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A double-label time-resolved fluorescent strip for rapidly quantitative detection of carbofuran residues in agro-products

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ABSTRACT

A rapid and quantitative time-resolved fluorescent immunochromatographic assay (TRFICA) for detecting carbofuran residues in agro-products was reported in this paper. This assay was developed based on double-label immunoprobes, one of which was a carbofuran-specific antibody coupled with europium microbeads for the test (T) line signal while the other was mouse IgG coupled with europium microbeads for the control (C) line signal. Quantitative relationships between carbofuran concentrations and T/C ratios were established to determine the analyte concentration. To increase assay accuracy, four standard curves were established for the agro-products (green bean, cabbage, apple, and pear). The limits of detection (LODs) ranged from 0.04 to 0.76 mg L^{-1}. The spiked recoveries of carbofuran in the agro-products were in the range of 81–103%, which was in good agreement with a standard HPLC method. Therefore, we provided a new and reliable method for determination of *N*-methylcarbamate pesticide carbofuran residues in agro-products including vegetables and fruits.

1. Introduction

Carbofuran (or furadan), one of the most toxic *N*-methylcarbamate pesticides, has been used worldwide for agricultural and domestic pest control for many years. Considering its high toxicity to humans, birds, livestock, poultries and fish, and its long half-life in environment (Evert, 2000; Gupta, 1994; Yang et al., 2007), carbofuran residues in agro-products are a threat to consumer safety and human health. To eliminate this threat, many countries set rigid regulations on carbofuran residues in agro-products. For example, China set its maximum residue limit (MRL) at 0.1 or 0.2 mg kg^{-1} in grains and oilseeds on August 1, 2014 (GB 2763 – 2014, 2014), and the United States Environmental Protection Agency (EPA) set its MRL at 0.1 mg kg^{-1} in selected agro-products including green bean, banana, coffee and rice on April 17, 2015 (Environmental Protection Agency, 2015). Therefore, effective analytical methods become the last defense for safety of agro-product consumers.

An immunoassay has become an important on-site monitoring tool because of its high sensitivity, simplicity, cost-effectiveness and efficiency (Li et al., 2012). In particular, immunochromatographic methods for detecting agricultural contaminants have been developed quickly in both developed and developing countries, and many strips and even readers have been reported (Dzantiev, Byzova, Urusov, & Zherdev, 2014). The dip strip test is quite a nice screening tool for the target analyte, and is usually considered as a qualitative or semi-quantitative assay.

As for carbofuran, a nanocolloidal gold based immunochromatographic assay has also been developed for qualitative detection, whose sensitivity reached 0.25 mg L^{-1} (Zhou et al., 2004), but it is only used for detecting carbofuran residues in water samples.

To find new methods to improve quantitative detection of an immunochromatographic assay, we used europium particles to decrease the background signals because of their time-resolved fluorescence feature (Cummins et al., 2006). Besides, we used carbofuran as a target analyte to develop a rapid and quantitative double-label time-resolved fluorescent immunochromatographic assay (TRFICA) for detecting carbofuran residues in agro-products, which may provide a new immune research approach for other chemical pesticides.

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2. Experimental

2.1. Main reagents and instruments

Europium microbeads were provided by Shanghai Uni Biotechnology Company. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), boric acid, rabbit anti-mouse IgG, bovine serum albumin (BSA) and carbofuran standards were all purchased from Sigma-Aldrich. Nonspecific mouse IgG were prepared in our lab. Nitrocellulose membranes, sample pads, and absorbent pads were purchased from Millipore Corp. (Bedford, MA, USA). An XYZ3050 Dispensing Platform, CM4000 Guillotine Cutter and LM4000 Batch Laminator (Bio Dot, Irvine, CA, USA) were used to prepare test strips. Sonicator 3000 (Misonix, USA) was used to synthesize immunoprobes. The vacuum freeze drier (Thermo Electron Corporation, Rockford, IL, USA) was used to dry immunoprobes that were divided into small bottles. A portable reader previously described by Zhang et al. (2015) was used to read fluorescence signals of both the T and C lines on a strip.

2.2. Preparation of carbofuran-specific antibody and nonspecific mouse IgG

As double labels, two antibodies were prepared, which were carbofuran-specific antibody and nonspecific mouse IgG.

The carbofuran-specific antibody was prepared according to the reported reference (Zhang et al., 2008). Briefly, two female New Zealand white rabbits were immunized with the conjugates of BSA and carbofuran-*N*-caproic acid which were previously synthesized in our laboratory (Qu, Chen, & Liu, 2013). A total of six injections were carried out, and then the antisera were obtained and purified. An indirect competitive ELISA was used to characterize the specificities of the antibodies, indicating less cross-reactivity to other *N*-methylcarbamate pesticides.

The antibody for the control line which does not bind to carbofuran or carbofuran conjugates was prepared using our hybridoma cell line 10C9 (previously developed for aflatoxin) (Li et al., 2009).

2.3. Preparation of time-resolved fluoresce probes

Double immunoprobes were respectively prepared using the above two antibodies conjugated to europium microbeads. The microbeads with an average size of 190 nm have a carboxyl acid group which can bind to a carrier protein through a dehydration reaction.

For conjugation, the beads (200 μL; 1.9%, w/v) were mixed with 800 μL borate buffer (pH 8.18, a mixture of 65 mL 0.2 mol L^{-1} boric acid solution and 35 mL 0.05 mol L^{-1} sodium borate solution) using a vortex mixer, and then treated by a sonicator for 12 s at room temperature. EDC, *N*-1-((ethylimino)methylene)-*N*-3,*N*-3-dimethylpropane-1,3-diamine and 40 μL of 15 mg/mL solution were added, mixed fully, and then shaken for 15 min. The activated beads were then centrifuged at 13,000*g* for 10 min at 10 °C. After the supernatant was removed, 1 mL borate buffer (pH 8.18) was added and the precipitate was resuspended. Then, the carbofuran-specific antibody or nonspecific mouse IgG (60, 80 or 120μg from 1 mg mL^{-1} solution in 0.01 mol L^{-1} phosphate buffer at pH 7.4) was added. The mixture was shaken overnight at 250 rpm at room temperature, and then centrifuged at 19,000*g* for 10 min at 10 °C. After the supernatant was removed, 1 mL borate buffer (pH 8.18) with 0.5% BSA was added to resuspend the precipitate. The resuspended beads were shaken at 250 rpm at room temperature for 2 h, and the final solution was stored at 4 °C for use.

2.4. Preparation of immunochromatographic strips

Along with the probes, an immunochromatographic strip containing only a test (T) line and a control (C) line was prepared. On a nitrocellulose membrane (Millipore HF095, Millipore HF135 or Millipore HF180), the T line was previously coated with the carbofuran-ovalbumin prepared (Qu et al., 2013) and the C line was coated with the rabbit anti-mouse IgG prepared in our laboratory. During the coating step, an XYZ3050 Dispensing Platform (Bio Dot, Irvine, CA, USA) was used with the spray speed set to 0.6 μL cm^{-1}. Besides the type of membrane, the following previously prepared blocking formulas were also investigated according to the previous description (Zhang, Li, Zhang, & Zhang, 2011):

1) 0.01 mol·L^{-1} pH 7.4 PBS + 2% BSA
2) 0.01 mol·L^{-1} pH 7.4 PBS + 2% BSA + 0.5% polyvinyl pyrrolidone (PVP-K 30)
3) 0.01 mol·L^{-1} pH 7.4 PBS + 2% BSA + 0.1% Trion X-100
4) 0.01 mol·L^{-1} pH 7.4 PBS + 2% BSA + 0.5% PVP-K 30 + 2.5% sucrose
5) 0.01 mol·L^{-1} pH 7.4 PBS + 2% BSA + 2.5% sucrose + 0.02% NaN_3

Finally, the prepared strips were dried at 37 °C before use.

2.5. Establishment of standard curves

To establish standard curves for determining carbofuran residues, we also optimized the compositions of the sample extraction solution and dilution solution as well as the most suitable dilution times for analysis.

To select a suitable sample extraction solution, we compared effects of acetone-water, acetonitrile-water, and methanol-water on carbofuran recoveries.

To select a suitable dilution solution for the extraction, we investigated the phosphate buffer (pH 7.4, 0.01 mol L^{-1}), 0.4% Tween-phosphate buffer, 0.5% TEGOTENS® FG 40-phosphate buffer, borate buffer (pH 8.18), 0.4% Tween-borate buffer and borate buffer (pH 9.2), and compared their effects on the assay sensitivity.

To establish standard curves, we used a standard high performance liquid chromatographic (HPLC) method to make sure that the samples of green bean, cabbage, apple and pear were free from detectable residues with the limit of quantification (LOQ) being ~0.02 mg/kg (NY/T 761-2008, 2008). Then, we used these samples to prepare extraction solutions, with which serial concentrations of carbofuran standards were prepared. The above optimized conditions were used for sample extraction, dilution and competitive reaction in the application with the dipstick. After obtaining the results with a TRFICA reader previously described by our group (Zhang et al., 2015), quantitative relationships between analyte concentrations and T/C ratios were established.

2.6. Validation

Four samples free from detectable residues, including green bean, cabbage, apple and pear, were used for validation experiments. These samples were cut into small pieces and carbofuran standards in methanol were spiked into each sample (25.0 g). The optimized extraction solution (40.0 mL) was added into each sample and homogenized for 2 min. The supernatants were then diluted for competitive immunoreactions on TRFICA strips. On the same day, the spiked samples were also detected using the standard HPLC method (NY/T 761-2008, 2008).

3. Results and discussion

3.1. TRFICA principle

An immunochromatographic assay usually has only one labeled antibody (immunoprobes), which is single-label assay, and the immunoprobes usually need to be slightly excessive to create a clearly visible control line for qualitative analysis (Zhang et al., 2011). We have developed a new, rapid and quantitative assay, which is, double-label time-resolved fluoresce immunochromatographic assay (TRFICA).

The TRFICA procedure includes sample extraction, standing time, and supernatant processing (diluting the supernatant and adding it into a small bottle containing dried immunoprobes). The strip is dipped into the small bottle and the reaction is allowed to proceed for 10 min. The strip is then removed from the bottle and immediately read by a portable TRFICA reader (Fig. 1).

In this double-label system (Fig. 1), Immunoprobe 1 is a carbofuran-specific antibody conjugated to europium microbeads while Immunoprobe 2 is a nonspecific mouse IgG conjugated to europium microbeads. Carbofuran conjugates (coating antigen) are immobilized on the T line and the rabbit anti-mouse antibody on the C line. Therefore, Immunoprobe 2 will be consistently captured by the C line while Immunoprobe 1 that is not bound to free carbofuran will be captured by the T line. Immunoprobe 1 bound to free analytes will flow past both T and C lines. Therefore, quantitative relationships can be established between analyte concentrations and T/C ratios and put into a portable reader for automatic quantitative calculations.

Since independent Immunoprobe 2 was used for coloring the control (C) line of the strip, the C-line signal is constant. In the single-label immunoprobe system, the C-line signal changes with the analyte concentration. Therefore, the amount of used Immunoprobe 1 (carbofuran-specific immunoprobe) could be reduced relatively. As a result, the double-label system may theoretically increase both assay sensitivity and accuracy based on the relationships between carbofuran concentrations and T/C ratios.

In the double-label TRFICA, time-resolved fluorescence was used to decrease the background signals mainly from the sample matrix, which was shown on the portable reader previously reported by our group (Zhang et al., 2015; Majdinasab et al., 2015).

3.2. Optimization and establishment of the TRFICA method

To develop the TRFICA method, several factors were investigated, including the most proper working concentration of Immunoprobes 1 and 2 (carbofuran conjugate and rabbit anti-mouse IgG), immunoreaction environment (membrane type, blocking formulas, compositions of extraction solution and dilution buffer) and lateral flow reaction time.

(1) The most proper working concentration of immunoreagents (Immunoprobe 1 and 2). With non-overdose applied, three combinations of immunoreagent amounts were tested and their sensitivities (IC_{50}) were compared. According to the result (Table S1), No. 2 combination showed the highest sensitivity of 0.16 mg L^{-1}. Therefore, we selected the amount of No. 2 immunoreagent combination to optimize other conditions.

(2) Effects of membrane types on strip signals. Membrane is as important as solid carrier materials for immunochromatographic assays. We compared three types of Millipore membranes. The result (Table S2) showed that the test (T) line signal is the clearest on Millipore HF095 strips. Therefore, Millipore HF095 was selected for further researches.

(3) Optimization of blocking formulas. Five blocking formulas were compared. The data (Fig. 2) showed that the T-line signal was the clearest when No. 5 blocking formula (0.01 mol·L^{-1} pH 7.4 PBS + 2% BSA + 2.5% sucrose + 0.02% NaN_3) was used.

(4) Optimization of extraction solutions. Three extraction solutions were compared by their effects on assay recoveries. The result (Fig. S1) showed that the carbofuran recovery was the highest (96.3%) when 80% methanol-water was used as the extraction solution.

(5) Optimization of dilution solutions. Six dilution solutions were compared by their effects on assay sensitivity. The result (Table S3) showed that the assay sensitivity was the highest when 0.4% Tween-phosphate buffer was used to dilute the extraction solution.

(6) Effects of reaction time on TRFICA assay sensitivity. Four reaction time lengths were compared. The result (Table S4) indicated that too long or too short reaction time decreased

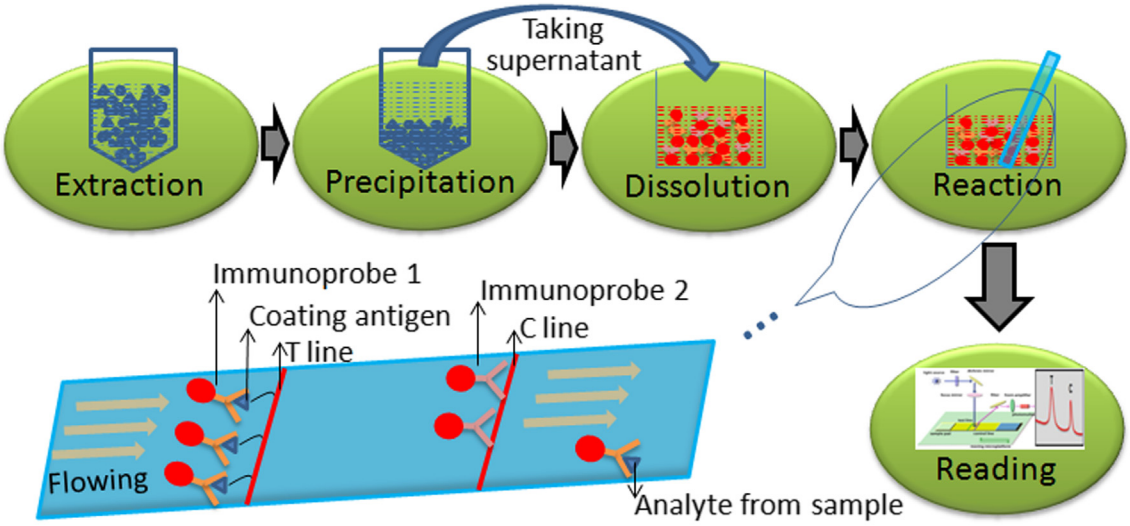

Fig. 1. Main procedure and principle of quantitative double-label TRFICA, where Immunoprobe 1 is a carbofuran-specific antibody conjugated to europium microbeads while Immunoprobe 2 is a nonspecific mouse IgG conjugated to europium microbeads. Carbofuran conjugates (coating antigen) are immobilized on the T line and the rabbit anti-mouse antibody on the C line. Therefore, Immunoprobe 2 will be consistently captured by the C line while Immunoprobe 1 that is not bound to free carbofuran will be captured by the T line. The bound species will flow past both the T and C lines. Therefore, quantitative relationships between analyte concentrations and T/C ratios can be established and put into a portable reader for automatic quantitative calculations.

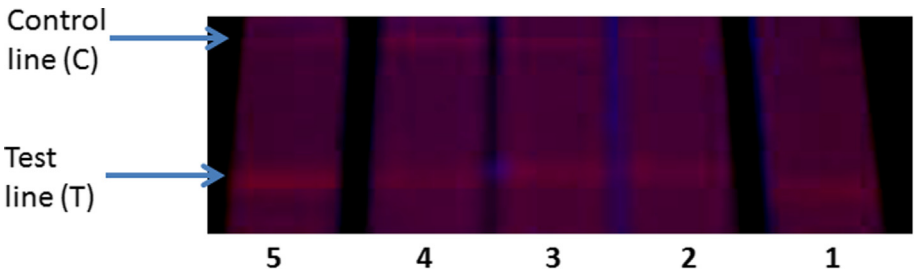

Fig. 2. Comparison of five blocking formulas. 1) 0.01 mol·L^{-1} pH 7.4 PBS + 2% BSA; 2) 0.01 mol·L^{-1} pH 7.4 PBS + 2% BSA + 0.5% polyvinyl pyrrolidone (PVP-K 30); 3) 0.01 mol·L^{-1} pH 7.4 PBS + 2% BSA + 0.1% Trion X-100; 4) 0.01 mol·L^{-1} pH 7.4 PBS + 2% BSA + 0.5% PVP-K 30 + 2.5% sucrose; 5) 0.01 mol·L^{-1} pH 7.4 PBS + 2% BSA + 2.5% sucrose + 0.02% NaN_3.

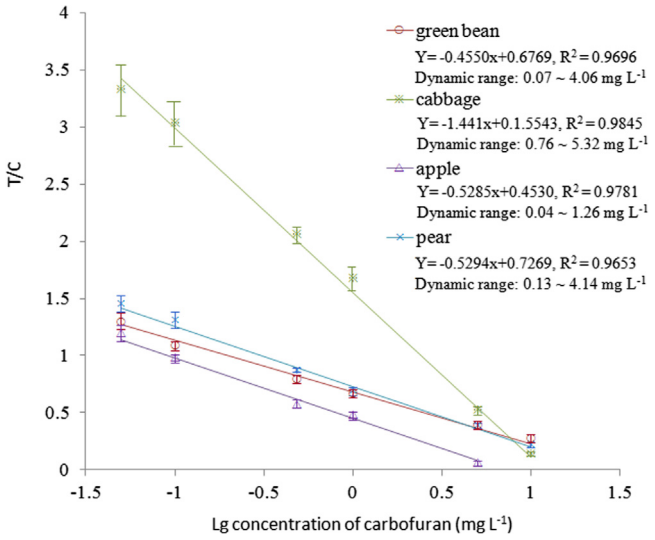

Fig. 3. TRFICA standard curves for determination of carbofuran residues in agro-products. The spiked samples with carbofuran standards were extracted using 80% methanol. Then, the supernatants were diluted to 5 times of the original volume using the dilution buffer (PBS containing 0.4% Tween-20) for the test and the corresponding curve establishment. The strip-to-strip standard deviations were under 20%, and day-to-day ones were under 30%.

the assay sensitivity significantly. Taking both sensitivity and time-effectiveness into account, 10-min reaction time was selected for the developed TRFICA.

Finally, the main parameters of the optimized TRFICA method were summarized in Table S5, in which 10-min lateral flow reaction inferred the potential "rapid" feature of the newly developed method for determination of carbofuran residues in agro-products.

To increase assay accuracy, we established standard curves respectively using the above optimized conditions. Briefly, clean agro-product samples including green bean, cabbage, apple and pear were spiked with a series of carbofuran standards and extracted using 80% methanol, and then the supernatants were diluted to 5 times of the original volume using the dilution buffer (PBS containing 0.4% Tween-20) for the test and the corresponding curve establishment. The results (Fig. 3) showed an LOD range of 0.04–0.76 mg L^{-1} (calculated by three times of the standard deviation). This indicated that the significant difference among the tested samples might result from different sample matrices and that the cabbage matrix had the largest impact on the TRFICA results. The results inferred that the TRFICA standard curves for different kinds of agro-products should be separately established.

Compared to ELISA (Table 1), TRFICA showed a more rapid, simpler and more reliable (with the use of the control line) method for quantitative assays on test strips. Although TRFICA was not as sensitive as earlier reported ELISA (0.056–10 μg L^{-1}) (Jourdan, Scutellaro, Fleeker, Herzog, & Rubio, 1995; Mickova et al., 2003; Yang et al., 2008), it was slightly more sensitive than nanocolloidal gold based immunochromatographic assays (0.25 mg L^{-1} in the water sample, Table 1) (Zhou et al., 2004) and similar to Guo's gold immunochromatographic method (Guo, Liu, Gui, & Zhu, 2009), revealing that using europium microbeads and time-resolved fluorescence was a good alternative label for development of immunochromatographic assay.

3.3. Validation of the TRFICA method

To validate a new analytical method, we usually carried out both the spiked recovery test and standard method. Here we selected four clean agro-products: green bean, cabbage, apple and pear. The carbofuran-spiked samples were detected using the developed TRFICA and a standard HPLC method on the same day. The results in Table 2 showed a good recovery range of 81.0–103.3% based on the TRFICA method. These recoveries were

Table 1
Comparison of the developed TRFICA to the reported ELISA and nanocolloidal gold strip methods for detecting carbofuran residues.

Reference	Method	Sensitivity	Accuracy (RSD or correlation to HPLC)
In this article	TRFICA	0.04–0.76 mg L^{-1} in green bean, cabbage, apple and pear	0.7–18.8% r = 0.995, slope = 1.09
Zhou et al. (2004)	Gold immunochromatographic assay	0.25 mg L^{-1} in water samples	no data no comparison
Guo et al. (2009)	Gold immunochromatographic assay	32 μg L^{-1} in water	no data no comparison
Jourdan et al. (1995)	ELISA	0.056–5.0 μg L^{-1} in water and soils	no RSD r = 0.967, slope = 1.18
Mickova et al. (2003)	ELISA	0.4–10 μg L^{-1} in apple–strawberry baby food	3.6–40.0% r = 0.995, slope = 0.85
Yang et al. (2008)	ELISA	0.11 μg L^{-1} in lettuce, cabbage, water and soils	no data no comparison

Table 2
Spiked recoveries of carbofuran in agro-products using the TRFICA and a standard HPLC method.

Sample	Spiked concentration mg kg^{-1}	TRFICA		HPLC		Relative deviation[a] %
		Tested mg kg^{-1}	Recovery %	Tested mg kg^{-1}	Recovery %	
Green bean	0.8	0.76	94.6	0.845	105.6	10.1
	16	15.61	97.5	16.24	101.5	3.9
	32	31.78	99.3	32.05	100.2	0.8
Cabbage	6.4	5.18	81.0	6.38	99.7	18.8
	18	18.36	102.0	18.24	101.3	0.7
	40	34.40	86.0	39.92	99.8	13.8
Apple	1.6	1.51	94.6	1.58	98.8	4.2
	4.8	4.96	103.3	4.86	101.3	2.0
	9.6	9.11	94.9	9.56	99.6	4.7
Pear	1.6	1.63	102.0	1.65	103.1	1.3
	16	16.52	103.0	16.23	101.4	1.8
	32	29.81	93.0	31.25	97.7	4.6

[a] Since the repeated results were from three disposable strips, these data also meant the strip-to-strip reproducibility.

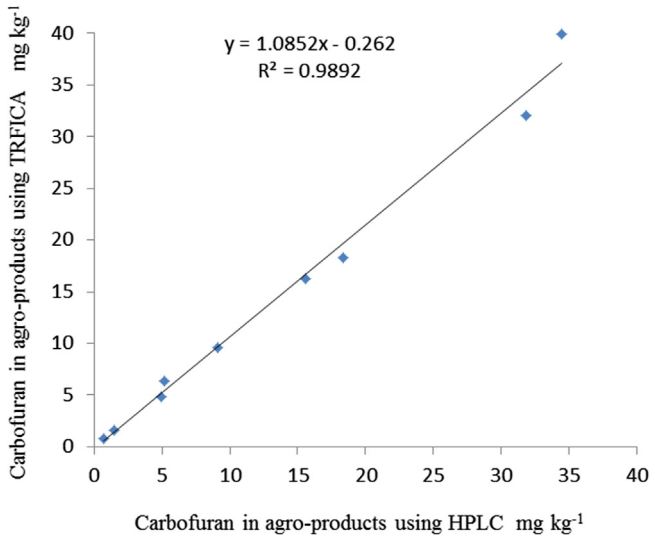

Fig. 4. Correlation between carbofuran concentrations as determinated by TRFICA and HPLC methods. n = 9, r = 0.995.

very similar to those (97.7–105.6%) obtained using the standard HPLC method. After statistical analysis, the relative deviations between both methods were not greater than 10.1% except cabbage (up to 18.8%), which further proved that the cabbage samples had the highest matrix effect on the TRFICA results. The comparison result indicated that the recoveries of both methods were in high accordance with each other, and that the developed TRFICA could meet the requirement for quantitative detection of carbofuran residues in agro-products including vegetables and fruits. Therefore, a new reliable method was provided to determine *N*-methylcarbamate pesticide carbofuran residues in the agro-products.

According to the data in Table 1, the correlation between carbofuran concentrations as determined by TRFICA and HPLC methods was calculated. The regression analysis yields a correlation of 0.995 (r value) and a slope of 1.09 between two methods (Fig. 4), which indicates that the detection results of the developed TRFICA are agreed by the standard HPLC method.

4. Conclusions

An immunochromatographic method for detecting agricultural contaminants is usually thought as qualitative or semi-quantitative assays although many strips and even readers have been reported. Therefore, in order to improve quantitative detection of an immunochromatographic assay, we employed carbofuran as a target analyte. As one of the most toxic *N*-methylcarbamate pesticides, carbofuran heavily threatens the safety of agro-product consumers and human health. To address this issue, we developed a rapid and quantitative double-label time-resolved fluorescent immunochromatographic assay (TRFICA) for detecting carbofuran residues in agro-products. To determine carbofuran in green bean, cabbage, apple and pear samples, their LODs were from 0.04 to 0.76 mg L^{-1} and the spiked recoveries of carbofuran in the agro-products were in the range of 81–103%, which agreed well with a standard HPLC method. Therefore, we provided not only a new reliable determination method for *N*-methylcarbamate pesticide carbofuran residues in agro-products including vegetables and fruits, but a new research approach for other chemical pesticides. However, the sensitivity of this method still requires improvement in our future work.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.02.016.

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