not possible. Each substance seemed to produce specific metabolic changes, since different biochemical pathways are involved; this specificity was reflected in the separate models described above.

The specificity of the models was further computed according to the DmodX criterion and the critical value (Dcrit) with 0.95 confidence intervals. These parameters show the distance of each sample to the model in the **X** space.[47] Taking as reference the lycopene model, a Dcrit of 1.273 was computed. All samples of lycopene, as well as blank samples, were within this limit. Considering samples of the other xenobiotics as test sets, it was found that most of them were considerably outside the critical limit. In Figure 8 (upper graph) an example of oleuropein samples fitted on the lycopene model is shown. These results were further supported by Cooman's plots. In Figure 8 (lower graph) the Cooman's plot resulting from lycopene and oleuropein models is demonstrated. In this plot two models were compared using the Dcrit generated for each of them. Four

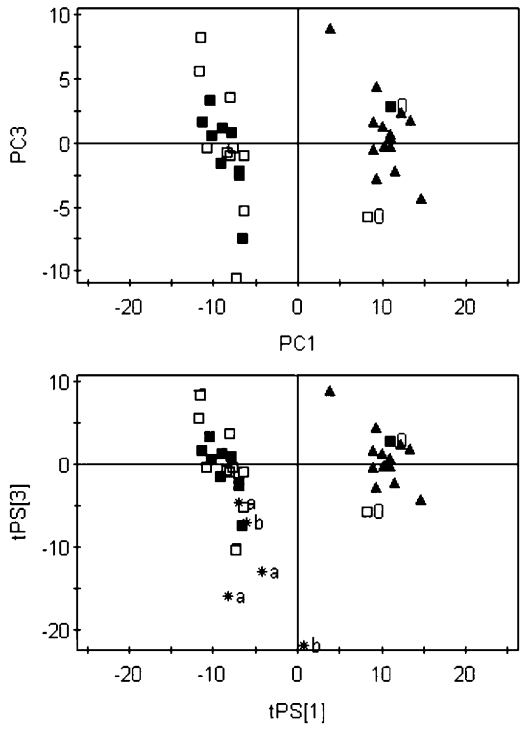

Figure 7. PC score plots of CPMG ^{1}H NMR spectra of canine plasma mapping samples collected prior to administration of two Adalat capsules (▲) and after administration of two Adalat capsules in milk to dog #1 (■) and dog #2 (□). Plots resulted after the application of OSC using as response vector **Y** two dummy variables (0 and 1, prior to and after administration, respectively). The upper graph contains the training set. In the lower graph the extra symbols (*) correspond to the test set. Key: ■0, sample prior to the administration of two Adalat capsules to dog #1; □0, sample prior to the administration of two Adalat capsules to dog #2; *a, sample after administration of two Adalat capsules to dog #1; *b, sample after administration of two Adalat capsules to dog #2.

quadrants were generated corresponding to the following memberships: (a) membership of lycopene samples only (upper left corner), (b) membership of oleuropein samples only (lower right corner), (c) common area of the two models, and (d) exclusion of membership to both models (upper right corner). As shown in the plot, the common area (quadrant c), was occupied by blank samples and by only three oleuropein samples. One lycopene sample entered the oleuropein area (quadrant b), and one oleuropein sample entered the lycopene area (quadrant a), while another oleuropein sample was marginally located in quadrant d. These results show little overlap. Similar results were obtained for ketoconazole and nifedipine. All different combinations of Cooman's plot were performed, and no overlap was observed.

PLS-DA and Predictions of Classes. Pre-OSC PLS-DA for lycopene, ketoconazole, and nifedipine led to models with $Q^2 \approx 0.2$ with poor discrimination. For oleuropein a relatively better model was obtained with $Q^2 = 0.5$. In order to improve discriminations, PLS-DA was repeated after OSC. Post-OSC PLS-DA was performed for each of the above training sets.

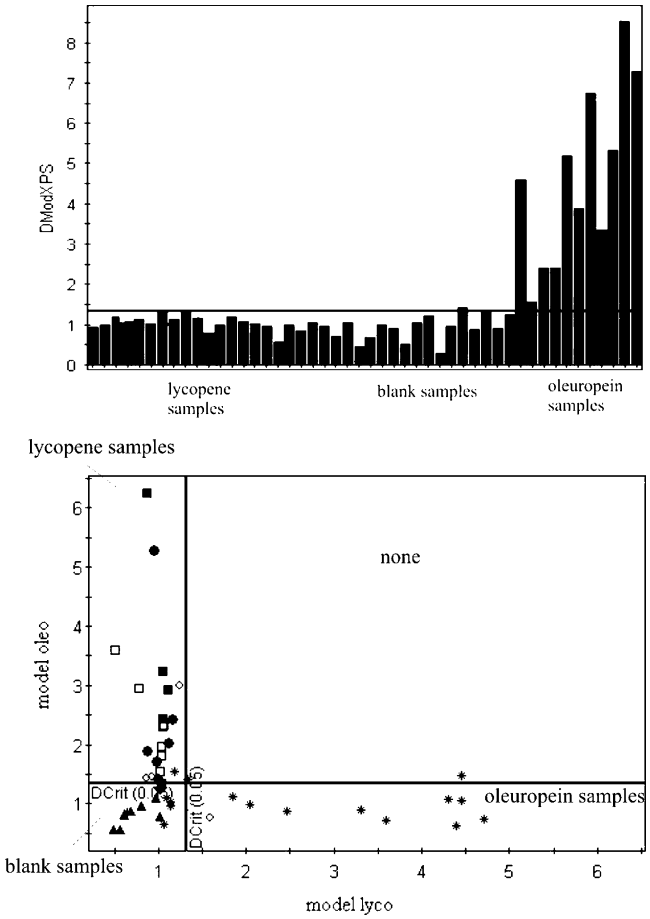

Figure 8. Distance to the model (DmodXPS) plot from CPMG ^{1}H NMR spectra of canine plasma after application of OSC for blank, lycopene, and oleuropein samples (upper graph). Cooman's plot showing the discrimination of samples prior to and after the administration of lycopene and oleuropein (lower graph). Upper left corner, membership of lycopene sample; lower right corner, oleuropein samples only; lower left corner, area occupied by blank samples; upper right corner, exclusion of membership to both models. Key is the same as in Figure 3 (upper graph) with the following addition: *, samples collected after administration of 750 mg of oleuropein in milk.

One component models were derived for each case with $R^2\mathbf{Y} > 0.871$ and $Q^2 > 0.854$. Membership probability was computed for 95% confidence limit for both training and test sets according to the default option of SIMCA software. All lycopene and nifedipine samples were correctly classified with a membership probability >0.6. An example of the discrimination is demonstrated in Figure 9 (upper graph), concerning the lycopene model. In the case of oleuropein model, one sample of the test set showed a class membership (p) on the cutoff value of 0.5. In the case of ketoconazole model two ambiguous classifications were observed with $p = 0.49$ and 0.53, respectively. The first concerned a blank sample in the training set which therefore was considered as false positive, and the second one concerned a sample after treatment in the training set. These results are displayed as a confusion matrix (Table 2) which shows correct classifications and ambiguous classifications for both training

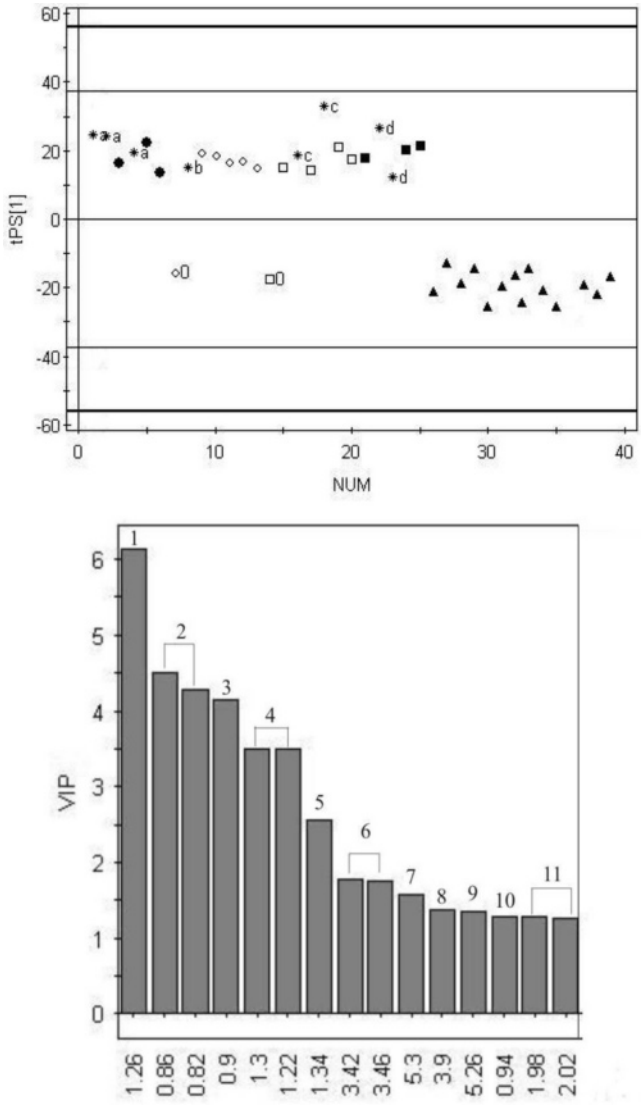

Figure 9. Lycopene PLS-DA model. Upper graph: Score plot of the principal component against sample identity. Symbols and key are the same as in Figure 3. Lower graph: Variable importance plot. Key: 1, LDL, VLDL $(CH_2)_n$; 2, LDL C**H**$_3(CH_2)_n$ and VLDL C**H**$_3CH_2CH_2CH$═; 3, cholesterol C21; 4, LDL, VLDL $(CH_2)_n$; 5, lactate, CH_3; 6, overlapping signals from sugars and amino acids CH_2; 7, unsaturated lipids CH═CH; 8 (probably) creatine signal CH; 9, α-glucose CH; 10, leucine, isoleucine, valine CH_3; 11, lipid CH_2C═C.

and test sets. True positive (TP), true negative (TN), false positive (FP), and false negative (FN) are the four different possible outcomes of a single prediction for a two-class case with classes 0 (no treatment) and 1 (treatment). According to the variable importance plot (VIP) (Figure 9, lower graph), many of the 10 most important variables (VIP > 1.5) were in agreement with ANOVA results. However, some other signals not found statistically significant according to the ANOVA test were also included in the different models, indicating that multivariate statistics can exploit more subtle differences among variables. As an example, in the lycopene model, signals corresponding to lipid residues are the most important variables (VIP > 3), while the lactate signal (δ 1.34) and the signal probably referring to creatine resonance (δ 3.9) are also included (Figure 9, lower plot).

Table 2. Confusion Matrix for the Validation of the PLS-DA Models Resulting for Each of the Four Xenobiotics[a]

		predicted							
		lycopene model		oleuropein model		ketoconazole model		nifedipine model	
		TR	BL	TR	BL	TR	BL	TR	BL
actual	TR	23	0	10 (1)[b]	0	19 (1)[c]	0	24	0
	BL	0	16	0	15	1[d]	15	0	16

[a] Key: TR, samples after treatment with a xenobiotc; BL, blank samples. [b] One test sample was ambiguously predicted with a class membership probability $p = 0.5$. [c] One sample of the training set was ambiguously predicted with $p = 0.53$. [d] One blank sample with $p = 0.49$.

Conclusions

NMR spectroscopy combined with OSC, PCA, or/and PLS-DA was successfully applied to treat subtle alterations in the metabolic profile in canine plasma after single oral administration of two food components and two drug products. This approach permitted a discrimination of samples obtained after treatment from the blank samples, independently from measured plasma concentrations, indicating that these variations persist even if the xenobiotics are not quantified in the plasma. Moreover, models were found to be specific for each substance, with practically no overlap.

Since ^{1}H NMR spectroscopy requires little or no sample preparation, is rapid and nondestructive, and uses small sample volumes,[1] our results described in this work are promising in the perspective of developing models suitable to identify the fingerprint of a xenobiotic prior to its detection or after its disappearance in the bloodstream avoiding time-consuming conventional biochemical approaches.

Acknowledgment. The authors thank the Hellenic Institute of Scholarships for the financial support given to M.A.C. They are grateful for the partial financial support of the General Secretariat of Research and Technology (EPET II, CSF II and PABET 2000), Ministry of Development, Greece, and University of Athens (program Kapodistrias). They also thank Professor L. Skaltsounis and Lecturer P. Magiatis (National and Kapodistrian University of Athens, Greece) for providing *all-trans*-lycopene and oleuropein.

MP060069Z