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Detection of chemical residues in tangerine juices by a duplex immunoassay

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ABSTRACT

A rapid duplex ELISA for the simultaneous determination of two of the most widely used organophosphorous insecticides in tangerine juices is described. To accomplish this aim, two individual enzyme-linked immunosorbent assays for chlorpyrifos and fenthion pesticides were integrated into one ELISA test. The strategy uses 96-well plates with specific wells coated with the corresponding haptenized conjugate. The optimized duplex ELISA was accomplished within 40 min achieving a detection limit of 0.20 ± 0.04 μg/L and 0.50 ± 0.06 μg/L, for chlorpyrifos and fenthion, respectively in tangerine juice samples. The determination of residues of both pesticides was carried out by simple sample dilution, without any extra sample clean-up procedure. Results of testing precision, stability, and selectivity demonstrated that the assay provided reliable analytical performances for the simultaneous determination of residues of chlorpyrifos and fenthion in fruit juice samples below the established European maximum residue limits (MRL). In addition, the accuracy and reliability of this duplex bioanalytical method is demonstrated by analyzing blind spiked juice samples and the results, correlated well with those achieved using a well-established GC/MS method (recoveries between 95% and 106%).

1. Introduction

Fresh vegetables, fruits and juices are the important part of a healthy diet because of the presence of significant amount of nutrients and minerals in them. However, at the same time, they can also turn out to be a source of toxic chemical substances as pesticides [1]. Among various pesticide classes, organophosphorous pesticide (OPPs) group is the most widely used class of agricultural pesticides [2–4]. In recent years, many studies have proven OPPs to be mutagenic, carcinogenic, cytotoxic, genotoxic, teratogenic and immunotoxic [5–10]. In addition, OPPs have tendency to bind to the enzyme acetyl cholinesterase, and to disrupt nerve functioning which further result in paralysis and death [11–12]. In this sense, accidental exposure of humans and animals to such chemicals may result in a potentially lethal cholinergic poisoning [13].

The increasing public concern about the possible health risk of pesticide residues in the diet has profoundly modified crop production strategies with emphasis on food quality and safety. Apart from this, the widespread concern for health of society has led to the strict regulation of MRL of pesticide residues in food [14].

Chlorpyrifos (CLP), *O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate and fenthion (FTN), *O*,*O*-dimethyl-*O*-[3-methyl-4-(methylthio)phenyl]phosphorothioate are two of the most used OPPs insecticides to control a variety of soil insects and arthropods, on a wide range of crops including peaches, nectarines, vegetables, cereals, and citrus fruits. Since these are absorbed by the fruits and vegetables and turned out to be noxious when consumed by human beings, both gas and liquid chromatography techniques have been traditionally applied to the detection of organophosphorous residues in fruits and juices [15] coupled to selective detectors [16,17] or mass spectrometer detectors [18,19]. Although chromatographic techniques provide low limits of detection, preliminary sample treatment is the bottle neck, making the analytical procedure laborious, and the number of samples analyzed is few due to economic issues. In this context, there has been increased interest in the development and implementation of rapid and cheaper screening methods, including immunoassays, for detection of pesticide residues. Having in mind that children consume relatively large quantities of fruit juices and that they are more susceptible to chemicals, since they are in the early stage of development, determining pesticide residues in fruit juices has a

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particular importance. In this sense, a rapid, sensitive and selective assay would be of great interest to detect pesticide residues in routine analysis as a screening method. Immunochemical techniques began recently to gain acceptance as a fast and cost-effective tools for separating and/or detecting trace amounts of chemicals such as pesticides [20]. ELISA methods are the most widely used immunoassays due to high sample throughput, although they are limited sometimes by their high specificity. Hence, immunoassay techniques are often used to detect a single analyte. On the other hand, orthogonal hapten–antibody/receptor combinations seem to be ideal for creating array structures with different immunoreagents for different analytes [21,22]. This kind of parallelization reaches its physical and economical limits with the increasing number of analytes that could be measured at the same time. The combination of generic immunoassays for simultaneous detection of CLP and FTN below the MRLs on a single assay could be an excellent tool for pesticide residue screening. Here we report the development and evaluation of a duplex ELISA combining immunoreagents specifically developed to detect two OPPs. The duplex ELISA has been evaluated by testing natural and commercial juice samples contaminated (or not) with both pesticides and the results discussed in comparison with those achieved using a well-established GC/MS method.

2. Material and methods

2.1. Chemicals

Analytical standards of chlorpyrifos, fenthion, chlorpyrifos-methyl, chlorpyrifos-oxon, fenchlorphos, bromophos, trichloronate, diazinon, dichlorvos, fenitrothion, fenthion-sulphoxide, malathion and the chlorpyrifos metabolite 3,5,6-trichloro-2-pyridinol (TCP) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stock solutions of pesticides were prepared in N,N-dimethylformamide (DMF) and kept at −20 °C until use. Standards were daily prepared in water by serial dilutions from the stock (6.90 mg/L for chlorpyrifos and 3.30 mg/L for fenthion) using borosilicate glass tubes. Peroxidase-labeled goat anti-rabbit immunoglobulins (GAR-HRP), poly(oxyethylensorbitan)monolaurate (Tween 20), *O*-phenylenediamine (OPD) and the proteins bovine serum albumin (BSA) and ovalbumin (OVA) were purchased from Sigma Chemical Co., (St. Louis, MO). Coating buffer (CB) was 50 mM sodium carbonate–bicarbonate buffer pH 9.6 and working buffer was 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, containing 0.05% Tween-20, pH 7.4 (PBS-T). The developing buffer was 25 mM sodium citrate and 62 mM sodium phosphate, pH 5.4. The enzymatic substrate solution was prepared in developing buffer dissolving OPD (2.0 mg/mL) and H_2O_2 (0.012%). All other reagents used were analytical or biochemical grade.

Polyclonal anti-chlorpyrifos (C2-II) and anti-fenthion (F7-I) antibodies and the coating conjugates OVA-C5 and OVA-F1 [23,24] were used as immunorreagents.

2.2. Apparatus

Polystyrene microtiter plates were purchased from Costar (Cambridge, MA, USA). Washing steps were carried out using a 12-channel microplate washer from Nunc (Roskilde, Denmark). Absorbance was read with a Multilabel Counter 1420 microplate reader from Wallac (Turku, Finland) at dual wavelength mode. The mean absorbance value of triplicate wells was processed. Using Sigmaplot software package (Jandel Scientific, Erkrath, Germany), ELISA absorbance values of standards were plotted vs. the logarithm of analyte concentration and fitted to a four-parameter logistic equation [25].

Magnetic stir bars (20 mm length × 0.5 mm film thickness) coated with poly(dimethylsiloxane) (PDMS), a desorption unit TDS-2 connected to a programmed temperature vaporization (PTV) injector (CIS-4), and a TDSA autosampler were all supplied by Gerstel (Mulheim/Ruhr, Germany). The PTV injector was installed in an Agilent 6890 GC-5973MS system (Agilent Technologies, Palo Alto, CA).

2.3. Duplex ELISA protocol

Flat-bottomed polystyrene ELISA plates were segmented in two sections (CLP and FTN) and coated with 100 μL/well of OVA-C5 at 0.1 mg/L and OVA-F1 at 1.0 mg/L for chlorpyrifos and fenthion analysis, respectively. After 16 h at 4 °C, plates were washed six times with 10 mM PBS-T pH 7.4, and then 50 μL of a mixture of pesticide standards or samples in PBS-T and 50 μL of a cocktail serum solution, containing specific polyclonal antibodies against chlorpyrifos and fenthion were added. After 15 min, the plates were washed as before and then 100 μL of GAR-HRP (1/4000 dilution in PBS-T) was added to each well and the solution incubated for 15 min. Next, the solution was washed away as above described and finally, 100 μL/well of substrate solution was added and the enzymatic reaction was stopped after 10 min by addition of 2.5 M H_2SO_4 (100 μL/well). The absorbance was read at 490 nm as test wavelength and 650 nm as reference wavelength.

2.4. Immunoassay optimization

The influence of several critical parameters such as ionic strength, pH, surfactant concentration, BSA and time of competition on assay performances was studied. Criteria used to evaluate the assay performances were sensitivity (IC_{50}), maximum absorbance (*A*), dynamic range (DR, established between the pesticide concentrations producing 20% and 80% colour inhibition) and limit of detection (LD, estimated at 10% inhibitory concentration).

To evaluate the specificity of polyclonal antibodies, different chemically related and unrelated compounds were tested. Four calibration curves for each compound were obtained in optimized ELISA conditions. The cross-reactivity (CR) values were calculated according to the following equation:

$$\mathrm{CR}\ (\%) = \frac{(\mathrm{IC}_{50})\ \text{chlorpyrifos or fenthion}}{(\mathrm{IC}_{50})\ \text{related} - \text{compound}} \times 100 \quad (1)$$

2.5. GC/MS determinations

GC–MS analyses were performed in a 6890 GC gas chromatography coupled to a 5973 MS system (Agilent Technologies, Palo Alto, CA), with electron impact ionization detection. A HP5-MS capillary column (Agilent 19091S 433E) with a 0.25 mm i.d., 30 m and 0.25 μm film tickness was used for the determination of organophosphorous pesticides. The pollutants were extracted from 100 mL of mandarin juice samples using magnetic stir bars (20 mm length × 0.5 mm film thickness) coated with poly (dimethylsiloxane) (PDMS). The bars were previously conditioned in an empty thermal desorption tube at 300 °C for 4 h with a helium flow rate of 50 mL/min. After stirring the sample for 24 h at 900 rpm, the bars were thermally desorbed in the splitless mode at 280 °C for 6 min, transferring the analyte to the PTV injector at a 75 mL/min helium flow rate. Sodium chloride (20% m/v) was used as the matrix modificator. The temperature program was 2 min at 70 °C then, increased to 200 °C at a rate of 30 °C/min, hold for 1 min, and increased to 280 °C at 3 °C/min, with a final isothermal period of 2 min. The PTV injector and interface temperatures were held at 280 °C. The detection was made in the full scan mode m/z range 50–400, and for confirmation of the spectral data, the

characteristic ions for chlorpyrifos (m/z 314, 199, 197, 97) and fenthion (m/z 278, 169, 125, 109) were used.

2.6. Juice samples analysis

Fruit juices were purchased from local supermarkets and fresh juices obtained after squeezing raw tangerines. For matrix effects and recovery studies, any sample pre-treatment was used. Spiked juice samples containing chlorpyrifos or fenthion were analyzed by ELISA as described in Section 2.3. In order to avoid matrix effects, juice samples were simply diluted (1/10, v/v) in assay buffer and a set of standard curves were obtained and compared with those carried out in assay buffer. The pH value of diluted samples was checked and corrected if necessary.

The effect of thermal treatment of juices on pesticide concentration was evaluated using the optimized ELISAs. For that, 12 samples of 100 mL of natural or commercial juices were fortified with known amounts of pesticide (10, 40 and 60 μg/L) derived from stock solutions and heated (60, 90 and 120 °C) for 30, 40 and 60 s, respectively.

The samples were then analyzed by ELISAs and GC–MS. In order to assess assay reproducibility, triplicates of each fortification level were performed.

3. Results and discussion

3.1. Duplex ELISA conditions

Several points should be considered before using a single-target system in multiplexed assays. First, the antisera selectivity and sensitivity, and second, the carrier protein used in the coating conjugates is another variable to be marked. For these reasons, the development of a multianalyte ELISA requires an accurate selection of the suitable hapten structures. In this work, two specific haptens conjugates were immobilized by physisorption in distinct areas of a microplate, and a mixture of different specific polyclonal antibodies for chlorpyrifos and fenthion were used in an indirect ELISA-format, to determine simultaneously both analytes. An illustration of the working strategy is shown in Fig. 1.

In regard to the selectivity, both single-target systems were specific, since each antiserum only recognized its particular coating conjugate. In our case, since immunogens used to raise the antibodies were prepared with BSA, a different carrier protein was employed for coating the conjugate preparation, avoiding non-specific interactions and false positives. This criterion was matched by preparing ovalbumin based coating conjugates. In a second set of experiments, cross-reactivity studies were performed by measuring signal intensity variation to the presence of mixed analytes. In these experiments, single-target assays were carried out and cross-reactivity calculated as percentage of inhibition. In all cases, signal variation was below 0.01%, indicating high selectivity to the mixed analytes. This fact is essential when considering single assay candidates to be applied in multiplexed analysis.

Furthermore, the effect of pH, ionic strength and surfactant concentration and incubation time on assay performances (signal and IC_{50}) was studied at room temperature. First, the effect of pH of the working buffer was studied and observed that IC_{50} values and maximum signal (A) for the duplex ELISA varied 0.49–1.35 μg/L and 0.95–1.80 absorbance units (*A.U.*), respectively, within a pH range from 4.0 to 9.0, being pH 7.5 the optimum for keeping acceptable signal and good sensitivity for both analytes. Furthermore, the effect of the ionic strength (10–200 mM) on the assay performance was tested, showing that for the duplex assay, IC_{50} and absorbance values decreased gradually as buffer salt concentration increased, as it had been previously observed for other organophosphorus immunoassays [23]. In this study, the concentration of PBS selected as a compromise between signal response and IC_{50} was 10 mM. The effect of the addition of detergents in the duplex ELISA sensitivity was also studied. In this study (data not shown), a negative influence (lower signal and lower sensitivity) was observed if the surfactant concentration increased over 0.05%. According to these results, 10 mM PBS, containing 0.05% Tween 20 was the optimum competition buffer for duplex ELISA systems.

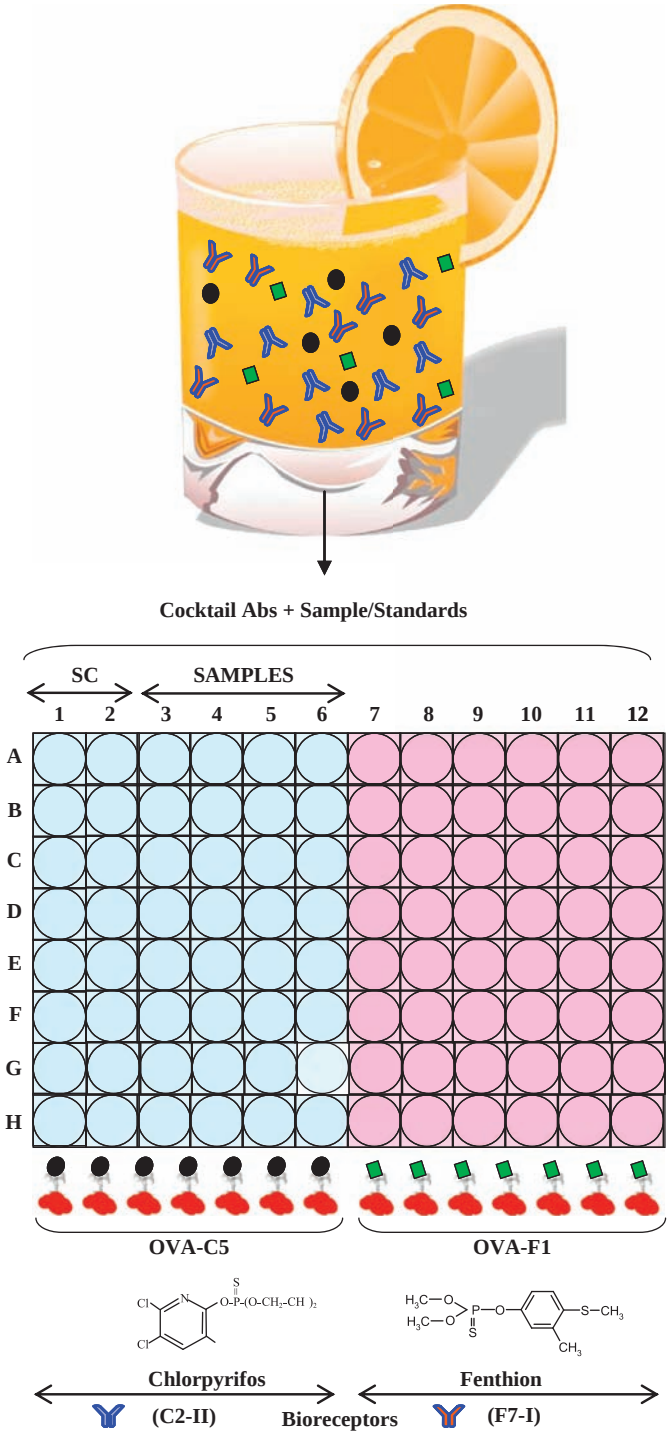

Fig. 1. Duplex enzyme-linked immunosorbent assay (dELISA). Different sections of the microtiter plate are coated with coating antigens of the two pesticides. The immunoreagents/bioreceptors and the analytes are then distributed through the whole plate as a mixture of reagents. Standard curves (SC) were carried out in columns 1,2 and 7,8 for chlorpyrifos and fenthion, respectively. Juice samples (32) were analyzed per duplicate.

Also, the influence of the incubation time on the competition step (15, 30, 45, and 60 min) was investigated. As can be seen in Figs 1–3, good analytical performances were obtained after 15 min competition in terms of sensitivity and signal response, taking 40 min the whole duplex assay.

Taking into account the selected conditions, a robust calibration curve ($n=50$) was constructed. Fig. 2 depicts the calibration graphs of the competitive optimized ELISAs. The errors bars—each point represents the mean ± standard deviation—at each calibration point, show the assay to assay variability expected from small differences in timing, temperature, or reagent age over the 50 assays. The assay for chlorpyrifos shows an IC_{50} of 3.0 ± 0.3 μg/L, a working range between 0.3 and 25.1 μg/L and a LOD of 0.2 μg/L. For fenthion, the analytical sensitivity was 6.6 ± 0.2 μg/L, a working range from 0.8 to 58 μg/L and a LOD of 0.7 μg/L.

3.2. Determination of matrix interference

Duplex ELISA is not completely free from interferences caused by unidentified compounds of the food matrix notwithstanding antibody's high selectivity. Thus, it is advisable to determine the importance of these matrix interferences before the application of the assay to real samples. The easiest and most immediate way to minimize and to overcome matrix interferences is sample dilution.

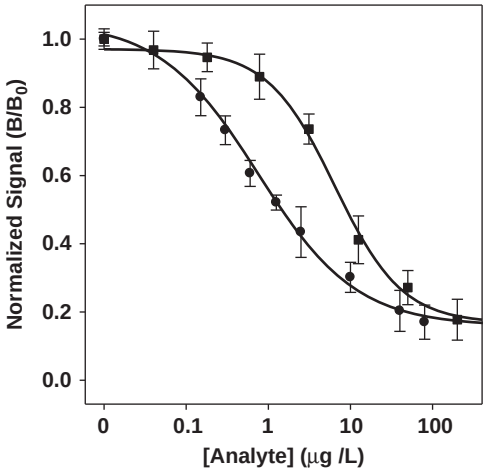

Fig. 2. Calibration curve ($n=50$) for the optimized duplex ELISA. (•) Chlorpyrifos, (▪) Fenthion.

As described in Section 2.3, juice samples were fortified and properly diluted in assay buffer (1/10, 1/20 and 1/50), and a set of standard curves were obtained and compared with those carried out in assay buffer. No significant matrix interference was observed only when filtered juice samples were 50-fold diluted and both calibration curves were very similar (data not shown). However, when diluting the sample 10-fold in PBS-T, significant matrix interference was found. Since our purpose was to optimize a rapid OPPs assay-omitting juice filtration step—a model matrix (MMx) with different commercial and natural tangerine juices from Murcia and Valencia was elaborated. As can be seen in Fig. 3, the curve parameter obtained for MMx correlated well with those obtained in both fresh and commercial juices, thus minimizing matrix interferences. As shown in Table 1, the standard curve in matrix show—for both pesticides—small differences for limit of detection and sensitivity (expressed as IC_{50}) values, with those obtained previously in assay buffer (PBS). Thus, to prevent erroneous results in the quantification of OPPs concentration, all juice samples were tested using a standard curve in matrix instead of that obtained with buffer (PBST), showing the potential to analyze tangerine juice samples by duplex ELISA, with a simple sample pre-treatment below the legislated levels.

The data correlated well with those obtained by Watanabe et al. [26], which evaluate matrix interference coming from juice samples in a commercial ELISA for imidacloprid. In apple and grape juice samples, no significant matrix interference was observed only when diluting at 10-fold (20-fold in the assay).

3.3. Cross-reactivity

The selectivity of the optimized ELISAs for CLP and FTN, was evaluated by assessing its response to several analyte-related compounds. As described in Section 2.4, cross-reactivity was defined as the percentage ratio between the IC_{50} value of the target analyte (CLP or FTN) and the IC_{50} value of the cross-reacting compound.

As shown in Table 2, none of the OPPs related compounds gave cross-reactivities for CLP higher than 0.2%, with the exception 2.5% fenchlorphos and 12% for chlorpyrifos-methyl. As it had been previously reported in other works, some authors have described ELISAs where chlorpyrifos-methyl is recognized to the same extent (CR 102%) as chlorpyrifos [25,27,28], presumably because the methyl groups were better accommodated by the antibody binding sites. Only the immunoassay described by Lawruk et al. [29] showed a lower CR (26%) for this compound—but higher than that

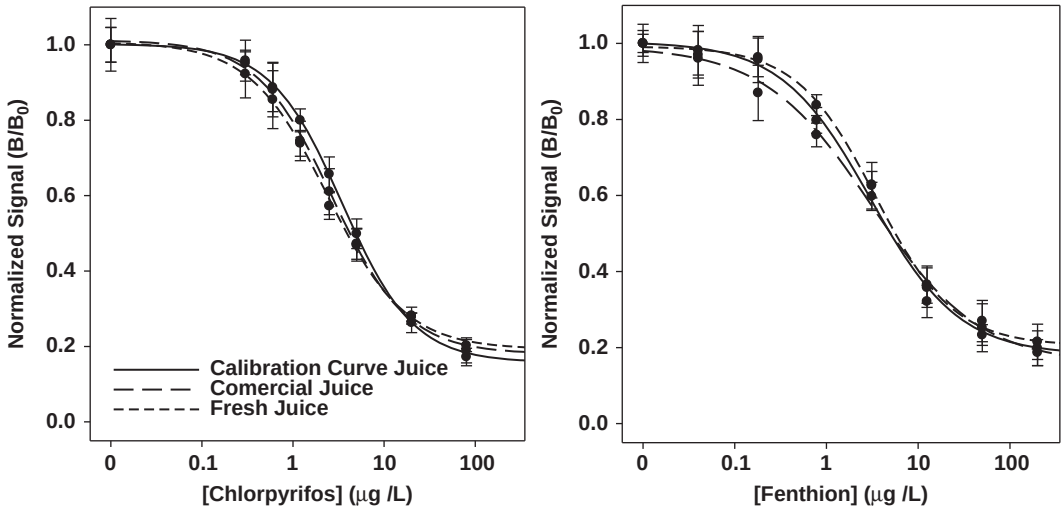

Fig. 3. Influence of matrix interference (Samples were analyzed without previous filtration) on the CLP and FTN assay in duplex ELISA.

optimized by us—probably because the hapten used for immunization maintains both ethoxyl groups in the phosphate ester. Also, a very specific assay was obtained for fenthion, since all CR values were lower than 4% -fenitrothion-, better than that the described in the literature for Zhang et al. (406%) [30] or Kim et al. (14%) [31], and in the same order as that reported by Brun et al. [23].

3.4. Analysis of juice samples by dELISA

To overcome a particular disadvantage of immunoassays—often used to detect a single analyte—the developed duplex ELISA was evaluated by analyzing juice samples for simultaneous determination of chlorpyrifos and fenthion in a single way, thus increasing the number of analytes that could be measured at the same time. In this sense, the accuracy of the proposed methodology for the determination of chlorpyrifos and fenthion in juice samples was studied using blind samples (see Section 2.6). Before addition step, samples were checked by GC–MS for incurred chlorpyrifos or fenthion residues. Each sample was evaluated in four separate assays, by triplicate, on the same day to verify reproducibility and minimize chlorpyrifos and fenthion degradation. Table 3 summarizes the accuracy of the chlorpyrifos and fenthion immunoassay in juice samples. In all cases, the amount of chlorpyrifos and fenthion added was completely recovered, with average percentage of recovery ranging from 85% to 97% for CLP and 96% to 117% for FTN, across the fortification range. For GC–MS the average recovery was in the range 89–104% (CLP) and 90–105% (FTN), very close to the results achieved by proposed duplex ELISA. Also, the accurate recovery of the fortified juice samples suggests that no matrix effects or interferences were present in the samples analyzed, and the proposed method was accurate across the OPPs concentrations tested. Such results are considered very well for residue methods [32], especially considering that the application of any immunoassay in complex matrices such as food and crops, is more difficult than to water samples [33].

Table 1
Values of more representative parameters of standard curves in assay buffer and juice for duplex ELISA for chlorpyrifos and fenthion.

dELISA		LOD	DR	IC_{50}	Scope	A_{max}	r^2
Chlorpyrifos	***PBST***	0.2	0.3–25.1	3.0 ± 0.3	0.98	1.30	0.981
	Juice[a]	0.2	0.3–9.0	2.2 ± 0.3	1.06	0.95	0.998
Fenthion	***PBST***	0.7	0.8–58	6.6 ± 0.2	1.08	1.86	0.992
	Juice	0.5	0.6–40	3.2 ± 0.2	0.90	1.42	0.997

Values are expressed in μg/L.

[a] Juice diluted 1/10 (without previous filtration).

Table 2
Cross-reactivity (%) of duplex ELISA.

Compound	Duplex ELISA	
	Chlorpyrifos	***Fenthion***
Chlorpyrifos	100	1.0
Fenthion	< 0.2	100
Diazinon	0.2	2.0
Malathion	< 0.2	4.0
Chlorpyrifos-methyl	12	< 0.2
Chlorpyrifos-oxon	< 0.2	< 0.2
Dichlorvos	< 0.2	< 0.2
Fenchlorphos	2.5	< 0.2
Bromophos	< 0.2	< 0.2
Fenitrothion	< 0.2	3.0
Fenthion-sulphoxide	< 0.2	1.0
TCP	0.2	< 0.2
Trichloronate	< 0.2	< 0.2

3.5. Effect of thermal treatment of juices on pesticide concentration

The citrus juices, mainly orange has been an economically important food product in the world, around 50% people take one a day. However, the application of OPPs has resulted in pesticide residues in fruit and deterioration of juice quality. Several methodologies have been developed for dissipation of OPPs residues in citrus concentrates, however the effect of heat treatment (pasteurization) on the dissipation of pesticides in tangerine juice, to the best of our knowledge, has not been reported. In this sense, 12 samples of 100 mL of natural or commercial juices were fortified with known amounts of CLP and FTN (10, 40 and 60 μg/L) derived from stock solutions and heated (60, 90 and 120 °C) for 30, 40 and 60 s, respectively. The samples were then conditioned by diluting 1/10 (v/v) in assay buffer and checked for chlorpyrifos or fenthion with both duplex ELISA and GC–MS methods. As can be seen in Table 4, the chlorpyrifos and fenthion concentrations determined by duplex ELISA or GC–MS were well correlated. Regarding the effect of heat treatment, we observed that the behaviour of both pesticides was similar, since the concentration slightly declined in the same way under the same conditions. Although some evidences supported the positive effect of increased heat power and treatment time on the degradation of OPPs pesticides [34,35], for each thermal treatment there were no changes in pesticide concentration over time, maybe due to slight time variations (20 s) tested, according to a soft pasteurization process. In this sense, in Table 4 we set the average value—pesticide concentration—for each thermal treatment.

Table 3
Determination of chlorpyrifos and fenthion residues in blind samples by duplex ELISA and GC–MS techniques.

Concentration (μg/L)	Chlorpyrifos				Fenthion			
	dELISA		***CG-MS***		***dELISA***		***CG-MS***	
	Value ± SD	*R* (%)	Value ± SD	*R* (%)	Value ± SD	*R* (%)	Value ± SD	*R* (%)
0	< LOD		< LOD		< LOD		< LOD	
10	8.5 ± 1.0	85	8.9 ± 0.5	89	9.7 ± 3.2	96	9.0 ± 1.3	90
40	38.2 ± 1.1	96	43.2 ± 0.9	108	46.8 ± 2.3	117	44.3 ± 0.8	110
60	58.1 ± 0.8	97	62.1 ± 1.1	104	64.5 ± 1.4	107	63.2 ± 1.0	105
Mean recovery		**93**		**100**		**107**		**102**

Values as mean ± S.D. (n = 12); n.d. non-detected; and *R* recovery.

Table 4
Recovery values obtained for chlorpyrifos and fenthion in tangerine juices (with or without heat treatments), by ELISA and GC–MS techniques.

Pesticide	*OPP added* (μg/L)	Duplex ELISA								GC–MS							
		ST		TT (60 °C)		TT (90 °C)		TT (120 °C)		ST		TT (60 °C)		TT (90 °C)		TT (120 °C)	
		Mean value	R (%)	Mean value	R (%)	Mean value	R (%)	Mean value	R (%)	Mean value	R (%)	Mean value	R (%)	Mean value	R (%)	Mean value	R (%)
Chlorpyrifos	10	12.6 ± 0.5	126	12.2 ± 0.3	122	11.5 ± 0.2	115	11.2 ± 0.3	112	12.5 ± 0.1	115	11.4 ± 0.2	114	10.9 ± 0.1	109	9.6 ± 0.2	96
	40	44.2 ± 0.1	110	44.8 ± 0.3	112	42.3 ± 0.1	106	38.6 ± 0.2	96	45.1 ± 0.2	113	44.1 ± 0.2	110	43.9 ± 0.2	110	40.2 ± 0.1	100
	60	69.6 ± 0.1	116	67.3 ± 0.6	112	63.2 ± 0.2	105	61.5 ± 0.5	102	66.2 ± 0.4	110	66.0 ± 0.6	110	63.6 ± 0.3	106	58.9 ± 0.5	98
Mean recovery			***117***		***115***		***109***		***103***		***113***		***111***		***108***		***98***
Fenthion	10	11.7 ± 0.4	117	11.2 ± 0.2	112	10.8 ± 0.5	108	9.2 ± 0.1	92	11.5 ± 0.2	115	11.6 ± 0.4	116	11.4 ± 0.5	114	9.8 ± 0.1	98
	40	47.5 ± 0.2	119	48.0 ± 0.2	122	47.0 ± 0.4	118	46.6 ± 0.2	117	44.2 ± 0.4	111	44.6 ± 0.2	112	44.0 ± 0.2	110	38.2 ± 0.3	95
	60	67.5 ± 0.3	112	62.2 ± 0.1	107	63.1 ± 0.4	105	58.3 ± 0.4	97	64.6 ± 0.3	108	63.3 ± 0.2	106	63.9 ± 0.3	107	58.4 ± 0.2	97
Mean recovery			***116***		***114***		***110***		***102***		***112***		***111***		***110***		***97***

Values as mean ± S.D. ($n=12$); n.d. non-detected; R recovery; ST without thermal treatment; and TT. thermal treatment.

4. Conclusions

The performance of the developed duplex ELISA shows the analytical potential for the rapid and quantitative determination of chlorpyrifos and fenthion in tangerine juices. The methodology allows the analysis of a dozen of samples in about 40 min, reducing the total time required in traditional ELISA immunoassay, also providing selective and sensitive responses. The duplex ELISA performs very well in juice samples without any prior sample treatment other than dilution 1/10 (v/v). The analytical performances (accuracy and precision), were comparable to those obtained by the gas chromatographic method for the analysis of juice samples, with mean recoveries for both pesticides close to 100%. In addition, the soft heat treatment (low temperatures and short exposition times) does not suppose a significant influence in the reduction of pesticide concentration. Further work will address the employ of generic immunoreagents, to test in the same plate, different insecticide families, as well as, the miniaturization of the developed and proposed immunochemical multiplexed protocols on a microarray system. Moreover, these results will be the base for developing a powerful multianalyte immunosensor system.

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