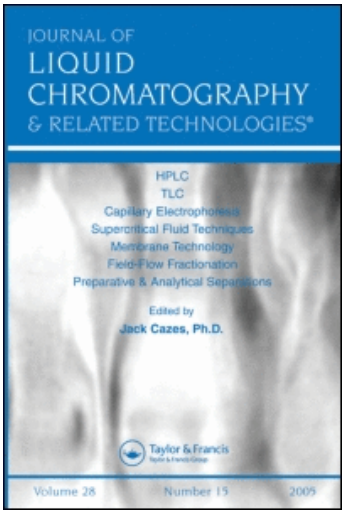

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INTERCALIBRATION OF CHROMATOGRAPHIC METHODS IN MULTIRESIDUE PESTICIDE DETERMINATION

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ABSTRACT

An Ion-Interaction RP-IIR-HPLC method was developed, able to simultaneously separate neutral, basic, and acidic pesticides in a single run. The proposed method was validated by comparison of the results obtained for the same sample by conventional RP-HPLC and GC/MS methods. For this purpose parametric *t-*, *F-*, *t-paired, and t- for multiple samples* and non parametric *Wilcoxon matched-pair signed-rank* tests were employed. The accuracy of the proposed IIR-RP-HPLC method was evaluated with respect to a lab-made reference sample of spiked tap water.

INTRODUCTION

The pesticides in use for weed control in agriculture and forestry are many hundreds and characterized by very different functionalities. Many of them present low biodegradability and low volatility; thus herbicides and their residuals can easily cumulate in soils and become phytotoxic for sensitive crops cultivated in rotation. Sensitive multiresidue methods are, therefore, required for the analysis of surface waters and soils, to simultaneously determine as many residues as possible.

The EC Directives on the Quality of Waters intended for Human Consumption states that the concentration of pesticides must not exceed the level of 0.1 μg/L for each compound and 0.5 μg/L for total pesticides.[1] For surface water a total amount of 30 μg/L is generally accepted.

A standard reference method, or a generally-recognized standard method, does not exist and the choice of the method to be used is often up to the analyst, as a function of the composition of the water sample, the kind of pesticide of particular interest, and the availability of the instrumentation. Anyway, chromatographic methods are generally suggested and, in lack of a standard reference method, the comparative use of two independent methods or, at least, of two different stationary phases characterized by different polarities, is recommended. HPLC and GC methods are generally employed but none of them are able to simultaneously separate neutral, acidic, and basic pesticides, such as the nowadays widely used phenoxyacids, phenylureas, and triazines, due to their different lipophilicity.

GC appears to be the major analytical technique due to its high efficiency and the availability of sensitive and selective detectors. In particular, GC methods for the analysis of ureic-,[2-4] triazinic-,[5-14] and phenoxyacidic- (after derivatisation)[15,16] pesticides are reported. Few GC methods concern the simultaneous separation of pesticides belonging to different classes, such as triazines and phenylureas.[17] On the other hand, HPLC application is suitable for the analysis of non-volatile, thermolabile or highly polar compounds, such as phenoxyacids.[18-26] Multiresidual methods using HPLC were developed in the last years thanks also to the introduction of the hyphenation with mass-spectrometry.[27-33] Anyway, to our knowledge, none of the multiresidual methods developed up to now allows the simultaneous separation of the two main groups of acidic- and neutral-basic pesticides.

In ion-interaction mode a reversed stationary phase is dynamically modified by a suitable Ion-Interaction Reagent (IIR) present in the eluent. Typically the IIR is a salt formed by a lipophilic cation that is adsorbed onto the ODS and by an anion that is in turn retained onto the stationary phase surface

through electrostatic forces. Thus an electrical double layer is formed onto the original surface and the modified column becomes able to retain, even simultaneously, both cationic and anionic species. In addition, taking into account that not all the original RP sites are modified, also neutral species can be retained under the same conditions.

These properties were experimented here in the separation of a mixture of pesticides with predominantly basic (as triazines and urea-derivatives), neutral (as bromacil) and acidic (as cresol-derivatives with pK 9-10 and, mainly, phenoxyacids characterized by pK values around 3-4) character. The structures of the analytes are reported in Figure 1.

The proposed method was validated by: i) the comparison of the results obtained with the new method with those obtained for the same sample by conventional RP-HPLC and by GC/MS methods, with the help of statistical parametric and non parametric tests and ii) the evaluation of the results obtained with the new method for a lab-prepared spiked sample of tap water, used as a reference standard.

EXPERIMENTAL

Instrumentation

HPLC analysis were carried out with a Merck-Hitachi (Tokyo, Japan) Lichrograph Chromatograph Model L-6200, equipped with a two channel D-2500 Chromato-integrator and interfaced with a UV-visible detector L-2450 of the same firm. Gas-chromatographic analyses were performed by a GC HP 5890, Series II (Hewlett Packard, PA, USA) interfaced with a quadrupolar MS detector HP 5972.

A Metrohm 654 pH-meter (Switzerland) equipped with a combined glass-calomel electrode was employed for pH measurements and a Hitachi (Tokyo, Japan) Model 150-20 spectrophotometer for absorbance measurements.

Chemicals and Reagents

Ultrapure Milli-Q water (Millipore) was used for the preparation of all the standard solutions. Hexylamine was Fluka analytical grade chemical and dichloromethane was Merck analytical grade chemical. Ortho-phosphoric acid was C. Erba chemical and acetonitrile BDH analytical grade chemical. The pesticides were supplied by Lab Service (Bologna, Italy).

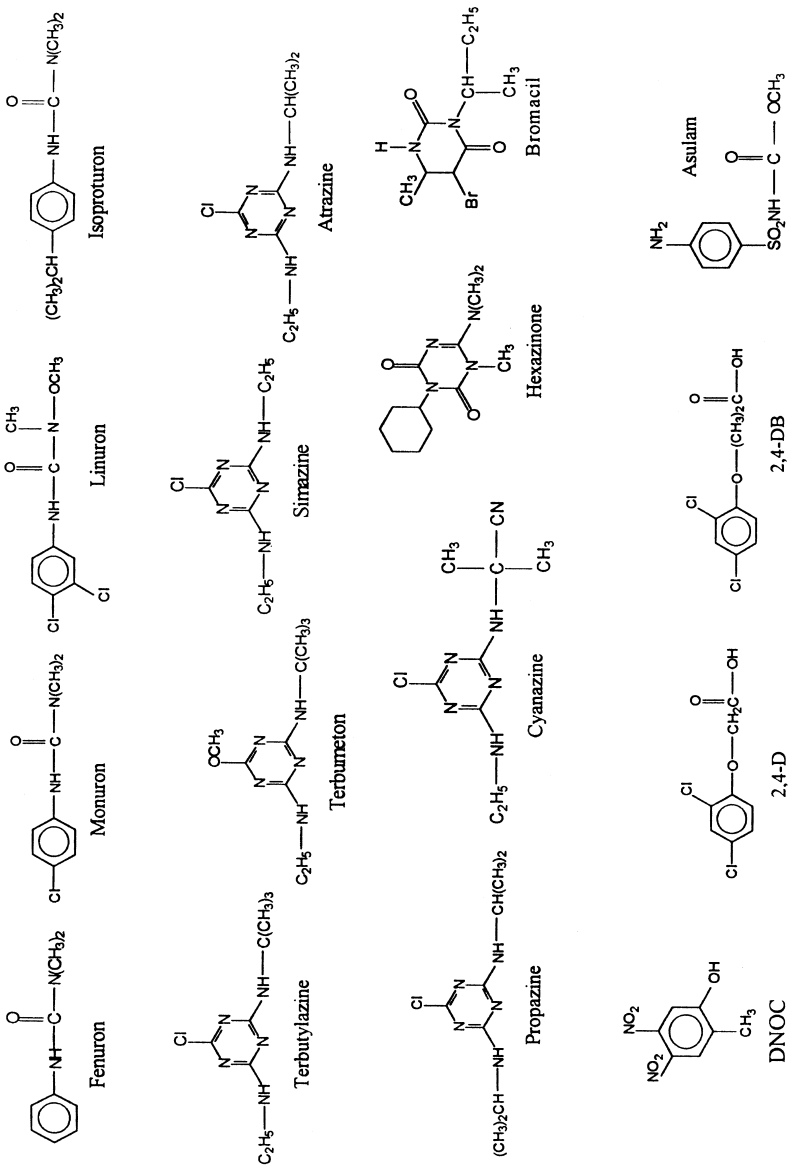

Figure 1. Molecular structures of the 15 pesticides considered.

IIR-HPLC Analysis

The stationary phase was a Merck LiChrosorb C18 (250 × 4.6 mm ID, 5μm) column fully end-capped, used together with a guard pre-column Merck Lichrospher RP-18, 5μm, dynamically modified, in isocratic conditions, by an alkylammonium salt present in the mobile phase. The optimized eluent was a 5.0 mM hexylamine -ACN (73:27, v/v) mixture brought to an operational pH = 6.4 ± 0.2 by ortho-phosphoric acid.

In order to shorten the total analysis time the following flow-rate (F) program was employed: 0-15 min, F = 1.0 mL/min; 15-20 min, F from 1.0 mL/min to 3.0 mL/min; 20-60 min, F = 3.0 mL/min.

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline was obtained; about one hour at 1 mL/min was necessary. After use the column was washed by flowing water (0.50 mL/min for 15 min), a water-acetonitrile 50:50 (v/v) mixture (0.50 mL/min for 30 min) and finally 100% acetonitrile (0.5 mL/min for 5 min). Spectrophotometric detection was performed at 240 nm.

RP-HPLC Analysis

The same stationary phase as for the IIR-HPLC analyses was employed.

The elution was carried on at flow-rate of 1.0 mL/min with the following concentration gradient: 0-20 min: ACN-water (25:75, v/v); 20-40 min: ACN-water from (25:75, v/v) to ACN-water (50:50, v/v); 40-60 min: ACN-water (50:50, v/v).

GC-MS Analysis

Gas chromatographic analyses were performed in the following conditions:

Capillary column: HP-5MS, (30 m ×0.25 mm ID, 0.25 μm thin layer).
Oven: 40°C (5 min); 40°C to 160°C at 20°C min^{-1}; 160°C hold 14 min; 160°C to 280°C at 20°C/min; 280°C hold 5 min.
Carrier: helium, pression pulse to 35 psi during the injection, then constant flow-rate = 0.7 mL/min.
Injection: splitless, 1 μL, 250°C.
MS scan program: 50-400 *m/z*.

Reference Sample

The reference sample was prepared by spiking 1.00 L of tap water with 0.1 μg/L of each of the 15 pesticides. The solution was then filtered through Millipore 0.45 μm, brought to pH <3 by HCl and extracted with two 25.0 mL aliquots of dichloromethane. The combined organic extracts were evaporated to dryness on a Rotovapor at 30°C under vacuum and diluted with the mobile phase to a volume of 0.5 mL. As a blank, 1.00 L of not-spiked water was treated in the same way and analyzed.

RESULTS

Conventional RP-HPLC Method

As mentioned, literature reports examples of HPLC multiresidual separations of neutral and basic pesticides, while no example of the separation including acidic pesticides too is reported. Anyway, all the possibilities offered by the conventional RP-HPLC to get the separation of bromacil (neutral), triazine- and urea- derivatives (basic) and acidic phenoxyacids were explored.

With a water/ACN (73:27, v/v) solution as the mobile phase, neutral (bromacil) and basic (ureic and triazines) species can be separated, but the hydrophilic phenoxyacids are not retained and co-elute with the solvent front. Also, when bringing the pH value of the mobile phase to values around 3, in order to enhance the lipophilicity of acidic analytes, it was impossible to find out conditions of organic solvent composition in the mobile phase suitable for all the pesticides investigated. At around pH 3 phenoxyacids require organic solvent concentration much higher than triazinic and ureic species; also the use of gradient elution was of no help. No other possibility to intervene is offered by the RP-HPLC mode.

The quantitation levels of the pesticides investigated, evaluated through the standard calibration plots and for a signal to noise ratio =3, range from 2 μg/L for fenuron to 20 μ/L for bromacil.

Ion-Interaction RP-HPLC Method

The IIR-HPLC technique offers more opportunities of optimization, due to the larger number of factors affecting the retention. Recently in our laboratories the use of alkyl phosphate salts as ion interaction reagents (IIR) was shown to be

particularly versatile in the separation of many cationic and anionic analytes[34,35] and the effect on resolution of i) IIR alkyl chain length, ii) mobile phase pH, iii) IIR concentration and iv) organic solvent concentration was studied.

To find the optimum resolution conditions for the pesticides considered here the use of IIR as phosphate salts of hexylamine, heptylamine, and octylamine was experimented. The effect of the pH of the mobile phase was studied in the range between 3 and 8 and the ACN concentration was varied between 20 % and 50%.

The use of hexylammonium phosphate at pH=6.4 (ACN concentration 27%) permitted the separation, with good resolution, of the 15 pesticide mixture considered. In the presence of the ion-interaction reagent, acidic and basic pesticides are retained as ion-pairs onto the modified stationary-phase surface, as shown by the different retention times obtained in the presence and in the absence of IIR in the mobile phase. On the contrary, bromacil (the must lipophilic one), even in presence of IIR, is retained through conventional reversed-phase mode and its retention time is practically the same as observed in RP-HPLC mode.

Figure 2 shows the chromatogram recorded under the optimized conditions. The quantitation levels, evaluated by the calibration plots and for a signal to noise ratio =3, are very similar to those obtained in RP-HPLC and range between 2.0 μg/L for fenuron and monuron to 19 μg/L for terbumeton.

Gas Chromatographic Method

In order to extend the comparison to another widely diffused method for the analysis of pesticides, a GC/MS method was employed. Obviously the gas-chromatographic behaviour is largely influenced by the chemical structure of the pesticide. In particular, the ureic-derivatives are characterized by very low sensitivity, since they undergo thermal decomposition in the injector. An initial pressure pulse of the carrier gas during the injection, that leads to a reduction of the residence time of the analytes in the injector, was of some help. For detection purposes, regarding isoproturon and monuron their decomposition products (substituted chloro-benzene isocyanate) were followed by the mass analyzer.

Due to their very low volatility the detection of 2,4-D, 2,4-DB and DNOC was not possible, as expected. Thus the comparison with the IIR-HPLC method regarded just 12 pesticides. Quantitation levels resulted much higher than those obtained in liquid chromatography, ranging between 0.5 and 1.0 mg/L.

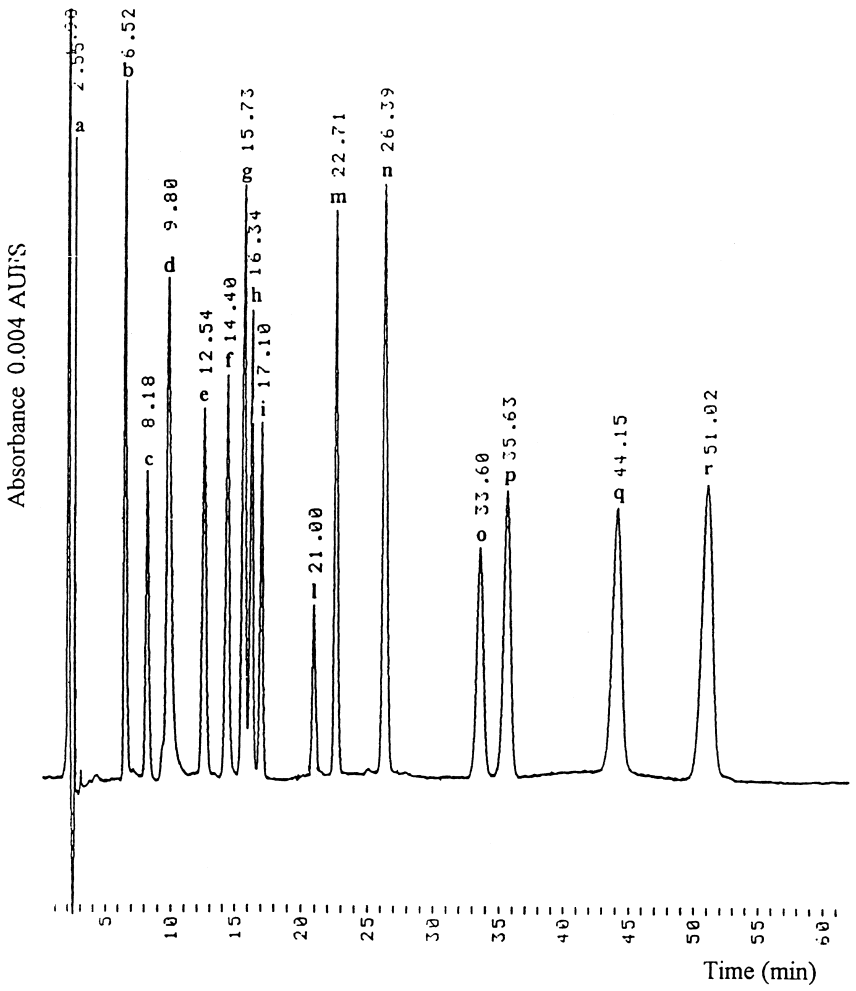

Figure 2. IIR-HPLC separation of the 15 pesticides: a) fenuron (0.15 mg/L), b) 2,4-D (0.50 mg/L), c) DNOC (0.50 mg/L), d) hexazinone (0.26 mg/L), e) bromacil (1.20 mg/L), f) simazine (0.40 mg/L), g) monuron (0.13 mg/L), h) cyanazine (0.40 mg/L), i) 2,4-DB (0.50 mg/L), l) atrazine (0.90 mg/L), m) isoproturon (0.40 mg/L), n) terbumeton (1.00 mg/L), o) propazine (1.00 mg/L), p) terbutylazine (1.00 mg/L), q) linuron (1.00 mg/L). Injected volume: 100.0 μL. Spectrophotometric detection at 240 nm. Injected volume: 100.0 μL. Stationary phase: Lichrosorb C18 (250 × 4.6 mm i.d., 5 μm). Mobile phase: 5.00 mM hexylammonium phosphate water solution-ACN (73:27, v/v), pH 6.4. Flow-rate program: 0-15 min: 1.0 mL/min; 15-20 min: from 1.0 mL/min to 3.0 mL/min; 20-60 min: 3.0 mL/min.

Table 1

Comparison Between RP-HPLC and IIR-HPLC*

	Conc. mg/L	$\bar{x} \pm s$ RP-HPLC	IIR-HPLC
Fenuron	0.100	0.102 ± 0.006	0.108 ± 0.002
Hexazinone	0.200	0.210 ± 0.007	0.201 ± 0.004
Bromacil	1.000	1.018 ± 0.019	1.016 ± 0.048
Simazine	0.400	0.404 ± 0.017	0.409 ± 0.012
Monuron	0.100	0.106 ± 0.003	0.100 ± 0.007
Cyanazine	0.400	0.411 ± 0.008	0.407 ± 0.011
Atrazine	0.500	0.509 ± 0.037	0.518 ± 0.020
Isoproturon	0.400	0.404 ± 0.014	0.415 ± 0.024
Propazine	1.000	1.005 ± 0.085	1.008 ± 0.077
Terbutylazine	1.000	1.003 ± 0.060	1.004 ± 0.047
Terbumeton	1.000	1.018 ± 0.085	0.998 ± 0.125
Linuron	1.000	1.009 ± 0.058	1.025 ± 0.095

* Average value of 4 replicates.

Ion-Interaction RP-HPLC Method Validation

The validation, as concerns the accuracy, of a new analytical method can be performed both 1) by statistical comparison of the data obtained for the same sample with other methods and 2) with respect to a certified standard material whose concentration is known.

Statistical comparison

Standard solutions containing the mixture of the 15 analytes investigated here were prepared and analyzed with the two techniques compared. When comparing IIR-HPLC and RP-HPLC, the analytes concentrations were in the range 0.10 - 1.00 mg/L, while when comparing IIR-HPLC and GC/MS, higher concentrations were considered (0.50-1.00 mg/L) due to the higher determination levels presented by the GC-MS method.

In each case four replicates of each measurement were collected. The average value and the calculated estimated standard deviation obtained with the proposed IIR-HPLC method and respectively the conventional RP-HPLC and the GC-MS modes are shown in the Tables 1 and 2.

Table 2

Comparison Between RP-HPLC and GC/MS*

	Conc. mg/L	$\bar{x} \pm s$ RP-HPLC	GC/MS
Fenuron	1.000	0.995 ± 0.008	0.991 ± 0.032
Isoproturon	0.500	0.505 ± 0.007	0.509 ± 0.018
Linuron	1.000	1.015 ± 0.009	0.959 ± 0.040
Monuron	1.000	1.005 ± 0.009	0.981 ± 0.018
Bromacil	0.500	0.487 ± 0.007	0.522 ± 0.014
Hexazinone	1.000	1.001 ± 0.007	1.022 ± 0.016
Atrazine	0.500	0.517 ± 0.021	0.519 ± 0.007
Cyanazine	1.000	1.014 ± 0.008	1.033 ± 0.022
Propazine	0.500	0.479 ± 0.013	0.499 ± 0.019
Simazine	0.500	0.485 ± 0.003	0.507 ± 0.007
Terbumeton	0.500	0.516 ± 0.034	0.507 ± 0.007
Terbutylazine	0.500	0.496 ± 0.012	0.514 ± 0.013

* Average value of 4 replicates $\bar{x}$ and estimated standard deviations.

The data obtained for the analytes quantified by two independent techniques can be subjected to statistical treatment by parametric and non parametric tests, namely the *t-test*, the *F-test* and the *t-paired test.*[36] The *t-test*, that evaluates accuracy and precision with respect to the expected value, at a prefixed Confidence Interval (CI) and *n* (degrees of freedom), is usually used to compare a result with a standard one or with a certified value. To compare the variances of two different methods and to evaluate if the difference is statistically relevant the *F*-test is performed. The *paired-t test* compares two sets of data through a t_p value based on the s_p (pooled standard deviation) calculated for two sets of data.

The results obtained in the comparison between IIR-RP-HPLC and RP-HPLC methods from the *F*-test, the *t-test* and the *t-paired test* indicate that the two methods can be considered as statistically equivalent for all the analytes compared.

On the contrary, some statistical discordance is present for some analytes when the results of IIR-HPLC and GC methods are compared. It must be underlined that the tests used are able to compare the results of only one analyte at a time so that when comparing two methods for a multicomponent mixture, it

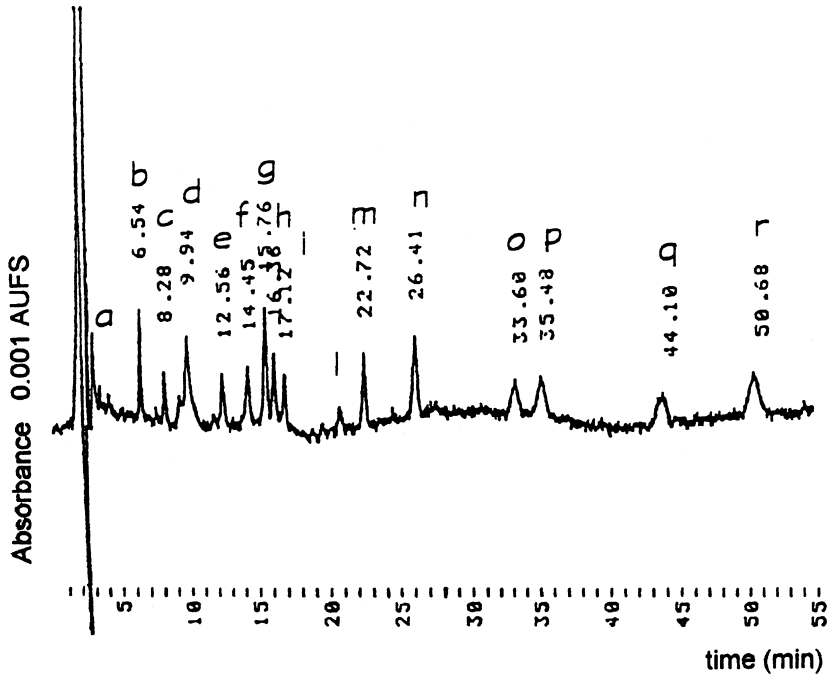

Figure 3. IIR-HPLC analysis of spiked (0.1 μg/L each) tap water sample after 2000-fold preconcentration. Conditions as in Figure 2. a) fenuron, b) 2,4-D, c) DNOC, d) hexazinone, e) bromacil, f) simazine, g) monuron, h) cyanazine, i) 2,4-DB, l) atrazine, m) isoproturon, n) terbumeton, o) propazine, p) terbutylazine, q) linuron.

may happen that the methods can be said to be statistically equivalent just for some of the analytes present in the mixture. To evaluate the whole performance of the method for all the analytes involved, the use of *t-test for multiple samples* and the *Wilcoxon matched-pair signed-rank test* is suitable: the results of these tests indicated that the GC-MS and the IIR-HPLC lead to statistically equivalent analytical results. The new IIR method here presented can be therefore considered validated with respect to both RP-HPLC and GC-MS methods.

Validation by comparison with a spiked real sample

A spiked sample to be used as a reference standard to test the IIR-RP-HPLC method was prepared. A sample of tap water was spiked with the 15 pesticides, 0.1 μg/L each. An extraction step with pre-concentration factor 2000, described above, was performed. A typical chromatogram, obtained under the developed IIR-RP-HPLC conditions, is shown in Figure 3. The results

compared with those obtained from the analysis of a standard solution of the pesticides, 200.0 μg/L each, in ultrapure water permitted evaluation of the recovery % , which always resulted in greater than 70%, with reproducibility (four replicates) within 8%.

CONCLUSIONS

In conclusion, the ion-interaction chromatographic method developed here offers the possibility of simultaneously separating a mixture of acidic and neutral / basic pesticides. This represents the biggest advantage of the method. Quantitation levels are of the same order of those obtained in RP-HPLC mode and lower than those which can be obtained in GC-MS.

The performance of the method has been checked by validation process performed with respect to a lab-prepared tap water sample and through a statistical intercomparison of the results obtained for the same mixture of pesticides with other chromatographic methods.

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