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Assessment of Residuality of Hymexazol in Strawberry (*Fragaria* × *ananassa*) Crop by a Modified QuEChERS Method and Liquid Chromatography Tandem-Mass Spectrometry

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Abstract: Hymexazol (HYM) is an active ingredient commonly used in a wide variety of crops; however, to date, there are no publications on its dissipation and residuality in strawberry fruits and leaves. The objective of the research was to evaluate the dissipation and residuality of hymexazol in strawberry using a modified QuEChERS method with UHPLC-ESI/MS-MS. For this, several validation experiments using the chromatographic method were conducted. The strawberry crop was established in the field, and the content of the HYM was monitored through several applications. The method showed good linearity (correlation coefficients > 0.9995), accuracy (recoveries in 73.7–109.4%), and sensitivity (limits of quantification 0.017 mg kg^{-1}). Despite the two and four drench-treatments of HYM in the strawberry crop, the compound was not detected at levels above the LOD 24 and 48 h after the last treatment. This is due to various plants metabolizing hymexazol to glucose conjugates of its tautomers, i.e., its *O*-glucoside and *N*-glucoside, probably with low or null movement to the aerial parts and fruits of the crop.

Keywords: food safety; residues; LC/MS; hymexazol; dissipation; strawberry

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1. Introduction

Strawberry (*Fragaria* × *ananassa*) is one of the most appreciated fruit crops worldwide, its sensory and nutritional qualities are valued, and it is known to provide a large number of nutraceutical antioxidants compounds such as vitamins, minerals, and phytochemicals (mainly phenolic acids and flavonoids) [1,2]. Strawberry production is led by China (3,336,690 tons), followed by the United States (1,055,963 tons), Egypt (597,029 tons), and Mexico (557,514 tons) (estimated, imputed, and official values for 2020) [3].

It is estimated that strawberry production will continue to increase, owing to the great diversity of industrialized products on the market, as well as to the demand for functional foods [4–6]. However, during agricultural production, the crop becomes vulnerable to phytopathogenic microorganisms, mainly fungi, which are responsible for the main diseases that have an economic impact on the strawberry crop. The fungi can infect all parts of the plant, causing severe damage, even death, and considerably reducing its production. Some of the main phytopathogenic agents are *Colletotrichum fragariae*, *Sphaerotheca aphanis*, *Botrytis cinerea*, *Fusarium oxysporum*, *Phytophthora* spp., *Verticillium* spp., *Oidium fragariae*, and *Mycosphaerella fragariae*, causing anthracnose, powdery mildew, gray mold, leaf scorch, leaf spot, leaf blight, black spot, and crown rot, among other diseases [4,7–9].

The moderate and safe use of agrochemicals is vital in modern agriculture. These products maintain production and food sovereignty because they minimize pre-harvest and post-harvest losses. However, the control, supervision, monitoring, and quantification of these residues are important, because strawberries are usually consumed fresh or minimally processed; thus, they are potentially present and damaging to health (teratogenic, mutagenic, and carcinogenic). Crop protection regulatory agencies such as the

Food and Agriculture Organization of the United Nations (FAO) and the European Union (EU) monitor and stipulate maximum residue limits (MRLs) as well as acceptable daily intakes [7,8,10]. During strawberry production, it is necessary to apply repeated doses of agrochemicals; therefore, their accumulation can be increased. The dissipation (half-life) of active compounds refers to the time in which the product is decomposed to half the initial concentration administered to the plant. This phenomenon involves factors such as temperature, humidity, sunlight, ozone, type of pesticide, and crop, among others [11]. Compound degradation refers to the biotransformation of pesticides to their metabolites and/or accumulation products, which are biocompatible with the environment. It is used to measure the presence in fruits, soils and other matrices.

Previous research has found a large number of agrochemicals used in strawberry cultivation, highlighting their half-life and residuality. For example, kresoxim-methyl, tetraconazole, pyridaben, and procymidone have a half-life of up to 14 days [12]. The dissipation patterns of acephate, etofenprox, imidacloprid, indoxacarb, alpha-cypermethrin, zeta-cypermethrin, fludioxonil, and oxytetracycline, used in Brussels sprouts, indicate a residuality of between 8.2 and 15.8 days [13]. Dissipation patterns of metaflumizone and acrinathrin, applied to *Aster scaber* herb, indicate a half-lives of 4.5 and 9.2 days, changing according to the geographical site where it is cultivated [14].

Hymexazol (3-hydroxy-5-methylisoxazole) (Figure 1) was developed by the Sankyo Co., Ltd., Tokyo, Japan, in the 1960s. It is an excellent agricultural active ingredient widely used in an extensive variety of crops. It stands out for its efficacy, low toxicity, and broad antifungal spectrum, mainly against *Pythium*, *Phytophthora*, *Aphanomyces* sp., *Rhizoctonia*, *Fusarium*, *Mortierella* sp., and *Rhizoctonia solani*, among others. Applications can be on the seeds and aerial parts or by drenching [15]. HYM inhibits spore germination by interfering with fungal DNA and RNA biosynthesis. Once applied, it is absorbed by the roots and metabolized into two glycosides (2-(β-D-glucopyranosyl)-5-methyl-4-isoxazoline-3-one and 3-(β-D-glucopyranosyl)-5-methylisoxazole), the first is glycosylated at the *N* terminal and the second at the *O*, where both confer unique characteristics to the product [16]. Recent research has designed HYM-loaded supramolecular hydrogels, improving their release, absorption, and biodegradability [17]; likewise, the adsorption, release, and thermodynamic characteristics of HYM loaded in diatomite, an efficient vehicle for the release of this product, have been investigated [18]. An optimized synthesis process of HYM has been designed, allowing large quantities of this product to be obtained in less time [19]. Few studies have investigated the residuality of HYM in fruits and vegetables [20,21]. Chromatographic methods (LC) and preparation with a QuEChERS method (Quick, Easy, Cheap, Effective, Rugged, Safe) are the most widely used. They are accepted internationally and have the largest number of publications, so they are optimal tools for this use [22–25].

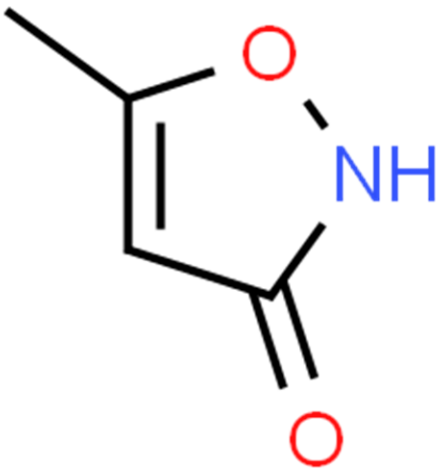

Figure 1. Chemical structure of Hymexazol.

The patterns of dissipation and residuality of agrochemicals in some crops have been extensively investigated; however, to the best of our knowledge, there are no studies on the dissipation and residuality of HYM in strawberries, HYM being one of the most successful agricultural active ingredients on the market, and strawberries a highly commercialized fruit worldwide. Therefore, the objective of the research was to evaluate the residuality and dissipation of HYM at different applied doses and sampling times in strawberry cultivation, using a modified QuEChERS and UHPLC-MS-MS method.

2. Materials and Methods

2.1. Reagents and Chemicals

MS grade acetonitrile (ACN), MS grade methanol (MeOH), Milli-Q water, HPLC grade ACN, ACS grade acetic acid, ACS grade formic acid, ACS grade ammonium formate, reagent grade magnesium sulfate, ACS grade ammonium acetate, and sodium chloride, Supelclean Envi-carb (graphitized carbon); Bondesil PSA 40 UM; 0.22 µm nylon membrane filter; hymexazol, lot SLBR6983V, purity 90%were purchased from Sigma Aldrich-Merck (St Louis, MO, USA).

2.2. Field Experiments

2.2.1. Crop Establishment

Strawberry cultivation was established in Bachigualato, Sinaloa, Mexico (24°46′58″ North and 107°27′30″ West. Altitude: 10 m asl) in direct planting on soil with a sandy loam texture, in beds 32 m long. The plantation was in a double row with a separation of 0.25 m between plants and 1.4 m between beds.

2.2.2. Treatments and Applications

Four HYM at 30% v/v treatments were applied at doses of 0 (control), 0.5, 1.0, and 2.0 mL/L of water with two replications per treatment. Each replication consisted of 80 plants (N = 160 plants per treatment). In each treatment, 20 mL per plant was applied via a drench, penetrating the injection rod 10 cm to the roots. Two applications of the treatments were made before leaf sampling and two other applications later before fruit sampling. The dates of application and sampling of leaves and fruits for residue analysis are shown in Table 1.

Table 1. Application of HYM and sampling of leaves and fruit of strawberries.

Event	Days after Transplant	Activity
Transplant	0	-
First application	35	Application in drench
Second application	48	Application in drench
	49	First leaf sampling
	50	Second leaf sampling
Third application	62	Application in drench
Fourth application	69	Application in drench
	70	First fruit sampling
	71	Second fruit sampling

2.2.3. Leaf and Fruit Sampling

From each replicate per treatment, 100 g of tender leaves (new leaves) were randomly taken at 24 and 48 h after the second application, corresponding to 49 and 50 days after transplantation. For sampling fruits from each replicate per treatment, 500 g of ripe fruit of uniform size and free of defects were randomly taken at 24 and 48 h after the fourth application, corresponding to 70 and 71 days after transplantation. In both samplings, each of the samples was transported in coolers at 10 °C and frozen at −15 °C until analysis.

2.3. Sample Pretreatment

A QuEChERS method was developed by Anastassiades et al. [26] with slight modifications suggested by Jiang et al. [20]. Representative 10 g portions of the thawed and re-homogenized samples were weighed into 50 mL plastic centrifuge tubes. Subsequently, 10 mL of ACN was added and sonicated for 10 min in an ultrasonic bath. After stirring, 4.0 g of $MgSO_4$ and 1.0 g of NaCl were added and stirred vigorously for 2 min, then centrifuged for 5 min at 5000 rpm. A total of 2.0 mL of the extract was transferred to a centrifuge tube containing 150 mg of $MgSO_4$ and 40 mg of graphitized carbon. The tube was vigorously shaken for 1 min and centrifuged for 5 min at 6000 rpm. The resulting supernatant was filtered through a 0.22 µm nylon membrane into a 1.5 mL vial for LC-MS/MS analysis.

2.4. Instrumental Parameters

Chromatographic analyses were conducted in an UHPLC Acquity Class H with Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA). Chromatographic separation was performed on an Acquity UPLC BEH^{TM} Shield RP18 column (1.7 µm, 2.1 × 100 mm). The mobile phase consisted of an aqueous solution of 7.5 mM ammonium acetate + 0.1% formic acid (A) and Acetonitrile (B). The elution gradient was as follows: 90% A (0 min), 80% A (0.7 min), 80% A (3.0 min), 90% A (3.1 min) and 90% A (5.0 min). The flow rate was 0.2 mL/min, and the column temperature was set at 30 °C. The acquisition parameters were collision gas, argon (Ar); nebulizing and drying gas, nitrogen (N_2); source temperature, 150 °C; desolvation temperature, 400 °C; cone gas flow, 200 L/h; desolvation gas flow, 650 L/h; collision gas flow, 0.15 mL/min; and capillary voltage, 3.2 kV. Ions were monitored using MRM (Multiple Reaction Monitoring) for at least two transitions (Figure S1) under the following tandem MS conditions (Table S2).

2.5. Analytical Method Validation

Validation of the method was conducted to establish the precision and recovery for HYM at different levels of fortification in the strawberry samples; the limit of detection (LOD) and limit of quantification (LOQ) were established with the repeatability test of the lowest level of fortification. For the analysis of matrix blanks (strawberry fruit), samples of commercial strawberries were analyzed in triplicate, applying the modified QuEChERS method to demonstrate that it was free of HYM residues and obtain the matrix extract for the preparation of standard dilutions. To determine system linearity, dilutions of the standard were prepared in the mobile phase and in the matrix extract at 5 and 6 concentration levels 0.01534, 0.5116, 0.10231, 0.2557, 0.51156 and 1.02312 mg/L, respectively. To establish the linear range of the system, HYM dilutions with 5 concentration points ranging from 0.02557 to 0.5166 mg/L were prepared in the mobile phase, and a curve with 6 levels ranging from 0.025 to 1.0231 mg/L was prepared in the matrix extract (only strawberry fruit).

The linearity of the method was established by fortifying control samples at five high (500 µL of mix at the concentration 10.2312 mg/L), medium (300 µL of mix at the concentration 10.2312 mg/L), and low (25 µL of mix at the concentration 10.2312 mg/L) concentration levels and plotting the average recovery amounts against the amounts of analyte added. In both cases, the acceptance criterion was $R^2 > 0.98$. To determine repeatability, control samples were fortified at five concentration levels. The recovery percentage (% R) and the precision under repeatability conditions were calculated. It was evaluated as a percentage of the coefficient of variation (% CV). The acceptance criterion for this test was % CV $\leq$ 20. To determine the accuracy (% R), the % R data obtained in the repetition of each level were taken and the average of the recoveries of the five levels was obtained. The acceptance criterion for this parameter was 70% < % R < 130%. To determine reproducibility, recovery percentage data were obtained from two repeatability tests on different days with 5 triplicate levels of HYM in a strawberry matrix; both tests met the criterion of 70% < % R < 130%. To calculate the detection and quantification limits, the repeatability data of the first fortification level were used, calculating the recovered

concentration and obtaining the standard deviation (SD) that was used to calculate the detection limits (LOD) and quantification (LOQ) of the method [27].

3. Results and Discussion

3.1. Method Validation

According to the verification protocol and validation guides CNRPyC/2017 and US-EPA 2000 [28,29], the methods were validated. Viñas et al. [30] found that using C_{18} columns is adequate for HYM analysis, in agreement with our research; however, Jiang et al. [20] and Kiljanek et al. [31] determined that retention is poor, so it is recommended to evaluate other types of columns, owing to the diverse characteristics of the brands of chromatographic columns. Different studies [7,10,32] have reported the usefulness and efficacy of LC/MS-MS to determine a wide variety of pesticides in strawberry cultivation, in agreement with what is reported below. In previous experiments (data no shown) we evaluated three columns Acquity C8 UHPLC 1.7 µm 2.1 × 100 mm, Acquity phenyl UHPLC 1.7 1.7 µm 2.1 × 100 mm, and Acquity UHPLC Shield C_{18} RP_{18}, 1.7 µm, 2.1 × 100 mm column. The latter was selected for the recovery and chromatographic conditions shown.

3.1.1. Linearity

Linearity was evaluated with five (solvent) and six (matrix effect) calibration points (0.025 to 1.0231 mg/L). The correlation coefficient (R^2) was higher than 0.98, and Figures S2 and S3. The mean recovery data and percentage values of the coefficient of variation (CV) are shown in Table 2. The recoveries for HYM ranged between 72.18% and 108.62%. The accuracy under repeatability conditions was 85.55 ± 6.8%.

Table 2. Linearity, % recovery and LOD/LOQ of the method.

	Linearity			Recovery and Intra-Laboratory Precision (%)			
Analyte	**Lineal Range of Method (mg kg^{-1})**	**Lineal Range of the System (mg/L)**	**Coefficient of Determination (R^2)**	**Recovery (%)**	**CV (%)**	**LOD (mg kg^{-1})**	**LOQ (mg kg^{-1})**
HYM	0.025–0.5	0.025–1.0231	0.9995	82.55 ± 6.8	8. 24	0.017	0.051

To determine the lineal range of the method 5 levels with three replicates were used.

3.1.2. Linear Range of Work

This parameter was evaluated by fortifying control samples at five high, medium, and low concentration levels, the second level being the LOQ, and in the case of the highest concentration level, it was the maximum residue limit (MRL) authorized in Japan. Each level met the precision under established repeatability and trueness conditions. For the experiment, the concentration range of 0.025 to 0.5 mg kg^{-1} was established, plotting the average of the recovery concentrations against the theoretical concentration added, obtaining the correlation coefficient (r) and determination (R^2) greater than 0.98, as observed in Figure 2.

3.1.3. Precision under Repeatability Conditions and Intermediate Precision

Precision was calculated as relative standard deviation or % CV at the low concentration level. As shown in Table S4 the precision criteria were met under repeatability conditions. Table S3 shows the evaluation of the recovery values of each experiment, where there is no effect on the results obtained at different times.

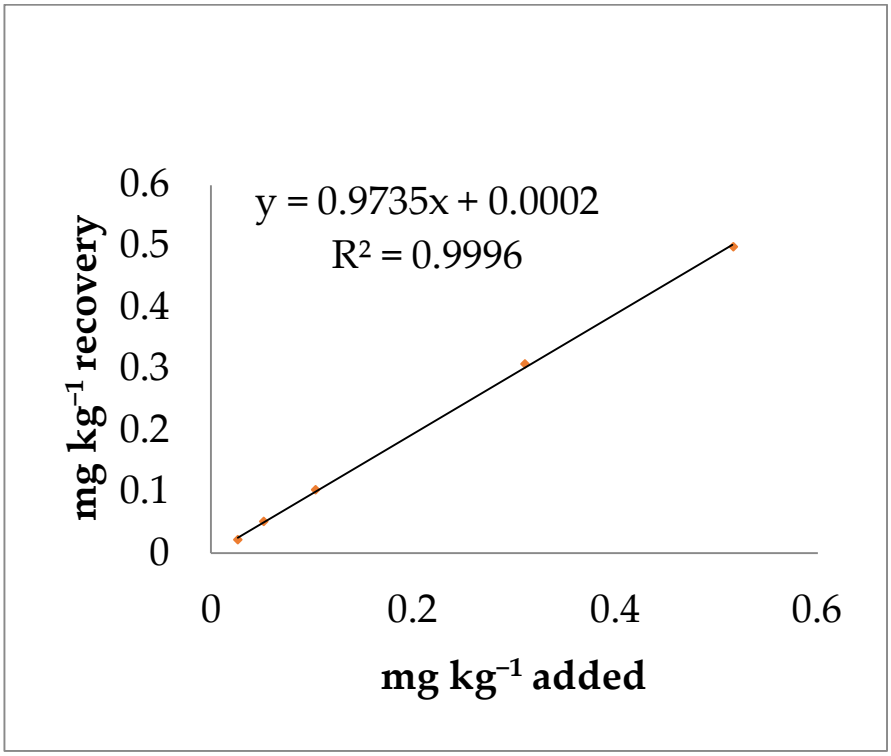

Figure 2. Linearity of the method (average of 3 repetitions of each level).

3.1.4. Accuracy (% R)

To evaluate this parameter, the % R data obtained in the two repeatability tests were taken, and the average of the recoveries of the five levels of each test was obtained. The accuracy of the method was 98%. The results of the accuracy evaluation are shown in Table S4.

3.1.5. Limit of Detection (LOD) and Limit of Quantification (LOQ)

This parameter was calculated by analyzing six blank matrices (fresh fruit) fortified at 0.0257 mg kg^{-1}, where the standard deviation (SD) obtained was used to calculate the LOD and LOQ using Equations (1) and (2) for t 0.99 = 3365 (See Table S5); one-tailed Student's t-table value with five degrees of freedom (n = 6) and 99% confidence level, the established LOQ was 0.05 mg kg^{-1} (lower than the MRL of 0.5 mg kg^{-1} for strawberries established in Japan). The quantification limit of HYM reported in the present study was 0.051 mg kg^{-1}; however, other studies have reported an LOQ of 0.01–0.02 mg kg^{-1} [15], and other studies and regulations higher LOQs [16,29,30].

3.2. Dissipation and Residuality of Hymexazol in Leaves and Fruits of Strawberry

After 24 and 48 h of the second HYM application, the residue content was quantified; however, it was not detected (<0.017 mg kg^{-1}). After 24 and 48 h of the fourth application of HYM, its content in the fruits was evaluated and not detected in the same way. Figures 3 and 4 show the chromatographic runs of the leaf samples at 24 and 48 h after applying HYM at different doses, contrasting these with the run of the HYM standard, demonstrating its non-detection.

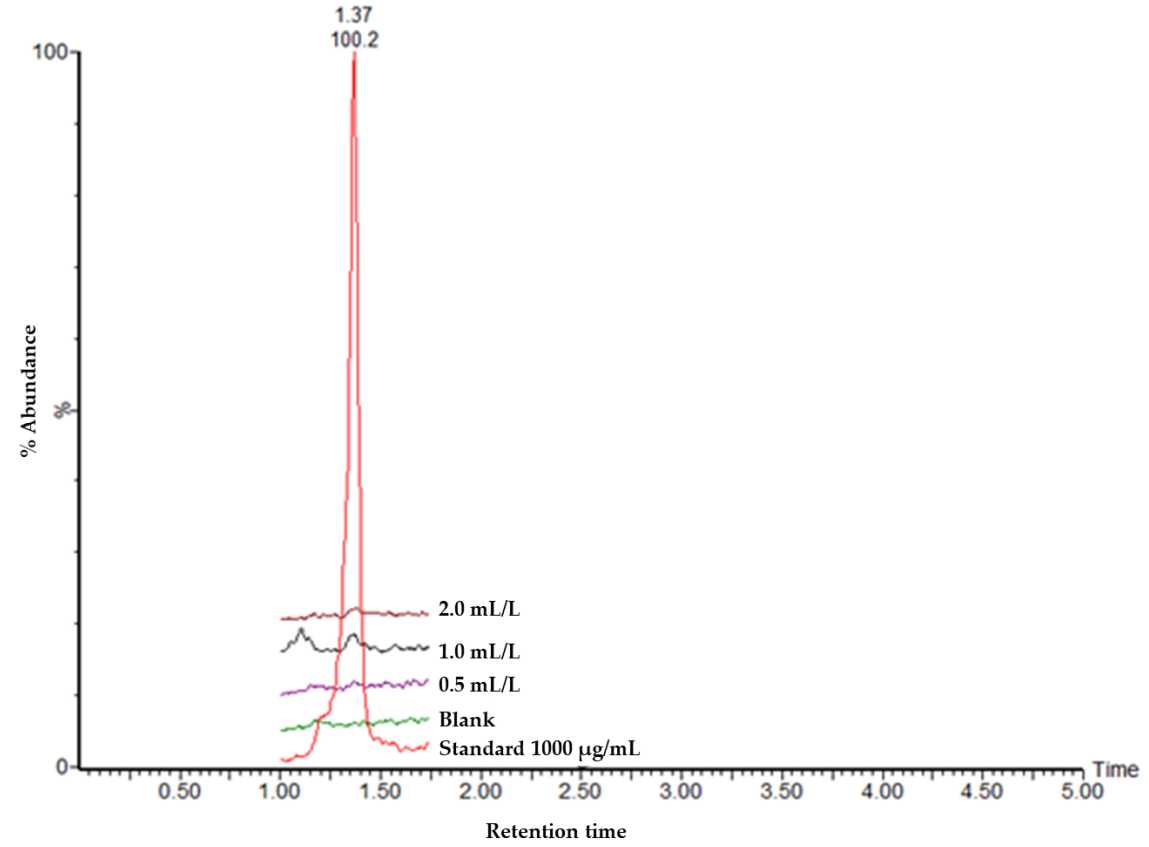

Figure 3. Chromatograms of leaf samples 24 h after two applications of HYM.

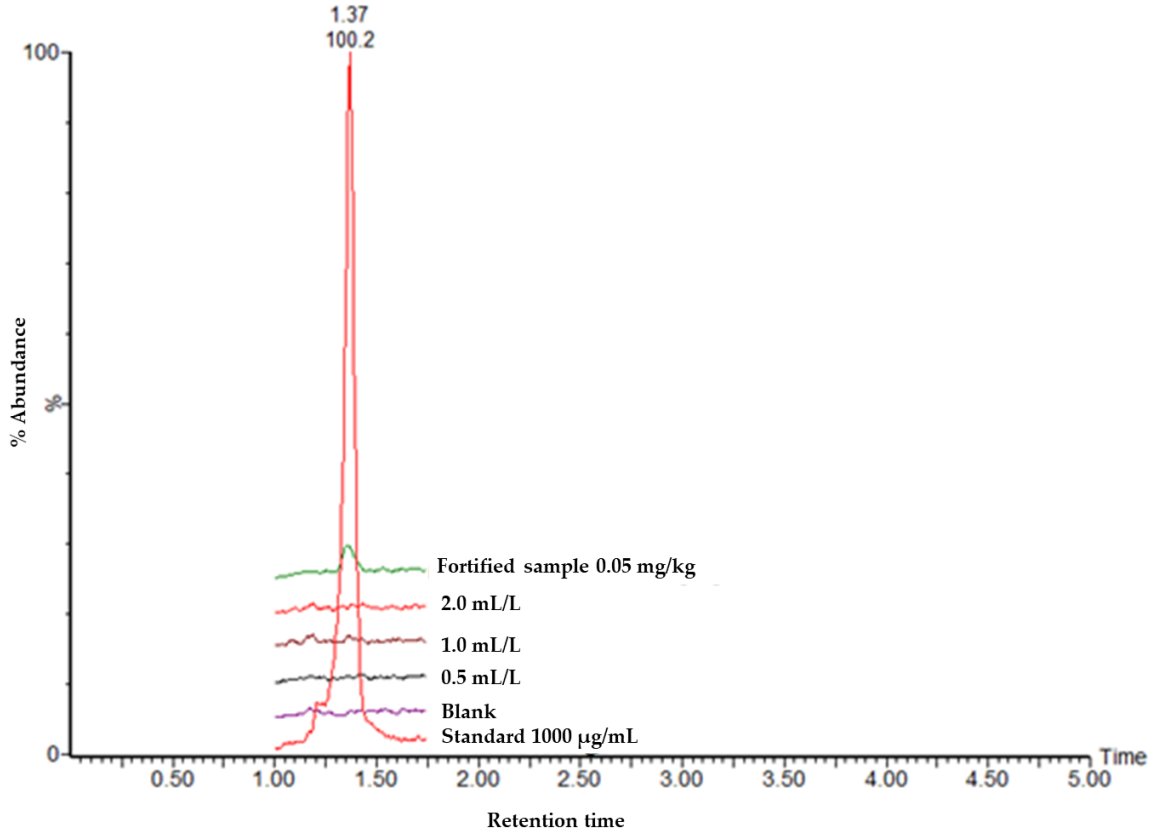

Figure 4. Chromatograms of leaf samples 48 h after two applications of HYM.

Figures 5 and 6 show the chromatographic runs of the fruit samples at 24 and 48 h after applying HYM at different doses, contrasted with the run of the HYM standard, demonstrating its non-detection.

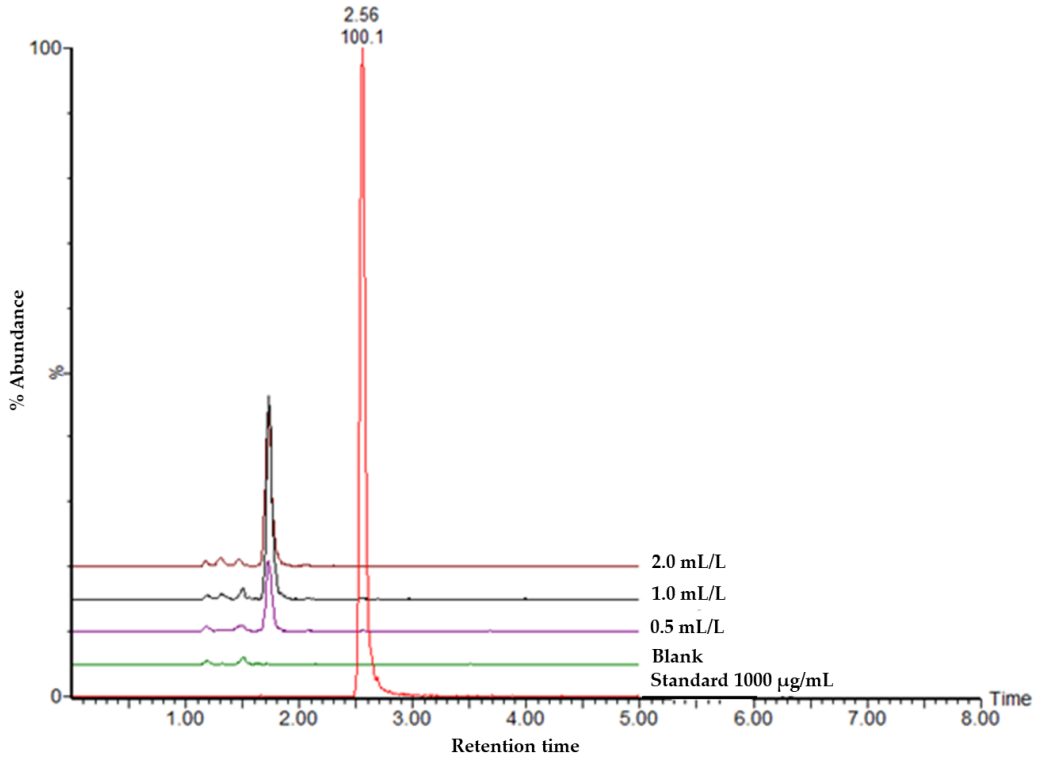

Figure 5. Chromatograms of fruit samples 24 h after four applications of HYM.

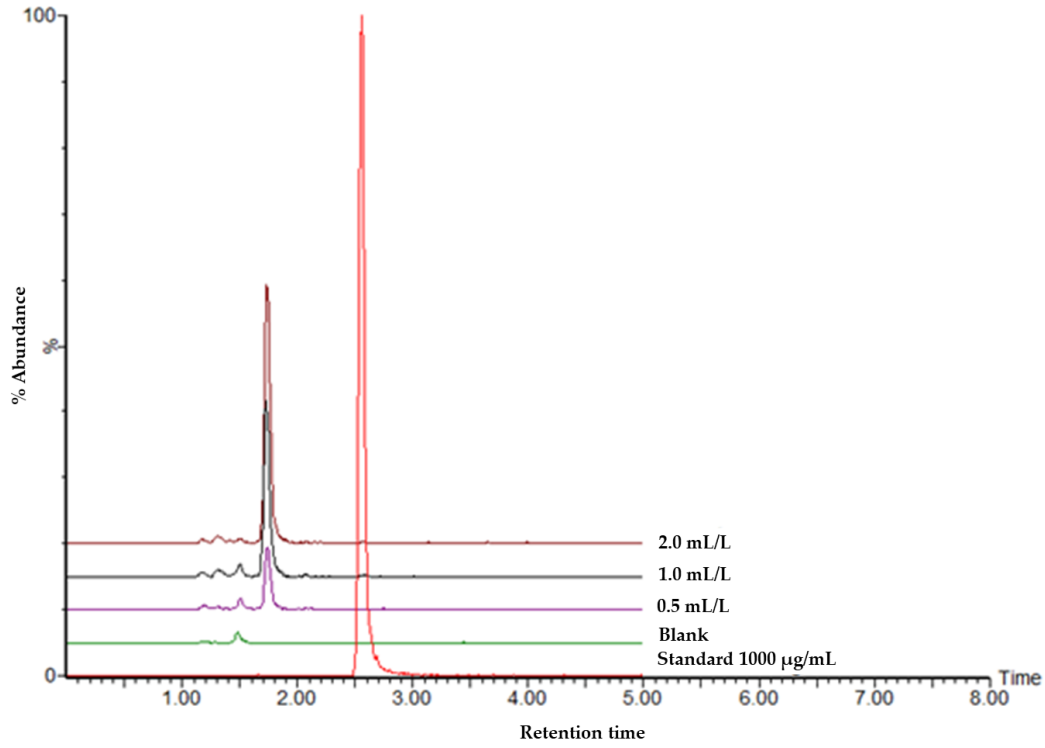

Figure 6. Chromatograms of fruit samples 48 h after four applications of HYM.

Other studies have investigated the presence of HYM in vegetables and fruits; for example, Sun et al. [21] evaluated sprayed applications of HYM on cucumber plants through GC-FPD, reporting contents of less than 0.5 mg kg^{-1} on days 7, 14, and 21 after its application. In contrast to our results, the authors applied the HYM in aerial parts of the cucumber so the product remained via contact; in our study, the HYM was applied via drench, so the only way to find it in the leaves and fruits was by HYM translocation. Jian et al. [20] evaluated 104 fruits (apples, grapes, oranges, peaches, pears, and watermelons, among others), of which only cucumber showed traces of HYM with 0.0085 mg kg^{-1}. These studies agreed with our results, reasserting the hypothesis that HYM has a low rate of translocation to fruits and aerial parts. Agreeing with our results, Viñas et al. [30,33] reported no HYM concentrations in several fruit samples and processed products (juices, wines, malt beverages), and the authors validated several experiments based on solid-phase microextraction and the GC/MS method. Only in a spiked canned pineapple was HYM found above their detection limits (0.042 mg/L) [34]. Agreeing with our results, in a technical study conducted in Culiacan, Sinaloa [35], applications through drip irrigation to tomato plants (*Solanum lycopersicum*) in doses of up to 2.0 L/ha of HYM did not show residuality in fruit samplings 0, 3, and 7 days after harvest. In contrast to our results, Martínez-Domínguez et al. [36] evaluated samples of nutraceutical products made with leaf extracts of ginkgo biloba, finding 0.01 mg kg^{-1} HYM only in one sample.

Other studies of the dissipation of commercial formulations used in strawberry crops in China have found that the half-life ranged between 3.8 and 15.8 days, with procymidone having the longest half-life, followed by fluxapyroxad and tetraconazole [12]. As HYM has no residuality after 24 and 48 h of application, this may be an advantage over others products. The dissipation of pesticides and agrochemicals is a relevant indicator when evaluating their behavior in the environment and possible residuality in foods. In the present study, four applications of HYM were evaluated, but no residue was found in any sampling; this may be mainly due to the glycosylation and minimal translocation of HYM and external factors such as climate, type of crop, microorganisms present in the soil, exposure to ozone, diffusion processes, and solar radiation [12,37–39].

The first unique studies of the translocation and metabolism of HYM determined that when HYM was applied to the roots of tomato, cucumber, and rice, it quickly translocated to the leaves (24 h), and more than 60% of it metabolized in two conjugated glucose products from their tautomer [40,41]. Kamimura et al. [41] found that when HYM is applied to the roots, they quickly absorb it and translocate it to the stems within approximately 3 h and to the foliage after 24 h, and if the HYM is applied to the leaves, it moves to the transpiration stream [41]. We hypothesized that in a drenching application, the strawberry roots absorb and possibly translocate it to other plant organs, but in their tautomers. For this reason, it was not possible to quantify HYM in the leaves and strawberry fruits. Another hypothesis was that the soil used for the experiment contained a large number of microorganisms and other fertilizers that quickly metabolized the HYM to the tautomers [41]. In these cases, it is necessary to investigate the translocation and metabolism of HYM and their metabolites in strawberry leaves and fruits.

4. Conclusions

According to the CNRPyC/2017 and EPA/US Validation Guides, the results of the performance verification of the modified QuEChERS procedure were satisfactory for the parameters of linearity, precision under repeatability conditions, intermediate precision, accuracy (% recovery), and limits of detection and quantification. Recoveries ranged from 73.78% to 109.49% with a CV of 8.24%. For this reason, the present study concludes that it is a fast, simple, sensitive, and reliable procedure for the determination of HYM residues in products of vegetable origin using the LC-MS/MS system. No residues (<0.017 mg/L) of HYM were detected on strawberry leaves after two applications of three doses. No residues (<0.017 mg/L) of HYM were detected in ripe strawberry fruits after four applications of three doses. The degradation of the HYM in the strawberry crop was clarified, standing out

for its rapid dissipation in the fruits and in the leaves. This indicates its low translocation to other plant tissues and organs, so it can potentially be used to treat specific root diseases. In future research, it is recommended to study the glycosylated metabolites of HYM to have a broader framework on the dissipation of this product and to be able to elucidate other aspects related to the safety of the product.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12123110/s1. Figure S1. Ion transitions. (A) Precursor (B,C) Products of hymexazol. A concentration of 1.0231 mg/L was injected. Figure S2. System linearity of matrix effect of strawberry fruit. Figure S3. System Linearity. Table S1. Conditions of MS/MS. Table S2. Evaluation of the Chi square statistic for repeatability. Table S3. Analysis of variance for one factor with random effects. Table S4. Method accuracy. Table S5. Equations for calculate the LOD and LOQ.

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Conflicts of Interest: The authors declare no conflict of interest.

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