QCAR models to predict wild mushrooms radical scavenging activity, reducing power and lipid peroxidation inhibition

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ABSTRACT

Wild mushrooms have become attractive as a source of physiologically beneficial compounds including antioxidants such as phenolic compounds and tocopherols. The concentrations of antioxidant compounds (phenolics and α-tocopherol) and EC_{50} values of antioxidant activity (concentration required to achieve 50% of radical scavenging activity and lipid peroxidation inhibition, or 0.5 of absorbance in reducing power) were analyzed by partial least square (PLS) regression analysis. Three QCAR (Quantitative Composition-Activity Relationship) models were constructed and their robustness and predictability were verified by internal and external cross-validation methods. Antioxidant activity correlated well with phenolics and α-tocopherol contents, the major antioxidants in wild mushrooms. The models proved to be useful tools in the prediction of mushrooms radical scavenging activity, reducing power and lipid peroxidation inhibition.

Keywords: Wild mushrooms; Phenolics; α-tocopherol; Antioxidant activity; QCAR; PLS

1. Introduction

An excess of reactive oxygen and nitrogen species (ROS and RNS) leads to oxidative stress, resulting in oxidative DNA, proteins and lipids damage which have been related to degenerative processes inherent to several diseases such as cancer, Alzheimer's, Parkinson's, arthritis, asthma, diabetes and cardiovascular diseases [1]. The increasing interest in human health, nutrition and disease prevention has enlarged consumers' demand for functional foods [2].

Mushrooms have become attractive as a source of physiologically beneficial compounds including antioxidants such as phenolic compounds (mostly phenolic acids) [3-6] and tocopherols [7-10]. Accumulating chemical, biochemical, clinical and epidemiologic evidence supports the chemoprotective effects of phenolic antioxidants against oxidative stress-mediated disorders [11]. The pharmacological actions stem mainly from their free radical scavenging activity and metal chelating properties as well as their effects on cell signaling pathways and on gene expression [11]. Due to its role as scavenger of free radicals, α-tocopherol is also believed to protect our organism against degenerative malfunctions, mainly cancer and cardiovascular diseases [12].

Numerous tests have been used for measuring the antioxidant capacity of mushrooms including 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (RSA), reducing power (RP) and inhibition of lipid peroxidation (ILP) [4,7,8,10]. The mentioned tests were applied to mushrooms from different countries such as Brasil, China, India, Korea, Spain, Taiwan, Turkey and Portugal [13].

In a previous report our research group established a Quantitative Composition-Activity Relationships (QCAR) model to predict the reducing power of mushrooms based on

their phenolics and flavonoids concentrations [14]. Nevertheless, despite our attempts it was not possible to construct a robust model to predict free radical scavenging activity and lipid peroxidation inhibition based on those parameters. Herein we describe the establishment of QCAR models to predict different aspects of antioxidant activity (RSA, RP and ILP) of wild mushrooms based on phenolics and α-tocopherol concentrations.

2. Methods

2.1. Data set

A total of seventeen samples from seventeen Portuguese wild mushroom species were used in this study (Table 1). The samples were collected in Bragança (Northeast of Portugal), in autumns of 2008. Phenolics and α-tocopherol concentrations, radical scavenging activity (RSA), reducing power (RP) and inhibition of lipid peroxidation (ILP) EC_{50} values were obtained from a previous report of our research group [10]. Phenolic contents were determined by Folin-Ciocalteu's spectrophotometer assay, while α-tocopherol concentrations were obtained for high performance liquid chromatography (HPLC) coupled to fluorescence detection.

For antioxidant activity data, the results of three *in vitro* assays were used: scavenging activity on DPPH radicals- RSA, reducing power- RP, and inhibition of lipid peroxidation- ILP. The RSA was calculated as a percentage of DPPH discolouration using the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The RP was obtained by measuring the

absorbance of the product obtained by the reduction of the ferric ion to the ferrous form; a higher absorbance indicates higher RP. The inhibition of lipid peroxidation was evaluated through β-carotene bleaching inhibition, using the equation: (β-carotene content after 2h of assay/initial β-carotene content) × 100.

To make RSA, RP and ILP data homogenous and comparable, all the values were reported as EC_{50} (expressed in mg/ml, concentration required to achieve 50% of RSA and ILP, or 0.5 of absorbance in RP).

2.2. QCAR models

To build the three QCAR models the complete data set (Table 1) and the Partial Least Square (PLS) method implemented in SIMCA-P v12 statistics software, were used [15]. The seventeen samples were first divided in two groups: training and test sets. The training set, representing about 3/4 of the total number of samples (13 samples), was used to build the QCAR models. The remaining 1/4 (4 samples) was assigned to the test set and used to validate the model. The division was made to cover all the antioxidant activity scale [16,17] and the samples included on the training set were randomly selected within each group [18].

The goodness of fit of the models was evaluated using the following statistical parameters: squared correlation coefficient (R^2), standard deviation of regression (S), significance of the model (P) and Fisher ratio value (F).

The predictive stability and robustness of the models was first verified by internal cross-validation calculating the following parameters: Q^2_{LOO} ("Leave-One-Out"; 1-PRESS/TSS were PRESS is the Predictive Error Sum of Squares and TSS the Total

Sum of Squares), permutation test of SIMC-P+ software and RMSE $_{(training\ set)}$ (Root Mean Squared Errors for the training set) [19-21]. Using the test set, the models were further checked by external cross-validation by calculating parameters: Q^2_{ext} (External, 1-(PRESS/n_{EXT})/(TSS/n_{TR})) and RMSE $_{(test\ set)}$ (Root Mean Squared Errors for the test set). PRESS is defined as the sum of the squared difference between the observed value and the predicted value for each compound in the training set, n_{EXT} is the number of observation in the external test set, TSS is defined as the sum of squared deviations from the data set mean and n_{TR} is the number of observations in the training set [22].

3. Results and Discussion

3.1. QCAR models validation

Three QCAR models were developed where the antioxidant activity of wild mushrooms, measured using different methods (RSA, RP and ILP), was correlated with phenolics and α-tocopherol content. To test the predictive power of all models a thorough statistical analysis was conducted and several parameters were calculated (Table 2). Special attention was paid to model validation and for this reason an external validation procedure was performed. This approach implied that the tested mushrooms species were randomly divided in training and test sets (Figure 1), with 13 and 4 species on each set respectively. Although a smaller number of species is used to build the models, the external validation approach is widely acknowledged as the best method to validate a predictive model, as it is usually immune to over-fitness and over-prediction. The robustness and predictive power of all models were confirmed by internal leave-

one-out (LOO) validation, as demonstrated by R^2 and Q^2 values, and by external validation as demonstrated by Q^2_{ext} value. Also, both the RMSE values for the training and test sets were low and similar thus validating the models.

Further validation was performed using a permutation test provided by SIMCA-P software (Figure 2) [19]. In this test the models were recalculated for randomly reordered response data (EC_{50} RSA, EC_{50} RP and EC_{50} ILP) and these permuted EC_{50} RSA, EC_{50} RP and EC_{50} ILP values were related to intact predictor data by refitting the model and including cross-validation. For each model the intercepts of the two regression lines (for $R^2_{permutation\ test}$ and $Q^2_{permutation\ test}$) indicate the degree of over-fit and over-prediction. Intercepts for $R^2_{permutation\ test}$ and $Q^2_{permutation\ test}$ below 0.30 indicate a valid model, as is the case for the three models. Figure 2 shows the results obtained from 100 permutations for each of the samples under study.

Figure 3 shows plots of predicted versus experimental EC_{50} values for the three models (RSA, RP and ILP), were mushrooms from both the training and test sets are included. A good agreement is observed between predicted and experimental EC_{50} values confirming the predictability of QCAR models.

Also, an analysis of possible outliers was conducted by plotting the residuals (predicted EC_{50}–experimental EC_{50}) versus experimental EC_{50} for the three models (Figure 4). The residual values in the three models were within the -3S to 3S standard deviation interval, usually considered the limits for spotting outliers, so none of the observation values was considered as an outlier. The random distribution of the residuals observed about zero on all models is also a measure of the robustness of the model.

3.2. QCAR models interpretation

A good correlation between the RSA, RP and ILP with phenolics and α-tocopherol was only observed when the values were

The correlation between RSA, RP and ILP with phenolics and α-tocopherol content was not linear rather exponential. This is an indication that higher contents of these compounds in mushrooms will significantly increase the antioxidant potential (lower RSA, RP and ILP EC_{50} values mean better antioxidant activity). Furthermore, the QCAR models confirm the powerful antioxidant properties of phenolics and tocopherols as also their significant contribution to the antioxidant properties reported for several wild mushrooms. Although the three models presented good statistical correlation, the RSA model presented the highest correlations both by internal (Q^2_{LOO}=0.895) and external (Q^2_{ext}=0.981) validation and can thus be considered the best model.

RSA assay measures the reducing capacity of antioxidants toward DPPH (stable organic nitrogen radical with a deep purple colour. Therefore, phenolics and α-tocopherol present in the mushrooms extracts, which have a high antioxidant activity, result in a rapid decline in the absorbance of the DPPH [23,24].

In the reducing power assay the Fe^{3+}/ferricyanide complex ($FeCl_3/K_3Fe(CN)_6$) is reduced to the ferrous form (Fe^{2+}). Therefore, in the presence of mushrooms extracts with phenolics and α-tocopherol, the yellow colour of the test solution changes to various shades of green or blue, and this can be measured at 700 nm [24].

Decolourization of β-carotene can be monitored at 470 nm and can be employed as an assay of inhibition of lipid peroxidation. The β-carotene undergoes a rapid discoloration

in the absence of an antioxidant since the free linoleic acid radical attacks the β-carotene molecule, which loses the double bonds and, consequently, loses its characteristic orange colour [25]. Mushrooms antioxidants (phenolics and α-tocopherol) can neutralize any free radicals formed within the system (*e.g.*, the linoleate free radical) and, consequently, may delay decolourization of β-carotene [24,26].

The antioxidant activity of phenolic compounds (ArOH) seems to be related with the phenolic groups and transference of electrons or hydrogen atoms according to $LOO^{\bullet}$ + ArOH → LOOH + $ArO^{\bullet}$ ($ArO^{\bullet}$ has to be stable enough to react slowly with LH, but rapidly with $LOO^{\bullet}$, interrupting the chain reactions) [27].

In the lipid peroxidation process, tocopherols can act as antioxidants by donating an hydrogen atom to peroxyl radicals produced from polyunsaturated fatty acids in membrane phospholipids or lipoproteins to yield a stable lipid hydroperoxide (LOOH) and a tocopheroxyl radical ($TO^{\bullet}$), which can react itself with other peroxyl or tocopheroxyl radicals to yield stable compounds: $LOO^{\bullet}$ + TOH → LOOH + $TO^{\bullet}$. Tocopherols can also react with alkoxyl radicals ($LO^{\bullet}$) formed in the propagation step ($LO^{\bullet}$ + TOH → LOH + $TO^{\bullet}$) or, in oxygen limited conditions and low hydroperoxides concentrations, can react directly with $L^{\bullet}$ radicals ($L^{\bullet}$ + TOH → LH + $TO^{\bullet}$) [12].

The three different QCAR models obtained for RSA, RP and ILP indicate that the mechanisms of action of phenolics and α-tocopherol in each assay is different, but probably of a synergistic nature in all the cases. Currently this integrated mode of action of mushrooms chemical compounds is acknowledged to the key element on several mushrooms beneficial biological activities. As investigation in the field of natural products starts to focus more on this synergistic view, cheminformatics studies

including QCAR modeling may become an important tool in understanding the active properties of natural extracts.

Overall, three predictive QCAR models for mushrooms free radical scavenging activity, reducing power and lipid peroxidation inhibition, using phenolics and α-tocopherol concentrations, were developed. Several internal and external validation protocols were used to confirm the predictive power of the QCAR models. Especially external validation is acknowledged to be the best rationale protocol to avoid over-prediction and over-fitting. These models will be useful tools in the study and prediction of antioxidant activity of more mushroom species.

Acknowledgements

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) and COMPETE/QREN/EU for financial support through the research project PTDC/AGR-ALI/110062/2009.

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Table 1. Phenolics (mg GAE/g extract), α-Tocopherol (μg/g of dry weight), experimental and predicted antioxidant activity (reducing power- RP, radical scavenging activity- RSA and inhibition of lipid peroxidation- ILP) values (mg/ml) of wild mushrooms from Portugal.[a]

Species	Phenolics	α-Tocopherol	Experimental EC_{50} RSA	Predicted EC_{50} RSA	Residues RSA	Experimental EC_{50} RP	Predicted EC_{50} RP	Residues RP	Experimental EC_{50} (ILP)	Predicted EC_{50} (ILP)	Residues ILP
Clitocybe alexandri	1.53	0.00004	28.72	26.77	1.95	7.01	5.25	1.76	4.45	3.14	1.31
Cortinarius glaucopus	2.8	0.00011	16.59	13.45	3.14	3.04	3.64	-0.60	1.73	1.53	0.20
Fistulina hepatica	4.44	0.00012	5.32	6.72	-1.40	2.24	2.46	-0.22	0.94	0.99	-0.05
Hygrophoropsis aurantiaca	7.9	0.0002	1.20	1.35	-0.15	1.34	1.01	0.33	0.71	0.54	0.17
Hypholoma capnoides[b]	1.71	0.00017	20.85	18.16	2.69	4.42	4.64	-0.22	2.90	2.24	0.66
Laccaria amethystina	2.85	0.00005	15.72	15.24	0.48	3.03	3.82	-0.79	1.23	1.72	-0.49
Laccaria laccata	1.59	0.00022	21.95	16.90	5.05	5.22	4.32	0.90	3.69	2.30	1.39
Lactarius aurantiacus	0.58	0.00003	30.00	40.43	-10.43	7.91	6.62	1.29	7.48	7.98	-0.50
Lactarius salmonicolor	4.14	0.00004	7.80	9.22	-1.42	2.39	2.86	-0.47	1.01	1.27	-0.26
Lepista inversa	3.6	0.00028	10.57	6.43	4.14	2.92	2.54	0.38	1.08	1.05	0.03
Lepista sordida[b]	4.1	0.00002	9.82	9.84	-0.02	2.95	2.58	0.37	1.03	1.43	-0.40
Mycena rosea[b]	3.56	0.00011	10.58	9.86	0.72	3.05	2.94	0.11	1.15	1.23	-0.08
Russula delica	2.23	0.00001	20.53	21.63	-1.10	4.41	4.60	-0.19	2.28	2.79	-0.51
Russula vesca	6.61	0.00002	3.91	3.53	0.38	1.53	1.64	-0.11	0.91	0.92	-0.01
Suillus collinitus	3.16	0.00006	14.05	13.11	0.94	2.97	3.52	-0.55	1.20	1.52	-0.32
Suillus mediterraneensis[b]	7.46	0.00004	2.90	2.38	0.52	1.32	1.47	-0.15	0.81	0.74	0.07
Tricholoma sulphureum	4.76	0.00006	4.69	6.82	-2.13	2.19	2.43	-0.24	0.93	1.04	-0.11

[a]Data obtained in previous work [10]. [b]Test set observations.

Table 2. Statistical parameters and formulas of the models radical scavenging activity (RSA), reducing power (RP) and inhibition of lipid peroxidation (ILP) using PLS method.

Model	N	R^2	Q^2_{LOO}	F	P	S	RMSE (training set)	Q^2_{ext}	RMSE (test set)
RSA	13	0.932	0.895	42.4	1.30×10^{-5}	2.32	3.999	0.981	1.416
PR	13	0.887	0.791	18.9	3.96×10^{-4}	2.18	0.824	0.974	0.234
ILP	13	0.890	0.84	26.3	1.05×10^{-4}	2.25	0.649	0.956	0.388

Formulas

RSA (mg/ml) = $10^{(-0.8991 * \text{Phenolics (mg GAE/g)} - 0.2238 * \alpha\text{-Tocopherol } (\mu g/g) + 2.49503)}$

PR (mg/ml) = $10^{(-0.89308 * \text{Phenolics (mg GAE/g)} - 0.16294 * \alpha\text{-Tocopherol } (\mu g/g) + 2.11326)}$

ILP (mg/ml) = $10^{(-0.913313 * \log(\text{Phenolics (mg GAE/g)}) - 0.162591 * \log(\alpha\text{-Tocopherol } (\mu g/g)) + 0.438538)}$

N- number of samples, S- standard deviation, R^2- squared correlation coefficient, P- significance, F- Fisher ratio, Q^2_{LOO}- "Leave-One-Out" correlation coefficient and RMSE- Root Mean Squared Errors for the training set and test set.

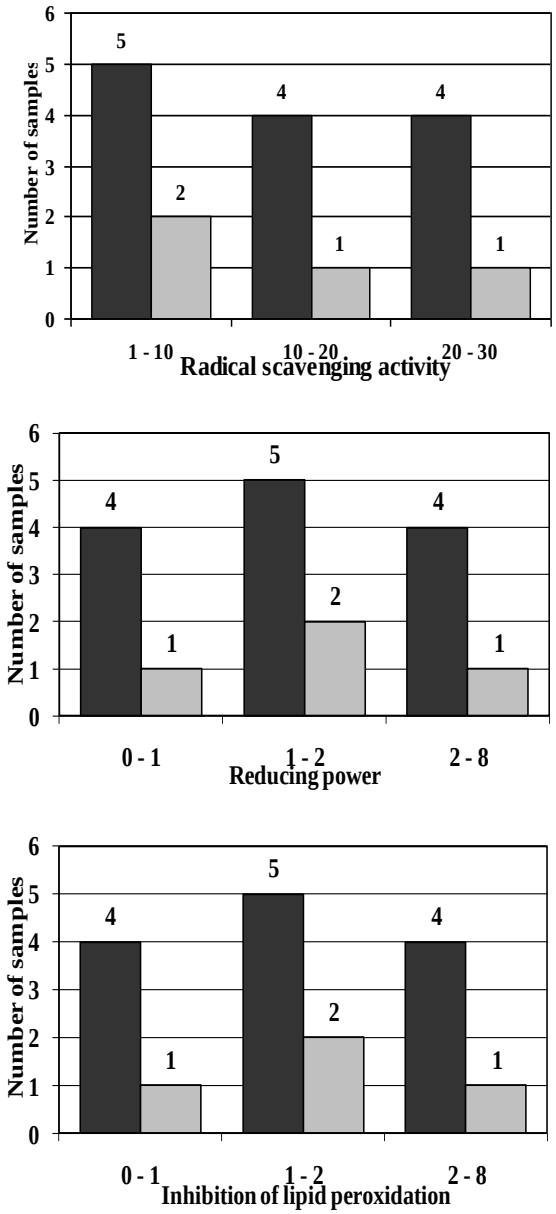

Fig. 1. Distribution of radical scavenging activity (RSA), reducing power (RP) and inhibition of lipid peroxidation (ILP) *versus* number of samples for the training set (black) and test set (grey) of the QCAR models.

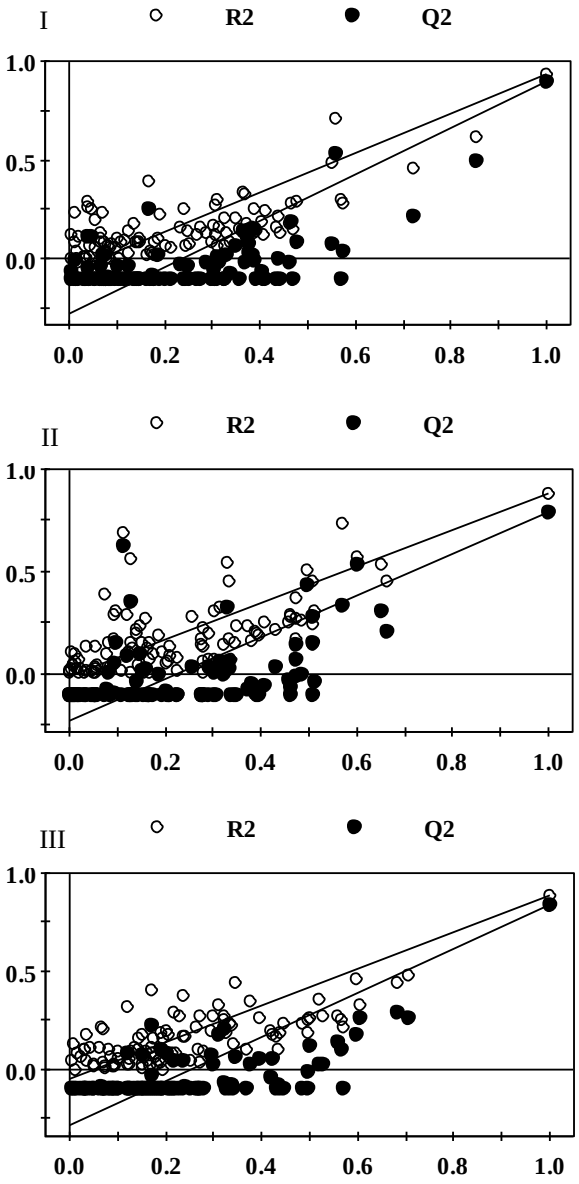

Fig. 2. Results of the permutation test. The R^2 and Q^2 values were obtained from 100 permutations for the three developed PLS models. Intercepts: (I) RSA: (R^2) $R^2_{\text{permutation test}}$=0, -0.0595, ($Q^2$) $Q^2_{\text{permutation test}}$ =0, -0.273; (II) RP: R^2=0, -0.0139, Q^2=0, -0.23; (III) ILP R^2=0, -0.0525, Q^2 =0, -0.285. The figure shows on the vertical axis, for each selected y-variable (RSA, RP or ILP), the values of R^2 and Q^2 for the original model (far to the right) and of the y-permuted models further to the left. The horizontal axis shows the correlation between the permuted y-vectors and the original y-vector for each selected y-variable. The original y has the correlation 1.0 with itself, defining the high point on the horizontal axis [15].

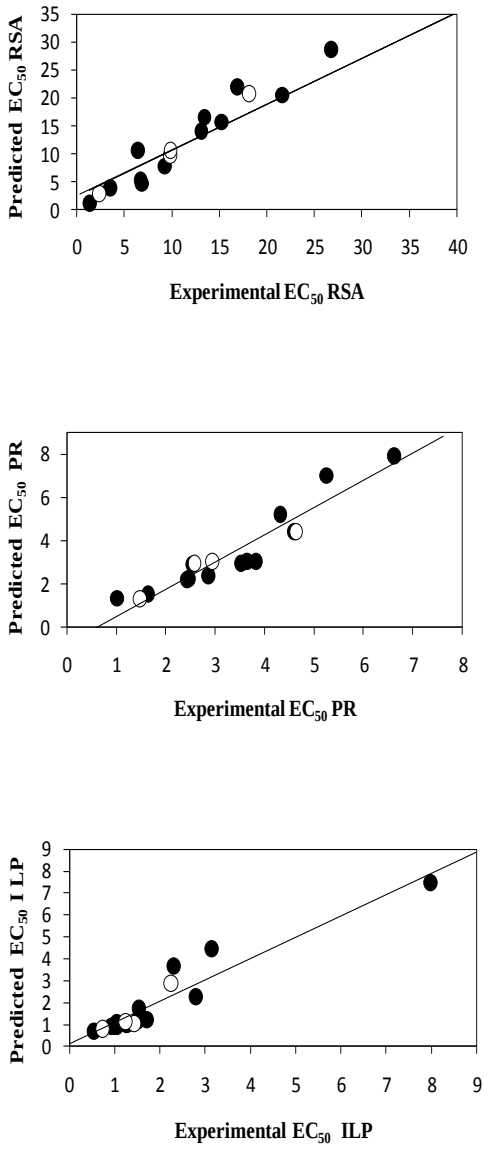

Fig. 3. Predicted versus experimental EC_{50} RSA, EC_{50} RP and EC_{50} ILP, for the training (•) and test sets (o).

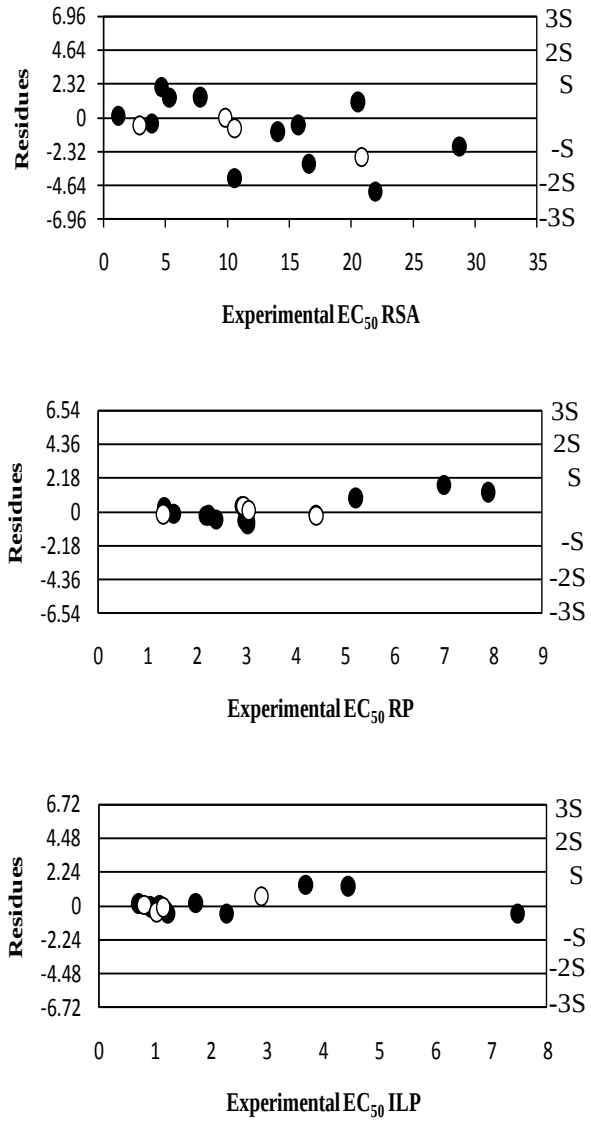

Fig. 4. Residual vs. Experimental EC_{50} RSA, EC_{50} RP and EC_{50} ILP, for the training (•) and test sets (o).