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Acidified QuEChERS method followed by GC-MS/MS on-cold injection for the determination of captan and folpet metabolites in strawberries and apple

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ABSTRACT

In this work, the on-cold injection GC-MS/MS approach's development and evaluation for the simultaneous determination of both captan and folpet metabolites with endpoint backflush had been evaluated. An acidified QuEChERS approach for sample extraction, accompanied by the on-cold GC-MS/MS approach, provides an accurate quantification of captan and folpet metabolites in apple and strawberries at reporting limit (LOQ= 10μg/kg), which was lower than the EU-MRL and NAFSA-MRL. The method shows the analytical matrix effect, which was higher in apples (134%, 152%) than strawberries (83%, 115%) for captan and folpet metabolites tetrahydro phthalimide (THPI) and phthalimide (PI), consecutively. However, apple and strawberries correlation characteristics at three spiking levels of 10, 50, and 100μg/kg of both captan and folpet indicated a linear relationship (R2>0.99) which proves the reduction of the matrix effect. The recovery of captan metabolite ranged from 97% to122% and 80% to107%, while folpet metabolite ranged from 77% to104% and 78% to 111% for apple and strawberries, respectively, with RSD pooled <10%. The result confirms both method robustness and fits for their intended use that permits the practice in routine analysis.

1. Introduction

Captan (1,2,3,6-tetrahydro-N-(trichloromethylthio) phthalimide), folpet (N-(trichloromethylthio) phthalimide) belong to thiophthalimide pesticides chemical group which are fungicides used on fruits, vegetables, ornamentals, and also on food crop packaging boxes, spoilage fungi on wood to treat fungal diseases [1, 2].

According to Egyptian Agricultural Pesticides Committee (APC) recommendations, captan used with application rate 200 gm/100 L and 150 gm/100 L on strawberry and apple **(M. domestica),** respectively, to inhibit **Botrytis and Venturia inaequalis** (apple scab), where the infected fruit becomes distorted allowing entrance of secondary organisms with preharvest interval of 7 days [3, 4]. Folpet used to control the appearance of reddish to small purple spots called leaf spots on the top side of strawberry leaves with an application rate of 200 gm/100 L and a preharvest interval of 5 days[4]. The maximum residue limits (MRLs) for sum of captan and THPI, represented as captan were 15 and 10mg/kg in apple, while in strawberries were 15 and 1.5mg/kg. On the other hand, the MRLs for sum of folpet and phtalimide, were represented as folpet 10 and 0.3mg/kg in apple and 5mg/kg in strawberries based on NAFSA-Egypt and EU-commission regulations, respectively [5–8].

The dissipation performance and residual levels of captan on apple based on Egyptian climatic conditions were studied. The residues (3mg/kg) were found to be lower than MRL after three days of application, where the half-life value ($t_{1/2}$) was 2.47 days. [9]. Treatments with Captan 80 WG in four varieties of apples were studied, and it found that residues varied from 3.19 to 4.76 mg kg^{-1} [9]. Numerous difficulties have been reported during the determination of captan and folpet at residue level by different authors due to thermal instability at higher temperatures of both [10], effect of different solvents, pH, and GC conditions [11], in addition to the possibility of degradation during the sample preparation using dry ice milling technique [12] to overcome difficulties throughout captan and folpet residues' assessment, applying supercritical fluid chromatography combined with mass spectrometry was conducted.

The findings showed that the recovery varied from 84% to105% with RSD < 8% at two concentration levels of 10 and 50 µg/kg. The PTV injector and GC-MS/MS utilization for the captan and folpet residues' quantitative determination in vegetables and fruit showed that the mean recovery results for captan at 0.01mg/kg was 96% with RSD 21% with minimal degradation to metabolite [13]. Another improvement on the assessment of captan, tetra-hydro phthalimide, captafol, folpet, and phthalimide by liquid chromatography-tandem mass spectrometry was tested, and the recoveries were found in the range of 70–120% and RSD<10% [14, 15].

Different extraction methods for the detection of captan in apple were applied; for example, acetonitrile QuEChERS extraction technique followed by GC-ECD for detection with GC injector temperature 280°C [16, 17]. The results found that the average recoveries of captan at 10, 50, and 500µg/kg were 80.5%, 86.2%, and 95.1%, respectively. Dissimilar approaches, such as using matrix-matched calibration, and analyte protectants were studied to compensate losses throughout analysis of captan and folpet due to degradation throughout homogenization, extraction, and GC-injection in fruits and vegetables by using 10mL acetonitrile containing 1% formic acid QuEChERS of extracts followed by GC-MS and GC-MS/MS with PTV solvent vent mode injector at 50°C [18].

One more detection approach for captan and folpet's simultaneous determination utilizing GC equipped with negative chemical ionization-mass spectrometry (GC-NCI-MS) and two-dimensional gas chromatography–time-of-flight mass spectrometry (GC × GC-ToFMS) with linear concentration ranged from 50 to 2500µg/L at 50µg/kg [19, 20]. Most of the previously mentioned quantitative determination techniques used more steps to stabilize captan and folpet form degradation or hydrolysis in all stages that increase both time and cost of analysis. The present work aims to validate and develop GC-MS/MS quantitative technique for simultaneous determination of both captan and folpet metabolites tetrahydrophthalimide (THPI) and phthalimide (PI), respectively, using on-cold multimode inlet injection containing ultra-inert dimpled liner (5190-4006, ultra-inert liner 2mm dimpled, split-less), with endpoint backflush.

2. Experimental

2.1. Pesticides reference standards

Captan, folpet, and aldrin certified reference materials were obtained from (Dr. Ehrenstorfer-LGC, Augsburg, Germany) with a 98% purity. Individual stock solutions were prepared at a concentration 1000µg/mL dissolved in toluene and kept at −20°C with purity correction 2%. An intermediate mixture of both was set at a level of 10µg/mL, and then calibration combinations were prepared via serial dilution form the intermediate mixture at 10, 50, 100, and 500 µg/L in toluene with using aldrin (IStd) as internal standard at level 100 µg/L, and then kept at −4°C.

2.2. Reagents and chemicals

Acetonitrile was obtained from Carlo ERBA (Marseille, France), while n-hexane was purchased from J.T. Baker, (Arnhem, Netherlands). Acetone, toluene, and formic acid were purchased from Merck (Darmstadt, Darmstadt, Germany). All the solvent and chemicals used are HPLC-grades. QuEChERs extraction packets containing salts were purchased form (Agilent, Santa Clara, USA) where the extraction tube containing 4gm anhydrous-magnesium sulphate ($MgSO_4$), 1gm sodium chloride (NaCl) (Part No: 5982-5550).

2.3. Apparatus

Ultra-Heavy Duty 3.75HP stainless steel Blender (Waring, New York, USA) for sample homogenization, deep freezer (Kirsch, Willstätt-Sand, Germany) with -20°C efficiency for sample storage, geno-grinder 2010 (SPEX Sample Prep, New Jersey, USA) as vertical sample shaker, refrigerated centrifuge TX-400 (Fisher Scientific, Loughborough, UK), ultrasonic bath (ELMA, Holzwickede, Germany), weighing balance (Mettler Toledo, Ohio, USA), rotary evaporator (Heidpolph Instruments GmbH & CO. KG) for specimen evaporation at 40 °C.

2.4. GC-EI (+) MS/MS analysis

The GC-EI (+) MS/MS system consists of a benchtop GC-7890A supplied with MS-7000B triple, quad system (Agilent, Santa Clara, USA) including ion source with positive electron ionization mode (70eV). Ultimate union assay was used to connect the two GC chromatographic columns (Agilent, Santa Clara, USA). The first was DB35-MS Ultra Inert (Part No: 121-3822UI) with dimension (30 m × 0.18 mm, 0.18 μm) utilized for separation, and the second column was Inert Fused Silica column (Part No: 160-7625-5) with dimension (0.7 m x 150 μm x 0 μm) used as a free inert bath to deliver the analyte from oven to mass system via thermal auxiliary at 280°C.

The injection volume was 1μl using a 10μl automated injection syringe through an ultra-inert inlet 2mm dimpled, spilt-less liner (Part No: 5190-4006). A multimode injection inlet system in split-less mode which was used at initial temperature 65°C, held for 0.35min, increased with a rate of 900°C/min to 280°C held for 18 min. Purity's Helium 99.9995% was utilized as the carrier gas at a constant flow rate of 0.9ml/min in the first column, while the second column maintained a constant pressure of 3psi during the run. The purge flow to the split vent was 50mL/min at 1.5min.

The oven temperature program was at the outset held for 1.5min at 60°C, ramped to 160°C with a rate of 40°C/min, then to 240°C with a rate of 4°C/min. Finally, the oven temp was manipulated to 310°C via 100°C/min. The total run time was 26.3min. End point-back flush option start for 2min, where inlet pressure is maintained at 1psi and ultimate union assay pressure at 25psi to reverse the flow though the first column that increases column lifetime via thermal cleaning at the end of the run time. When operating in electron impact ionization mode, the mass spectrometer MS-7000B triple quad system sustained an ion source temperature of 280°C, 150°C quadruple temperature, and a filament current of 35μA.

Perfluorotributylamine (PFTBA) is also utilized in the system's auto-tuning as a system suitability test for MS systems. A collision nitrogen gas of purity 99.999% was used on the collision cell at 1.5ml/min. Mass Hunter (ver. 10.0.368.0) was utilized for instrument control, data gathering, and data processing. The target pesticides were monitored using dynamic multiple reactions monitoring (dMRM) mode, where minimum two transitions per analyte were utilized for both the identification and quantification processes with S/N>10 [21].

2.5. Sample handling and homogenization

Apple and strawberries samples of two kilograms were collected then grinded with ultra-heavy duty 3.75HP stainless steel blender to obtain a homogenized sample, where the sample used as a blank was previously tested before free from target pesticides via quantitative analysis then stored at –20°C.

2.6. Sample preparation

Acidified QuEChERS method [18] was used for sample extraction, where ten grams of the homogenous specimen were prejudiced into a 50ml polypropylene centrifuge tube, accompanied by the addition of 10mL acetonitrile containing 1% formic acid. The sample was allowed to shake for 3 minutes using a geno-grinder at 900rpm, followed by the addition of 1g of sodium chloride and 4g of anhydrous magnesium sulfate, then shaking for one minute at 900rpm. Finally, centrifugation of the sample was done for 5 minutes at 2268rcf (Relative Centrifugal Force) at 4°C. The upper 2ml acetonitrile layer was evaporated near to dryness at 40°C and reconstituted with 2ml of aldrin (IStd) at a level of 100μg/L. Afterward, it was filtered via a 0.45μm membrane syringe filter into an obvious vial for GC-MS/MS analysis.

3. Results and discussions

3.1. Optimization of gas chromatography conditions

Using both on-cold injection mode at 65°C and ultra-inert dimpled liner decreases the degradation of captan and folpet metabolites due to GC-injection, and also reduce the activity of the liner active-sites, which provide reproducible inertness in GC-inlet system in temperature lower than degradation point for captan, and folpet metabolites at 173°C, 184°C respectively. Applying the GC temperature program accompanied with d-MRM acquisition parameters for folpet and captan summarized in Table **1**, the results show reproducible sensitivity with the linear response for target pesticides. Selected MRMs with bold font were used as quantifiers. The manufacturer recommends adjusting the filament off at the beginning of the run for 5 minutes during solvent.

3.2 Method validation

In this study, the method performance characteristics were evaluated from the validation results following the EC guidelines [20, 21]. The method was validated by evaluating the detection's linearity and limit, boundary of quantification, trueness, robustness, precision, and uncertainty. The anticipated approach was validated on two matrix "apple and strawberries". The linear method range was tested after evaluation of regression coefficient (R2) via plotting four-point calibration level response against concentration in the range of 10–500µg/L prepared as matrix matched calibration that contains natural product which acts as analyte protectant. The results found that 'R2' values were 0.999 and 0.998 for captan and folpet, respectively, indicating good linearity, as shown in Fig. **2**.

Also, the results show that there was linear correlation between expected concentration versus mean found concentration of both strawberry and apple fortified samples with regression coefficients 'R^2' more than 0.99 with accepted correlation for the method linearity as shown in Fig. **3,4**. Fig. **3** demonstrates chromatograms gathered when standard combinations of captan and folpet were added at 10, 50, 100, and 500µg/mL in green beans blank sample extract and fortified sample at 50µg/Kg. Captan and folpet completely break down into THPI and PI, consecutively. The chromatograms of folpet exhibited noticeably higher baselines and more background peaks throughout the run, despite the fact that folpet generally converted to more sensitive peaks than those derived from captan.

As soon as the matrix's lowest concentration of the target analytes was acceptable with appropriate accuracy and precision was found discovered, LOQs were set up [20]. Table **2** summarizes the repeatability results of validation that applied on both apple and strawberries. The recovery calculation was utilized to calculate the precision of the approach for a set of six repetitions at three fortification levels of 10, 50, and 100 µg/kg, where the fortified blank specimen was extracted using the acidified QuEChERS approach and then subjected for GC-MS/MS analysis. By combining the variances of the three distinct levels of concentration, reproducibility was determined [22].

$$RSDpooled = \sqrt{((rsd1^2\,(n1-1) + (rsd2^2\,(n2-1) + \cdots)/((n1-1)+(n2-1)} \quad Equation\ 1$$

RSD_{pooled} = Relative standard deviation, n = Number of samples

The findings exhibit that the recovery of captan was varied from 97 to122% and 80 to107% for apple and strawberries, respectively, while folpet was ranged from 77% to104% and 78 to 110% for apple and strawberries, respectively, with RSD_{pooled} <10% which confirms both method robustness and fit for its intended use. In order to prevent any false positive or negative results of both captan and folpet due to co-eluted matrix, both blank extract and matrix effect were examined during analysis by standard addition of known concentration of target analyte on blank extract via calculation of matrix effect percentage to compensate the effect, it was observed that at reporting limit (LOQ=10µg/kg) the matrix effect higher in apple (134, and 152%) than strawberries (83, and 115%) for captan and folpet respectively.

Intra-laboratory precision for the method was tested throughout the calculation of relative standard deviation (RSD %) from validation performed at three fortification levels 10, 50, and 100µg/kg, and the results show that for both captan and folpet in two matrices were RSDs%<15 indicating the reliability of the method. The inter-laboratory comparisons were checked by the recovery obtained from the analysis of spiked sample at concentration of 50 µg/kg applying the validated method in each working day and analysis of certified reference material EU-SRM strawberry puree containing both analytes. The reference of target analytes assigned value, fixed target relative standard deviation (RSD%) were extracted from EUPT-SRM12 report for z-score calculation [23].

$$Z = \frac{Ci - Crm}{rsd\%} \quad Equation\ 2$$

Where C_i the results of target analyte after method application, C_{rm} reference material assigned the value of target analytes, RSD% the target relative standard deviation of the target analytes. The results show a satisfactory z-score. The measurement uncertainty's validation for the method was calculated using Appendix C approach 1[21, 24] dependent on intra-laboratory assessment data. The results show MU dependent on intra-laboratory validation/QC data for one month at measurement results of 50µg/kg reported with recovery correction were 25 and 38% for captan and folpet, respectively.

3.3. Analysis of Real Samples

A total of 30 specimens, split equally between strawberries and apples, were bought from an Egyptian market and put through the suggested technique of analysis. Each batch of samples in the analysis process, included a blank sample, four matrix matched calibration levels, and a quality control sample spiked at 50 µg/kg. Table 4 shows the outcomes that were attained for the tested samples. The findings that captan was the sole contaminant found in all of the examined specimens. The permitted limits established by the NFSA and European Commission were not exceeded in any of the samples for captan as phthalimide residues. QC recoveries were obtained, showing the robustness of the method during several days of analysis and the estimation of measurement uncertainty.
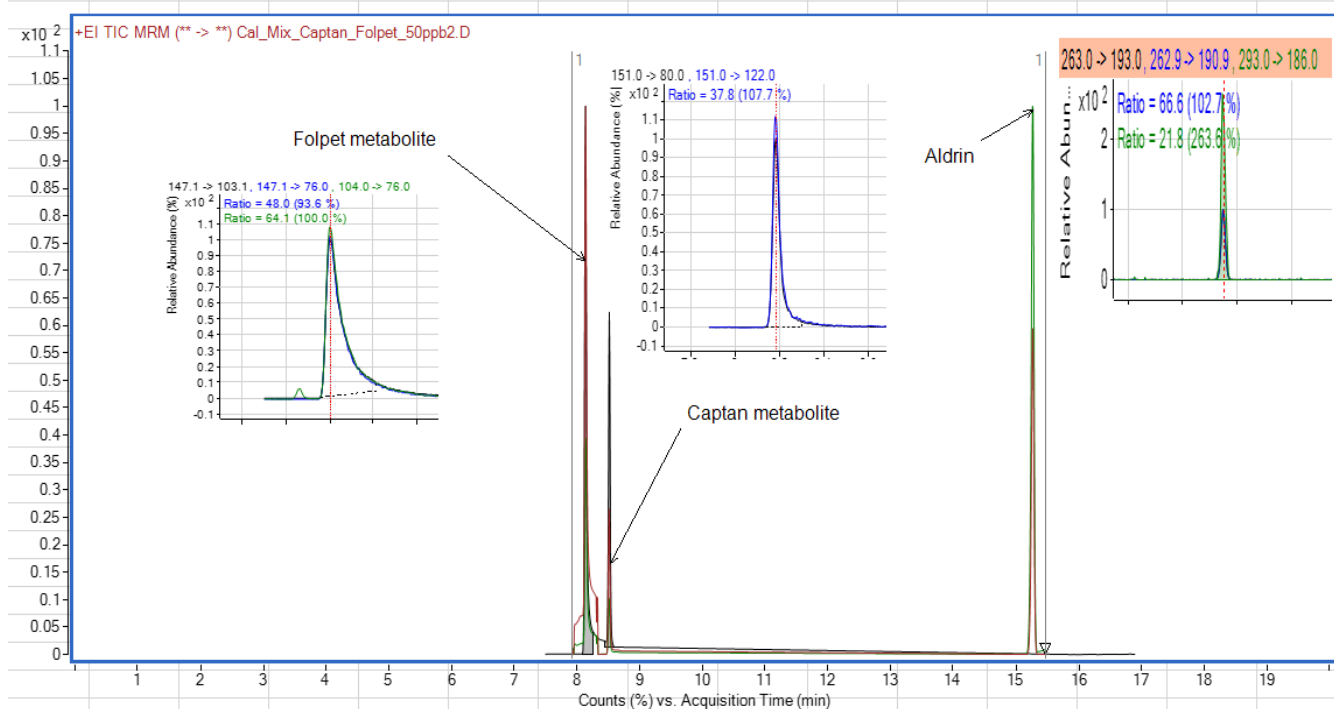

Fig. 1 represents the total ion chromatogram, retention time (RT), quantifier, qualifiers ions for aldrin (IStd), captan, and folpet metabolites at a concentration of 50µg/L

Table 1. summarized acquisition parameters, retention time (RT, min), MRM(m/z) and collision energy (ev) for captan, folpet metabolites and aldrin

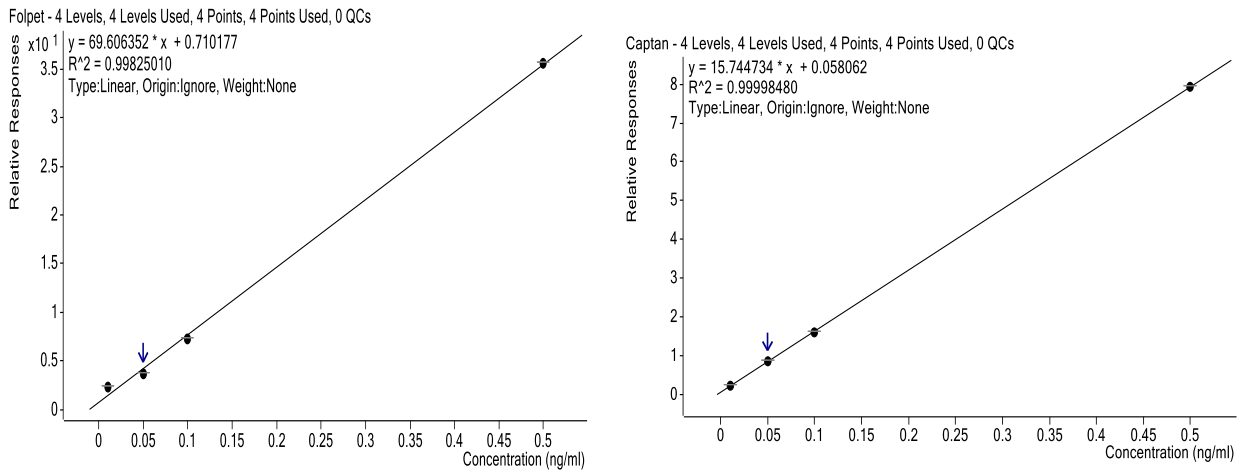| Compounds	RT (min)	MRM (m/z), CE (ev)
Aldrin	15.250	**263>193, (30)** 293>186, (25) 262.9>190.9, (40)
Captan as phthalimide (PI)	8.689	**151>80, (5)** 151>122, (8)
Folpet as tetrahydrophthalimide (THPI)	8.115	**147.1>103.1, (5)** 147.1>76, (25) 104>76, (10)

Fig. 2 represents the calibration curve, linear equation formula, and regression coefficient (R2) for captan and folpet metabolite

sample	Calibration levels				fortified sample
L1=10 µg/L	L2=50 µg/L	L3=100 µg/L	L4=500 µg/L		50 µg/Kg

Captan metabolite (PI)

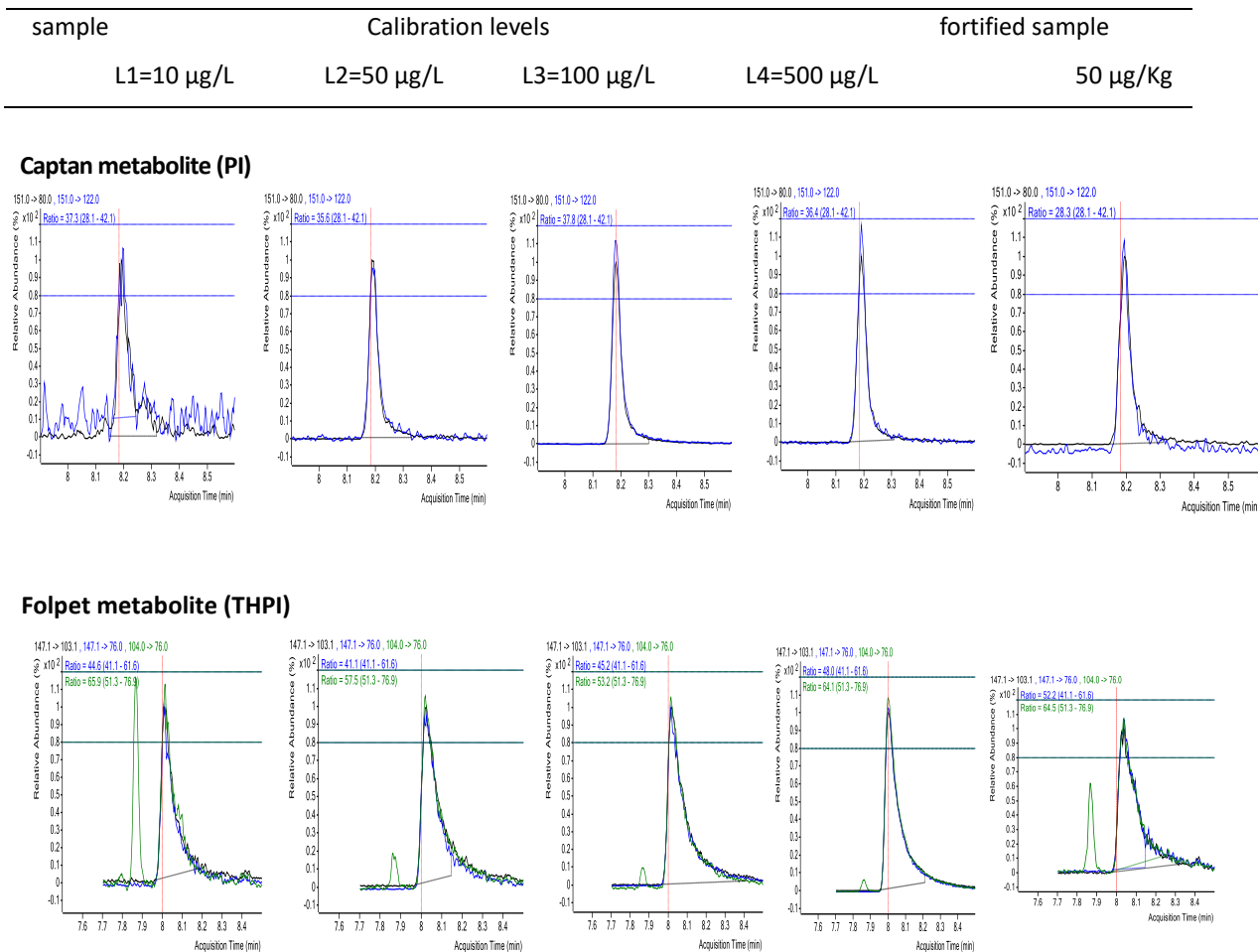
Folpet metabolite (THPI)

Fig. 3 Chromatograms of captan and folpet metabolites (PI and THPI) gathered from the concentrated standard mixtures' injection in the range 0f 10–500µg/L
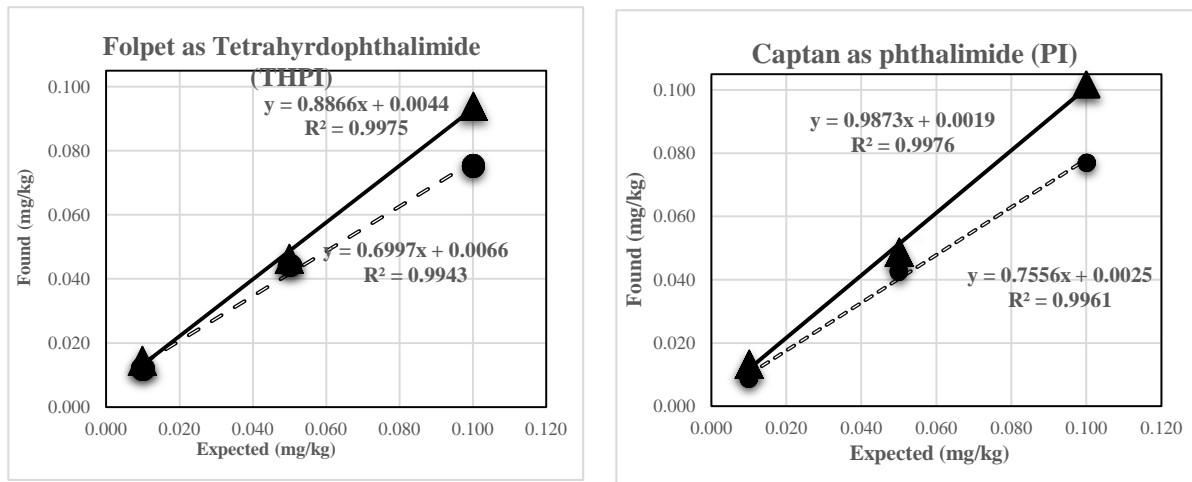
Fig. 4 represents apple and strawberries correlation characteristics for mean found at three spiking levels 0.01, 0.05, and 0.10 mg/kg of both captan and folpet as phthalimide and tetrahydrophthalimide, respectively.

Table 2. Validation data from repeatability (n=6), mean recovery ±RSD%, RSD_{pooled}%, applied on both apple and strawberries at three fortification levels 10, 50, 100µg/kg.

	Apple, n=6					Strawberries, n=6				
	10 µg/kg	50 µg/kg	100 µg/kg			10 µg/kg	50 µg/kg	100 µg/kg		
	Mean Recovery ± RSD			Q_{type}	RSD_{pooled}	Mean Recovery ± RSD			Q_{type}	RSD_{pooled}
Captan as (PI)	97±2%	102±9%	122±5%	94%	6%	107±7%	107±7%	80±9%	98%	8%
Folpet as (THPI)	104±6%	98±14%	77±5%	93%	9%	111±7%	111±7%	78±7%	100%	7%

Table 3. represents the results of certified reference material strawberry puree

	assigned value* (mg/kg)	RSD%*	results (mg/kg)	bias (mg/kg)	Z-score
Captan (SUM)	0.302	25.2%	0.265	-0.037	-0.15
Folpet (SUM)	1.195	21.1%	0.785	-0.41	-1.94

Table 4. results of real sample analysis, Range, Mean, Median, and MRL (mg/kg) of captan and folpet as phthalimide and tetrahydrophthalimide metabolites, respectively metabolites

		Range			MRL (mg/kg)	
		Min-Max (mg/kg)	Mean (mg/kg)	Median (mg/kg)	NAFSA_Egypt	EU
Captan as phthalimide	**Apple**	0.011 - 2.17	0.382	0.087	15	10
	Strawberries	0.022 - 0.884	0.176	0.075	15	1.5
Folpet as tetrahydrophthalimide	**Apple**	ND	ND	ND	10	0.03
	Strawberries	ND	ND	ND	5	5

N.D: not detected

4. Conclusion

Development and validation of the proposed method based on GC-MS/MS quantitative technique for both captan and folpet's simultaneous determination as phthalimide and tetrahydrophthalimide, respectively, in apple and strawberries byon-cold multimode inlet injection containing ultra-inert dimpled liner, with endpoint backflush has successfully been completed. Sample extraction was applied using an acidified QuEChERS method followed by on-cold GC-MS/MS method to provide acceptable performance with an accurate quantification of captan and folpet as phthalimide and tetrahydrophthalimide, respectively in apple and strawberries at a reporting limit of 10μg/kg which was lower than EU-MRL. This method showed analytical matrix effect at reporting limit (LOQ=10μg/kg) which was higher in apple (134, and 152%) than strawberries (83, and 115%) for captan and folpet respectively. However, apple and strawberries correlation characteristics at three spiking levels 10, 50, and 100μg/kg of both captan and folpet indicated linear relationship ($R^2>0.99$) which prove the reduction of the matrix effect. Therefore, this method can be used for routine analysis as the proposed method due its strong, accuracy and sensitivity which is achieved the regulatory needs.

5. Abbreviations

GC-MS/MS: Gas chromatography equipped with a tandem mass spectrometer, d-MRM: Dynamic multi-reaction monitoring, PI: Phthalimide, (THPI): Tetrahydrophthalimide, EU-SRM: European single residue method, LOQ: Limit of quantification, RSD: relative standard deviation, MRL: Maximum residue limit, NFSA: National Food Safety Authority, QC: quality control, MU: Measurement uncertainty, Egyptian Agricultural Pesticides Committee (APC).

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