Anal Bioanal Chem (2004) 378 : 484–489
DOI 10.1007/s00216-003-2242-1

ORIGINAL PAPER

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# Rapid immunoanalytical method for the determination of atrazine residues in olive oil

Received: 1 July 2003 / Revised: 8 August 2003 / Accepted: 21 August 2003 / Published online: 25 September 2003


**Abstract** A sensitive, simple and reliable method has been developed for the determination of atrazine in extra virgin olive oil. The analytical procedure involves direct extraction of the target analyte from oil matrix with methanol and a freezing clean-up step (–80 °C) followed by plate or sensor immunoassay determination. A detection limit of 0.7 ng/mL, with a dynamic range from 1.0 to 10.4 ng/mL, was reached. The method was highly selective for atrazine and propazine, showing little or no cross-reactivity to other similar compounds. The excellent recoveries obtained (mean value 91.3%) confirm the potential of this approach to detect atrazine in olive oil for application as screening and complementary method in pesticide regulatory and food safety programs. The proposed method correlates well with the reference gas chromatography (GC-MS) technique.

**Keywords** Immunoassay · Atrazine · Olive oil · Food analysis

## Introduction

Extra virgin olive oil is obtained from the fruit of the olive tree (*Olea europea*) solely by a cold press process without any further treatment. Beneficial effects of olive oil on health [1] have prompted a demand for this product worldwide [2].

The Codex Alimentarius Commission of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have established maximum pesticide residue limits in olives and olive oil [3]. The European Union also fixes maximum levels for pesticide residues in this product. Latest regulations point out the determination of nearly 100 different compounds with herbicides being one of the most widely spread out group in olive tree plantations [4, 5]. Also, other non-traditional olive oil producing countries such as USA and Australia are establishing guidelines for olive oil processing and quality assurance.

Different s-triazines (simazine, atrazine, terbuthylazine, etc.) are listed within the most extensively applied herbicides and their use on the soil may sometime leave detectable residues on olive fruits by direct contamination [6]. Therefore, these herbicides are included within the scope of targets to be determined in olive oil samples.

According to reported studies [7, 8, 9], the determination of pesticide residues in olive oil is a tough analytical procedure because of its hydrophobic nature and complex matrix.

Currently, clean-up methods applied to olive oil are based on liquid–liquid partitioning extraction with solvents of different polarity, solid phase extraction, size exclusion chromatography or adsorption column chromatography [10], followed by gas chromatography analysis with either electron capture, nitrogen-phosphorus or mass detection [7, 11]. Additionally, clean-up with an alumina column or the use of solid-matrix partition steps do not allow pesticides to be recovered well from olive oil [7]. Besides, elution of the target analyte from the solid phase makes it necessary to use organic solvents [12, 13]. Recovery depends on the nature of the analyte and extraction solvent [14]. Also, interferences caused by lipids co-eluting from clean-up adversely affect the analytical performance. In this sense, olive and other edible oils need a rigorous clean-up because of their particular matrices [7, 8].

Enzyme-linked immunosorbent assay (ELISA) and immunosensing technologies have proved to be fast and sensitive screening methods, as well as quantitative analytical tools useful for pesticide residue determination in food [15] and other matrices [16, 17, 18, 19]. So far, the major drawbacks for the application of pesticide immunoassay in food are related to bad recoveries and the removal of matrix interferences. Consequently, sample pre-treatment methods need to be developed to provide reliable data using minimum sample processing, or otherwise many of the potential advantages of immunoassays would be lost [20].

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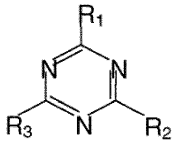
**Fig. 1** Chemical structures of the hapten 2d and s-triazine herbicides studied in this work




| Compound | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Hapten 2d | Cl | $NHCH(CH_3)_2$ | $NH(CH_2)_5COOH$ |
| Atrazine | Cl | $NH\ CH_2CH_3$ | $NHCH(CH_3)_2$ |
| Ametryn | $SCH_3$ | $NH\ CH_2CH_3$ | $NHCH(CH_3)_2$ |
| Deethylatrazine | Cl | $NH_2$ | $NHCH(CH_3)_2$ |
| Deisopropylatrazine | Cl | $NH\ CH_2CH_3$ | $NH_2$ |
| Cyanazine | Cl | $NH\ CH_2CH_3$ | $NHC(CH_2CH_3)_2CN$ |
| Irgarol 1051 | $SCH_3$ | $NHC(CH_3)_3$ | $NHCH(CH_2)_2$* |
| Prometryn | $SCH_3$ | $NHCH(CH_3)_2$ | $NHCH(CH_3)_2$ |
| Propazine | Cl | $NHCH(CH_3)_2$ | $NHCH(CH_3)_2$ |
| Simazine | Cl | $NH\ CH_2CH_3$ | $NH\ CH_2CH_3$ |
| Terbumeton | $OCH_3$ | $NH\ CH_2CH_3$ | $NHC(CH_3)_3$ |
| Terbuthylazine | Cl | $NH\ CH_2CH_3$ | $NHC(CH_3)_3$ |
| Terbutryn | $SCH_3$ | $NH\ CH_2CH_3$ | $NHC(CH_3)_3$ |

*ciclopropylamine

To our knowledge, the determination of pesticide residue content in olive and other edible oils using immunological techniques has not been previously reported in the literature.

The purpose of this paper was twofold. Firstly, the evaluation of novel extraction and clean-up procedures as easy sample pre-treatment methods and secondly, the optimization of ELISA and immunosensor methods for routine analysis of pesticide residues down to the ng/mL level in olive oil. The analysis of atrazine as a target in extra virgin olive oil is reported.

## Experimental

### Chemicals, immunoreagents and instruments

Atrazine and chemically related s-triazines herbicides were purchased from Dr Ehrenstorfer (Augsburg, Germany) and Riedel de Häen (Seelze-Hannover, Germany). Stock solutions of the herbicides were prepared either in *N*,*N*´dimethylformamide (DMF) or acetone and stored at –80 °C.

Hapten 2d (*N*-(4-chloro-6-isopropylamino-[1,3,5]triazin-2-yl)-6-aminohexanoic acid) was prepared as previously described [16]. Chemical structure of hapten 2d is depicted in Fig. 1. Anti-atrazine polyclonal serum R10 (freeze-dried powder) was reconstituted before use in 1:1 (v/v) 10 mM sodium phosphate buffer, pH 7.4 (PBS), and saturated ammonium sulfate.

All organic solvents, *o*-phenylenediamine (OPD), *p*-hydroxyphenylpropanoic acid (HPPA) and Tween 20 were purchased from Sigma-Aldrich (Madrid, Spain). Horseradish peroxidase (HRP), *N*-hydroxysuccinimide (NHS) and *N*,*N*´-dicyclohexylcarbodiimide (DCC) used for enzyme conjugate preparation were from Boehringer (Mannheim, Germany). Ultralink immobilized protein A/G was from Pierce (Rockford, IL). All other reagents were analytical or biochemical grade. The enzyme conjugate and the determination of atrazine by ELISA was carried out as previously reported [16]. The selectivity – ability to recognize atrazine structurally related compounds (see Fig. 1) – of the developed immunoassay in olive oil was also determined.

Polystyrene ELISA plates were from Costar Corporation (Cambridge, MA, USA) and the ELISA plate washer from Nunc Maxisorp (Roskilde, Denmark). Absorbance was read in a dual wavelength mode (490–650 nm) by means of a microplate reader (Victor model 1420 multilabel counter; Wallac, Turku, Finland).

For GC analysis a 6890 Hewlett-Packard device – automatic sampler – provided with 5% phenyl-methyl siloxane capillary column (HP-5MS) model 19091S-433 (30 m length×250 µm diameter×0.25 µm film thickness), and a 5973 mass selective detector operated in selected ion monitoring (SIM) mode was employed.

### Immunosensor

The flow manifold and the assay protocol used in immunosensor experiments have been described elsewhere [21]. In short, the assay protocol consisted of the following stages:

1. Competition step done in the syringe module (200 µL of R-10 antibody at 5 mg/L+200 µL of 2d-HRP tracer at 0.5 mg/L+800 µL of standard or sample solution)
2. Capture (1 mL of the previous mixture at 0.25 mL/min)
3. Washing step with buffer
4. Signal display (100 µL of a mixture containing 0.4 g/L HPPA and 0.006% v/v $H_2O_2$, incubated in the reactor for 3 min)
5. Sensor regeneration (2.5 mL of 0.1 M glycine/HCl solution, pH 2.0, injected at 0.5 mL/min, followed by washing with buffer)

All the solutions were prepared in 0.02 M sodium phosphate buffer, pH 8.0 (PB). The total assay time was 23 min.

### Extra virgin olive oil analysis

Eleven commercial extra virgin olive oil samples (A1 to A11) were collected from representative Spanish olive oil producing areas.

Samples were analysed for atrazine residues through ELISA after being pretreated following the different studied clean-up protocols. Competitive curves were mathematically analysed by fitting experimental points to a four-parameter logistic equation using the Sigmaplot software package (Jandel Scientific, Erkrath, Germany). Standards and samples were run in three replicate wells and the mean absorbance values were recorded.

For immunosensor analysis, the methanolic extracts were diluted in 20 mM PB and directly analysed by the automatic sensor system.

Prior GC-MS analysis, olive oil samples were extracted with methanol (1:1). Helium was used as carrier gas at a flow-rate of 1.2 mL/min and extracts were automatically injected into splitless mode. The oven temperature was held at 60 °C for 1 min, then increased 30 °C/min to 110 °C, 10 °C/min to 240 °C, 30 °C/min to 285 °C and held at this temperature for 10 min. Injector temperature was 250 °C. Atrazine was detected by selected ion monitoring of the characteristic fragment ions (m/z 200 173 138) [22]. Quantification was carried out using standards in matrix.

## Result and discussion

### ELISA conditions

For the determination of atrazine at ng/mL level through ELISA analysis, 1 mg/L rabbit polyclonal antibody R10 and 0.25 mg/L of enzyme conjugate were the optimum reagent

concentrations. Under these conditions, the determination of atrazine residues in aqueous media had a dynamic range between 0.14 and 4.50 ng/mL. The midpoint of the assay responses ($IC_{50}$) was 0.83 ng/mL.

## Study of the extraction process

For the evaluation of the extraction procedures, an olive oil standard sample was fortified with atrazine at 100 ng/mL.

The extraction procedure involved the mixture of 1 mL of sample with 1 mL of organic solvent (acetonitrile, ethyl-lactate, isopropanol, dimethylsulfoxide, or methanol) at room temperature for different contact time. Afterwards, the extracts were diluted in PBS before ELISA analysis to avoid solvent effect on assay performance, and recovery was determined. Regarding dilution of the extract before ELISA analysis, 1:5, 1:10, 1:25 and 1:50, corresponding to 10%, 5%, 2% and 1%, of organic solvent in assay, respectively, were tested. For all the organic solvents, concentrations above 2% in PBS had a disrupting effect on the assay performances. A clear decrease in maximum signal and sensitivity was observed. Similar results of the influence of organic solvent on the performance of ELISA have been reported in the literature [23, 24]. Therefore, 2% of solvent, which corresponds to 1:25 dilution (1:50 in the well) of extract, was the maximum solvent concentration tolerated by this ELISA.

As a starting point, extraction time was fixed at 15 min because, from our experience on extraction of other herbicides from foods, it was the minimum time with which satisfactory recoveries could be obtained [23].

When acetonitrile was used for extraction of atrazine from olive oil, recovery was below 20%. Extraction with dimethylsulfoxide did not increase recovery. Additionally, even 2% of dimethylsulfoxide in the ELISA prompted a fivefold decrease in sensitivity. Obviously, a higher extract dilution (1:100) diminished the effect of solvent. However, an additional loss of sensitivity occurs, making this solvent not feasible for ELISA analysis.

When using isopropanol, recovery values were not consistent because olive oil was partially dissolved on it. Besides, mean recovery value was lower than 19% even after dilution of the extract 25-fold (1:50 in the well). Concerning extraction with ethyl-lactate, some compounds, probably polyphenols and fatty acids, precipitated on dilution 1:25 in PBS, making the analysis of atrazine through ELISA difficult because of the huge interferent effect observed. As mentioned, dilution higher than 1:25 would have an adverse effect on assay sensitivity. In contrast, methanol was well tolerated since 2% methanol did not affect the assay performances (sensitivity and maximum signal were like those achieved in aqueous media). However, in terms of extraction efficiency, similar recovery values were obtained (mean recovery was 23%).

At this stage, we decided to check the efficiency of water on atrazine extraction from olive oil. These experiments consisted of mixing 1 mL of water at different temperatures (25 °C, 50 °C and 80 °C) with 1 mL of olive oil sample for 1, 5, 10, 30 and 60 min. Afterwards, the aqueous phase was pipetted out and directly applied to ELISA without further sample treatment. First of all, it is worth mentioning that no matrix effect was observed even though the extracts were not diluted before ELISA analysis. Furthermore, the higher temperature and longer extraction time yielded a slight better recovery (5 min with water at 80 °C yields a recovery of 1.6%). For instance, extraction of atrazine from olive oil with water at 80 °C for 5 min followed by ELISA analysis, led to a sensitivity of 2.3 ng/mL. In practice, the determination of atrazine content in olive oil samples would be limited to concentrations above 15 ng/mL because of the low recovery. However, taking into account that no dilution of the extract was required before ELISA analysis, the method could be advantageous. Nonetheless, extraction of atrazine with water at high temperatures (80 °C) may lead to the formation of hydroxyatrazine [25], which could be a potential drawback of the procedure.

When mixtures of methanol and water were used for extraction (methanol:water, 80:20, 60:40, 40:60, and 20:80), recoveries were even worst than using methanol alone. Therefore, methanol appeared the most appropriate solvent for direct extraction of atrazine from olive oil.

Irrespective of whether extraction was carried out by using 0.5, 1.0 or 2.0 mL of sample, with sample/solvent ratio being 1, similar recovery yield was obtained. When higher sample/solvent ratio was tested (1:5), a slight recovery enhancement (8%) was observed. However, a much more tedious clean-up procedure was required (rotary evaporation, reconstitution) in order to keep assay sensitivity, which made the assay less suitable for routine analyses. Thus, 1.0 mL of sample extracted with 1.0 mL methanol was chosen for further experiments.

In an attempt to improve clean up and enhance extraction yield, a novel procedure was studied. Before analysis, 1.0 mL olive oil was mixed with 1.0 mL methanol and homogenized by shaking samples on a vortex for 15 min. Then, the suspension was left for 1 h at –20 °C and –80 °C. Afterwards, methanolic extracts were pipetted out and conditioned by diluting 1:25 in PBS.

Freezing at –20 °C for 1 h did not collect the interferents into a single frozen mass and longer time was required for separation. This fact made the ELISA assay less reproducible than after freezing the mixture at –80 °C, which performs the best clean-up of the extracts. Using this sample pre-treatment method, mean recovery efficiency of 32% was achieved. When samples were homogenized for 5 and 10 min in the presence of the same amount of methanol and left at –80 °C for 1 h, similar recovery was obtained to that achieved by shaking for 15 min. Indeed, on the basis of the above tests, it was concluded that 5 min homogenization with methanol and clean-up at –80 °C for 1 h provided the best results.

## Matrix effect. Assay performances

In order to evaluate the influence of different olive oil samples on recoveries, the final extracts of the set of fortified

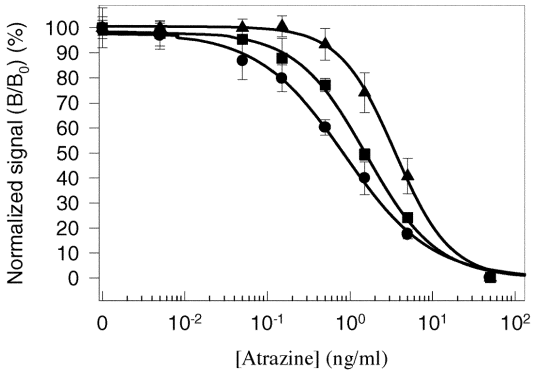



**Fig. 2** Calibration curves for atrazine in 2% methanol (●), in matrix extract (■) and in olive oil (▲). Each point represents the mean± standard deviation of 11 plates with three replicates per plate

samples were quantified using standards in 4% methanol (2% in the well), in matrix extract (ME) – samples are extracted before fortification – and in olive oil (OL) – samples are fortified and then extracted as described above. As is shown in Fig. 2, although extracts were diluted before ELISA analysis (1:50 in the well), a 1.84- ($IC_{50}$=1.5 ng/mL) and 4.24-fold decrease ($IC_{50}$=3.5 ng/mL) in assay sensitivity – compared to that achieved with standards in 2% methanol ($IC_{50}$=0.8 ng/mL) – was observed using ME and OL standards, respectively, suggesting a matrix effect.

Consequently, the use of standards in matrix extracts for the quantification of atrazine yields an assay with a dynamic range (20–80% inhibition) between 0.4 and 6.0 ng/mL and a limit of detection (10% inhibition; LOD) of 0.2 ng/mL.

On the other hand, the use of OL standards provided an assay with a dynamic range of 1.0–10.4 ng/mL, with a LOD of 0.7 ng/mL. Therefore, the residue of oil present in the final extracts affects the sensitivity of the assay.

An enhancement effect was observed for all samples, which led to recoveries significantly higher than when non-matrix-matched standards (2% methanol) were used. Overall recovery values of 49.4% and 92.0% were reached using ME standards and OL, respectively. Despite the excellent recoveries achieved when the quantification of fortified samples was carried out using OL standards, it should be mentioned that following the proposed extraction and clean-up procedures a mean percent recovery of 52% for atrazine in olive oil was reached. This apparent recovery is reported in percentage as the ratio of $IC_{50}$ value obtained with ME standards to the $IC_{50}$ value determined with OL standards. Indeed, the difference in assay sensitivity could be used as a clue to atrazine recovery.

In an effort to avoid matrix effects, the use of SPE methodology was also attempted. Methanolic extracts (3 mL) were loaded onto a C18 Sep-Pak cartridge with a syringe at the manufacturer's recommended rate (the cartridge was previously equilibrated with 5 mL methanol as suggested). The cartridge was then washed with 3 mL of methanol in order to eliminate a large part of the matrix. The residues were then eluted with water and methanol at the same flow-rate and later analysed by ELISA. Analysis of all collected fractions (cleaning and elution) indicated that atrazine was barely trapped on the solid phase under above experimental conditions, which confirm our outlook concerning the difficulties of pesticide residue determination in olive oil.

**Table 1** Cross-reactivity of some s-triazine herbicides in olive oil

| Compound | $IC_{50}$ (ng/mL) | Cross reactivity (%) |
|---|---|---|
| Atrazine | 3.5 | 100 |
| Ametryn | 117 | 3.0 |
| Deethylatrazine | 493 | 4.1 |
| Deisopropylatrazine | >3520 | <0.1 |
| Cyanazine | >3520 | <0.1 |
| Irgarol 1051 | >3520 | <0.1 |
| Prometryn | 62.9 | 5.6 |
| Propazine | 3.3 | 108 |
| Simazine | 220 | 1.6 |
| Terbumeton | >3520 | <0.1 |
| Terbuthylazine | 34.5 | 10.2 |
| Terbutryn | >3520 | <0.1 |

Cross-reactivity was calculated as CR=[$IC_{50}$(atrazine)/$IC_{50}$ (interferent)]×100

In summary, the performance of the developed method is comparable or slight better than that already reported by Lentza-Rizos et al. [26] where they detect atrazine and organophosphorus insecticides in olive oil using a low-temperature clean-up (–20 °C) and gas chromatography analysis (GC-NPD). That study reports an overall recovery for atrazine of 83% and 50 ng/mL as limit of quantification. Recently, Barrek et al. [27] have also reported the determination of 20 different pesticides in olive oil by GC-MS after extraction by size-exclusion chromatography with a limit of detection for atrazine of 300 ng/mL. In comparison, the proposed method showed a lower limit of quantification (LOD=33 ng/mL) making it more simple and sensitive for atrazine determination in olive oil.

Selectivity of the method was determined developing competitive calibration curves in olive oil for the most extensively used *s*-triazines. Cross-reactivity is defined as the percent ratio at the midpoint of the assay between atrazine and the cross-reacting compounds. As is shown in Table 1,

**Table 2** Immunosensor calibration parameters

| Dilution factor (%) | Standards in methanol $IC_{50}$ (ng/mL)[a] | OL standards | |
|---|---|---|---|
| | | Maximum signal (%)[b] | $IC_{50}$ (ng/mL)[a] |
| 10 | 66 (6.6) | 35 | n.d |
| 5 | 78 (3.9) | 72 | 246 (12.3) |
| 2 | 54 (1.1) | 75 | 164 (3.3) |
| 1 | 61 (0.6) | 87 | 234 (2.3) |

*n.d* not determined
[a]Values in brackets are the $IC_{50}$ concentrations in the final mixture
[b]Percentage of maximum signal as the ratio of the signal obtained for an olive oil standard to the signal obtained with a standard prepared by mixing pure methanol with buffer

**Table 3** Comparison of the recoveries for atrazine in olive oil using ELISA, immunosensor and GC techniques

| Sample | [Atrazine] added | [Atrazine] found | | | | | |
|---|---|---|---|---|---|---|---|
| | | ELISA | | SENSOR | | GC-MS | |
| | | value±SD | R(%) | value±SD | R(%) | value±SD | R(%) |
| A1 | 0 | <LOD | – | <LOD | – | <LOD | – |
| A2 | 10 | <LOD | – | <LOD | – | n.d | – |
| A3 | 40 | 32± 8 | 80 | 70±30 | 175 | n.d | – |
| A4 | 60 | 45±10 | 75 | 70±30 | 117 | 66± 7 | 110 |
| A5 | 80 | 71±16 | 89 | 99±11 | 124 | 73± 8 | 91 |
| A6 | 100 | 100± 4 | 100 | 200±20 | 200 | 103±10 | 103 |
| A7 | 120 | 100±13 | 83 | 130±50 | 108 | 92± 8 | 77 |
| A8 | 150 | 120±30 | 80 | 131± 7 | 87 | 119±11 | 79 |
| A9 | 200 | 210±20 | 105 | 270±90 | 135 | 166±13 | 83 |
| A10 | 400 | 470±30 | 118 | 480±30 | 120 | 453± 9 | 113 |
| A11 | 500 | 460±40 | 92 | 680±30 | 136 | 498±12 | 99 |
| Mean | | | 91.3 | | 134 | | 94.4 |

Atrazine concentration in ng/mL
*LOD* limit of detection as the concentration inhibiting 10% of the maximum signal, *n.d* not determined, *R* recovery

propazine was the only interfering compound (CR=108%) and able to be detected at low ng/mL level ($IC_{50}$=3.3 ng/mL). This result is in good agreement with previously reported data [13]. As propazine is scarcely used in olive groves, the assay is pretty selective for atrazine.

Besides, within analytical parameters, the reproducibility of the results is one of the most important factors. In order to study the reproducibility of the method, 11 subsamples of an olive oil (A7) were fortified at two atrazine levels (75 and 250 ng/mL) and processed using the optimized method and quantified with OL standards. A good reproducibility was obtained with mean recoveries of 85±17 and 87±15 at 75 and 250 ng/mL, respectively. These results demonstrate the suitability of the proposed method for rapid detection of atrazine residues in olive oil.

## Immunosensor

As the immunosensor can be used for the analysis of samples containing high percentages of methanol [28], the first experience consisted of the study of the calibration performances as a function of methanol content by the comparison of calibrations obtained with atrazine standards in methanol with those achieved with OL standards. The calibration parameters are shown in Table 2. It can be seen that the use of pure methanol leads to similar $IC_{50}$ values. Indeed, this result is consistent with the fact that an increase of methanol content in the final mixture leads to a lower sensitivity of the assay, but this effect is overcome by the higher concentration of analyte in the mixtures. More remarkable is the effect of the oil matrix co-extracted with the analyte. If the concentration of the matrix components is very high (10% dilution of the oil extract), the signals registered are too low, and the competition curve is not well defined, which makes necessary to use high dilution factors in order to achieve acceptable curves. The higher dilution factor, the higher signal obtained, which is consistent with the dilution of the interfering matrix components. On the other hand, the whole assay sensitivity depends not only on the matrix effect (oil components co-extracted and methanol) but also on the dilution of the extract. In this sense, the best conditions were found for a dilution factor of 2%. Using this dilution factor, a LOD for atrazine – in the native oil sample – of 50 ng/mL is achieved, and the dynamic range goes from 66 to 600 ng/mL. Also, the immunosensor could be used for more than 600 assay cycles without loss of sensor performances.

## Extra virgin olive oil analysis

Comparative studies were carried out through ELISA and GC-MS reference methods by analysing, as blind samples in different laboratories, a set of atrazine-spiked olive oils at ten fortification levels. In parallel, a non-spiked sample was checked for atrazine residue presence and used as control. As can be seen in Table 3, mean percentage recovery of 91.3% and 94.4% by ELISA and GC-MS, respectively, were reached with low RSD values (<22% in all samples). The proposed procedure correlated well with the reference method (r=0.97) indicating that there is no significant bias between the techniques (Fig. 3).

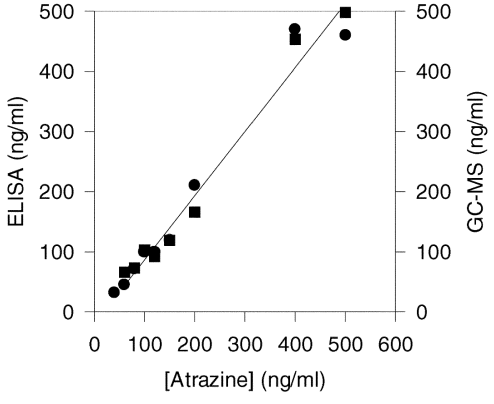



**Fig. 3** Correlation between the results for atrazine obtained using ELISA (●) and GC-MS (■) techniques

On the other hand, the results obtained with the immunosensor are higher than the spiking values, and also higher than those obtained through ELISA and GC-MS analysis. Also, the precision is bad. In this respect, it should be mentioned that even though calibration of the sensor with OL standards minimizes matrix effects, interferences still produce a positive bias, very significant in many cases (A3, A6, A9 and A11). Nonetheless, absence of atrazine or very low concentrations, were measured as "free" (<LOD) samples, i.e. no false positives have been found, so that the immunosensor applied on oil methanolic extracts can be used as a semiquantitative screening method and employed as an on-line control tool.

## Conclusions

The developed methodology was successfully applied to rapid detection of atrazine in virgin olive oil. Furthermore, this paper demonstrates the possibility of performing measurements of atrazine in oil samples in the field or oil mill without tedious extraction and clean-up procedures, reaching satisfactory quantitative yields.

This rapid method uses a low quantity of methanol and thus is a friendly environmental extraction procedure. Since most pesticides used in olive groves are very soluble in methanol, the proposed method could be applied for the determination of a wide range of pesticide residues.

Right now, agricultural authorities have established maximum residue limits for pesticides in olive oil for those applied to olives. For atrazine, 100 ng/g has been established as maximum residue limit. The proposed method reaches a detection limit of 0.7 ng/mL in the extract, corresponding to 33 ng/mL in oil (equivalent to 40 ng/g) which makes it very suitable for analysis under allowed MRLs. Furthermore, ELISA based methods have the advantages of real-time high throughputs and low price analysis.

We truly think that all these features ensure that this rapid, simple and sensitive method can be included as a useful complementary technique for the screening of pesticides in olive oil monitoring programs. In this respect, our research group is currently planning application of this methodology to the determination of high *n*-octanol-water partition coefficient (log $K_{ow}$) pesticides and metabolites, which are widely used in olive groves.

**Acknowledgments** This work was supported by the project RTA 01-115-C2. M. Garcés-García acknowledges a grant from Fundación Ramón Areces for carrying out PhD studies. The authors wish to thank Dr JA. Gabaldón for valuable GC-MS analysis. We would like also to acknowledge Dr MP. Marco, who kindly provided the polyclonal serum.

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