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Quantitative analysis of piperidin-1-ium {[5-(2-furyl)-4-phenyl-4*H*-1,2,4-triazol-3-yl]thio}acetate, substance of the veterinary drug «Tryfuzol», in poultry meat by LC–DAD–MS

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Key words: *Tryfuzol, Triazoles, Poultry, Chromatography, High Pressure Liquid, ESI Mass Spectrometry.*

Aim. Development of sensitive, acurate, reproducible HPLC-DMD-MS method for determination of the residual amounts of active drug substance «Tryfuzol» in poultry homogenate samples and application of this method for testing chiken groups is an important task for the confirmation of this drug safety.

Methods and results. The substance extraction conditions were optimized for methods development. It was established optimum extraction condition. Chromatographic conditions and mass spectrometric studies were optimized. As mobile phase, a flow rate of 0.4 ml min^{-1} isocratic elution 0.1% (v/v) formic acid in water (A) acetonitrile containing 0.1% (v/v) formic acid (B). The composition of the mobile phase of 70% / 30% (v/v). Total running time is 5 minutes. Wavelength of the detector is 276 nm. Control of substances a**vailability was carried by retention time and under UV and mass spectra.**

Conclusion. The technique complies with relevant regulations. The absence of said substance in the study chiken group was proven by using developed techniques.

Кількісне визначення піперидиній {[5-(2-фурил)-4-феніл-4Н-1,2,4-триазол-3-іл] тіо}ацетату, речовини ветеринарного препарату «Трифузол», в м'ясі птиці за допомогою ВЕРХ-ДМД-МС

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Розробка чутливої, правильної, відтворюваної ВЕРХ-ДМД-МС методики визначення залишкових кількостей активної субстанції препарату «Tryfuzol» у зразках гомогенату м'яса птиці та застосовування цього методу для випробувальних груп курей є важливим завданням для підтвердження безпеки використання вказаного препарату. З метою розробки методики оптимізовано умови екстракції речовини. Встановлено оптимальні умови екстракції. Оптимізовано умови хроматографічних і мас-спектрометричних досліджень. Як рухому фазу зі швидкістю потоку 0,4 мл $хв^{-1}$ з ізократним елююванням використали 0,1% (об/об) форміатної кислоти у воді (А), ацетонітрил, що містить 0,1% (об/об) форміатної кислоти (В). Склад рухомий фази 70% А/30% В (об/об). Загальний час роботи становить 5 хв. Довжина хвилі детектора – 276 нм. Контроль наявності субстанції здійснили за часом утримування, а також на підставі УФ і мас-спектрів. Розроблена методика відповідає вимогам відповідних нормативних документів. Також за допомогою цієї методики доведено відсутність указаної субстанції у групі курей, що досліджували.

Ключові слова*: трифузол, тріазоли, птиця, хроматографія, рідинна високого тиску, ESI мас-спектрометрія.*

Актуальні питання фармацевтичної і медичної науки та практики. – 2015. – № 2 (18). – С. 25–31

Количественное определение пиперидиний {[5-(2-фурил)-4-фенил-4Н-1,2,4-триазол-3-ил]тио}ацетата, субстанции ветеринарного препарата «Трифузол», в мясе птицы с помощью ВЭЖХ-ДМД-МС

Б. А. Варинский, Е. Г. Кныш, В. В. Парченко, А. И. Панасенко, А. Г. Каплаушенко

Разработка чувствительной, правильной, воспроизводимой ВЕРХ-ДМД-МС методики определения остаточных количеств активной субстанции препарата «Tryfuzol» в образцах гомогената куриного мяса и применения этого метода для испытуемых групп кур является важной задачей для подтверждения безопасности использования указанного препарата. С целью разработки методики оптимизированы условия экстракции вещества. Установлены оптимальные условия экстракции. Оптимизированы условия хроматографических и масс-спектрометрических исследований. В качестве подвижной фазы со скоростью потока 0,4 мл $мин^{-1}$ с изократическим элюированием использовалось 0,1% (об/об) формиатной кислоты в воде (А), ацетонитрил, содержащий 0,1% (об/об) формиатной кислоты (В). Состав подвижной фазы 70% А/ 30% В (об/об). Общее время хроматографирования составляет 5 мин. Длина волны детектора – 276 нм. Контроль наличия субстанции проводили по времени удерживания, а также на основании УФ и масс-спектров. Разработанная методика соответствует требованиям соответствующих нормативных документов. Также с помощью данной методики доказано отсутствие указанной субстанции в исследуемой группе кур.

Ключевые слова*: трифузол, триазолы, птица, хроматография, жидкостная высокого давления, ESI масс-спектрометрия.*

Актуальные вопросы фармацевтической и медицинской науки и практики. – 2015. – № 2 (18). – С. 25–31

Derivatives of 1,2,4-triazoles are potential medicinal substances with diverse biological activity. One of the newest and highly effective medicines from this chemical group is tryfuzol which along with immunostimulatory, hepatoprotective and anti-inflammatory actions exhibit antioxidant activity. It has antibacterial and antifungal properties [1]. The number of papers devoted to the HPLC determination of drugs in meat [2–6].

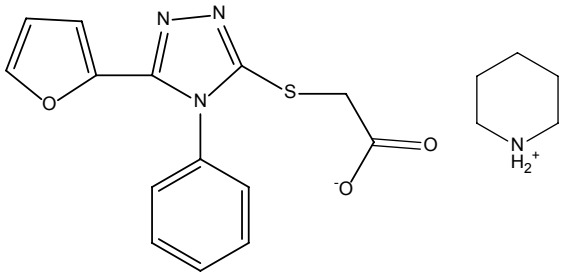
The aim of our study was to elaborate sensitive, accurate, reproducible HPLC-DMD-MS method of determination of active substance «Tryfuzol» residuals in the poultry meat homogenate samples and apply this method for the test groups of chiken.

Experimental

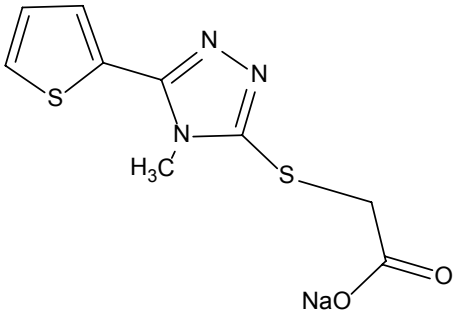
Chemicals and Reagents

The substances of piperidin-1-ium-{[5-(2-furyl)-4-phenyl-

4*h*-1,2,4-triazol-3-yl]thio}acetate (Tryfuzole) and {[4-methyl-5-(2-thienyl)-4H-1,2,4-triazol-3-yl]thio}acetic acid (internal standard, IS) were synthesized at toxicological and inorganic chemistry department (Head of the Department Doctor Pharm Sci, professor Panasenko O.I.) Zaporizhzhia State Medical University. The molecular structures of the analyte and the IS are shown in *fig. 1*.



m/z=302.1
Tryfuzole (1)



m/z=256.0
IS(2)

Fig. 1. Molecular structures and m/z for pseudomolecular ions of drug substance «Tryfuzole» (1) and sodium{[4-methyl-5-(2-thienyl)-4H-1,2,4-triazol-3-yl]thio}acetate (internal standard) (2).

Highly purified water (18 MΩ at 25°C) was made using a water purification system Direct Q 3UV Millipore (Molsheim, France). LC grade reagents (acetonitrile) were obtained from Lab-Scan (Gliwice, Poland), formiate acid (100%), Merck KGaA (Darmstadt, Germany).

Instruments

The device is LC MS: Agilent 1260 Infinity HPLC System (degasser, binary pump autosampler, single quadrupole mass spectrometer Agilent 6120 with ionization in electro-spray API-ES (ESI); OpenLAB CDS Software.

Chromatographic Conditions

An Agilent ZORBAX SB-C18 analytical column (30 mm x4.6 mm; 1.8 µm, Agilent Corporation) with guard column was used. The column temperature was set at 40°C and the injection volume was 2 µL. The mobile phase, pumped at a flow rate of 0.4 mL min^{-1} with isocratic elution, consisted of 0.1% (v/v) formiate acid in water (A), acetonitrile containing 0.1% (v/v) of formic acid (B). The mobile phase composition was 70% A/30% B (v/v). The total run time was 5 min. DAD wavelength was 276 nm.

Mass-spectrometric conditions and confirmation of tryfuzole and IS presence

Mass-spectrometer conditions was choosen to obtain maximal response: 1) scan mode for the identification of the peak m/z from 250 to 310; 2) positive polarity; 3) the drying gas rate (nitrogen) – 10 L/min; 4) capillari Voltage 4,000 V; 5)drying gas temperature, fragmentor voltage, nebulaizer preassure are presented at the *Table 1*. Optimization of the ion-source conditions was conducted by flow injection analysis (direct introduction of the sample into the ionization chamber without chromatografic separation) by full factorial design. Statistical analysis of the results was performed on a personal computer employing a Statistica Package v. 8.0 (StatSoft, USA) based on the values of full factorial design and the corresponding peak areas. The polynomial regression equations was determined. The optimal values of factors was found according calculated equations using Solver (Optimization tool for Excel, Frontline Systems, Inc., USA).

Table 1

Optimized mass spectrometric ionization parameters (T – drying gas temperature, U – fragmentor voltage, P – nebulaizer preassure)

Optimal conditions		
T, °C	U, V	P, psi
247	149	46

The ions for the analytes were monitored by (m/z): tryfuzole 302.1, IS 256.0.

Preparation of Solutions of Standard Calibration and Quality Control Samples

Standard solutions of the tryfuzole substance (1 mg mL^{-1}) were prepared by dissolving appropriate amounts of these reference substances in the mixtures of 0.1% HCOOH in CH_3CN and 0.1% HCOOH in H_2O – 30:70, 40:60 and 50:50 respectively.

Standard solutions of the tryfuzol substance for the determination of the recovery at SPE (0.05 mg mL^{-1}) were prepared by mixing of 50 µL of 1 mg mL^{-1} solutions of reference substance with appropriate amounts of the mixtures of 0.1% HCOOH in CH_3CN and 0.1% HCOOH in H_2O – 30:70, 40:60 and 50:50 respectively.

Non-extracted reference tryfuzol substance and IS for determination of the recovery at full extraction (SPE and liquid extraction) (0.07 mg mL^{-1}) were prepared by mixing of 70 µL of 1 mg mL^{-1} solutions of reference substances with appropriate amounts of the mixtures of 0.1% HCOOH in CH_3CN and 0.1% HCOOH in H_2O – 30:70, 40:60 and 50:50 respectively.

Standard calibration solutions of the tryfuzol substance (1.2; 1.6; 2.0; 2.3; 2.5; 6.0; 14.3 mg mL^{-1}) were prepared by the disolvation of the appropriate amount of the tryfuzole substance in the highly purified water.

Series of the tryfuzol calibration solutions were made by addition of 1 ml standard solution to the 30 g of homogenate and mixed. Then they were extracted by the 40 mL mixure of 0.1% HCOOH in CH_3CN and 0,1% HCOOH in H_2O – 40:60 (v/v) according to the procedure (*Fig.2 and Fig. 3*).

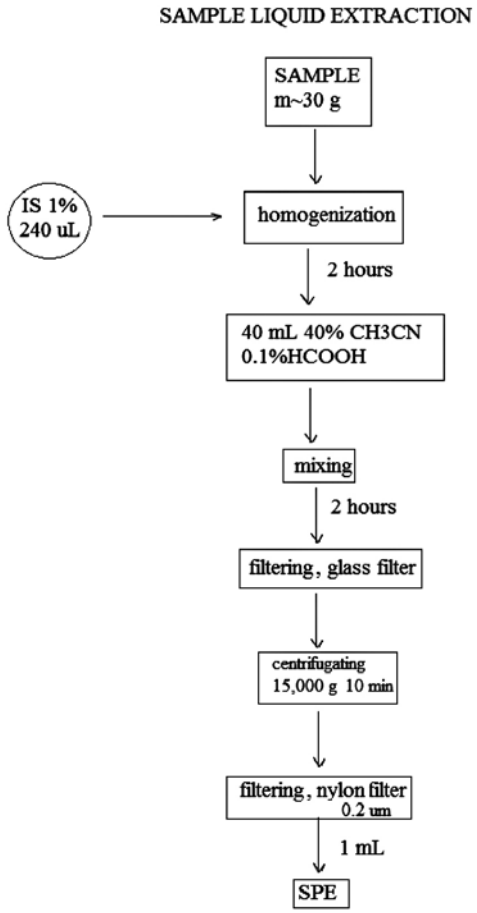


Fig. 2. Procedure of the sample liquid extraction.

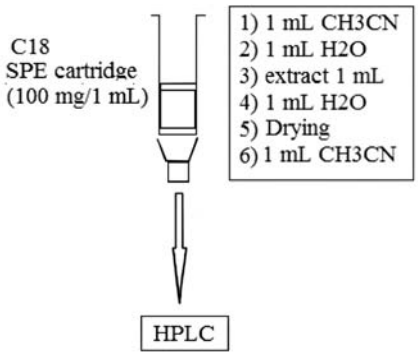


Fig. 3. Procedure of the solid phase extraction.

Quality control solutions (QC) were made by the same way, by the addition of 1 ml standard solution to the 30 g of homogenate and mixed. Then they were extracted by the 40 mL mixure of 0.1% HCOOH in CH_3CN and 0.1% HCOOH in H_2O – 40:60 (v/v) according to the procedure (*Fig.2* and *Fig. 3*). The final tryfuzol concentration were at the lower limit of quantification (LLOQ), within three times of the LLOQ (low QC), around 30–50% of the calibration curve range (medium QC), and at least at 75% of the upper calibration curve range (high QC). The final content of the tryfuzol substance in the standard gomogenate were 40, 70, 195 and 390 μg mL^{-1} for trifuzole.

All standard solutions were stored at 5°C, either for calibration curves of analyte or quality control (QC) in the pre-study validation and during the study.

Sample liquid extraction

30 gram of the meat was homogenized with a blender. 240 μL of the 1% IS was added and was mixed. After 2 hours 40 mL 40% CH_3CN with 0.1% HCOOH was added and was mixed. After 2 hours extract was filtered through a glass filter. It was centrifugated at 15,000 g 10 min and was filtered through nylon syringe filter ID 13 mm, pore size 0.2 μm, (*Fig.2*).

Solid phase Extraction

Procedure of the solid phase extraction was shown at the *Fig. 3*. SPE C18 cartridge (100 mg/1mL) was conditioned by the 1 mL CH_3CN and 1 mL of the extract obtained according to the procedure (*Fig.2*) was added into SPE cartridge

Validation of the Method

The specificity was confirmed by analyzing blank samples to determine the absence of interference with analyte.

Analytical signal LLOQ sample: the analyte signal of the LLOQ sample should be at least 5 times the signal of a blank sample [1,2].

Within-run precision and accuracy of drug «Tryfuzole» substance determination were determined by QC samples analyzing at four different concentrations: at LLOQ, low (within three times the LLOQ), medium (around 30–50% of the calibration curve range) and high (at least at 75% of the upper calibration curve range) concentrations.

Application of the Analytical Method

Determination of residual amounts of drug substance conducted according to procedure (*Fig. 2,3*), comparing with homogenate samples with the addition of the standard solution of the drug «Tryfuzol» substance.

Calculation of concentration

The calibration graph equation was calculated by the method of external standard that should be checked every time during the research conditions.

Results and Discussion

LC–UV and MS Optimization

Earlier authors [1–3] described HPLC conditions of the determination number of derivatives of 1,2,4-thiotriazoles. The acetonitrile was applied as the organic modifier. The authors suggested using acidic pH less than 3.0; while phosphoric acid chosen as acidifier. Low pH decrease ion-exchange mechanism of interaction of nitrogen-content bases with silanole groups and improve shape of the peaks, also increases retention due to interaction of protonated acidic molecules with reverse-phase sorbent [9–11]. We used prompted formiate acid as the more volatile and more convenient to use with mass spectrometer detector.

DAD detection wavelength was choosen according to the maximal adsorption and equial 276 nm. The ultraviolet spectra is showing at the *fig. 4,5*.

ES ionizatision is a soft method, so easy to receive un-fragmented ions. Analytes and IS respond best to positive ionization, so protonated molecular ions $[M+H]^+$ were present as major peaks for compounds. The mass spectra of the protonated molecules by m/z from 250 to 310 are presented in *Fig. 6,7*.

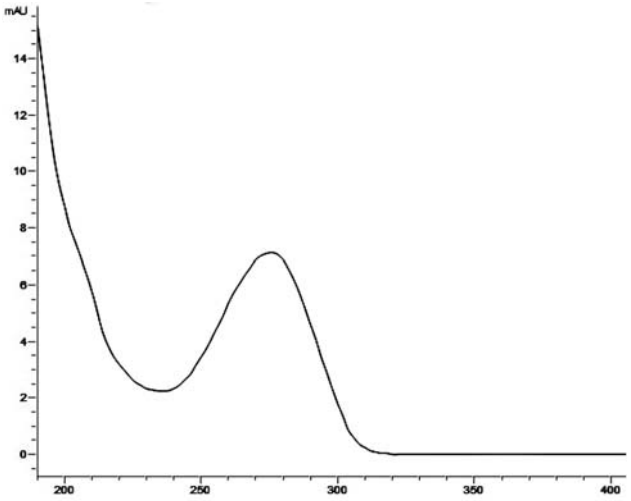


Fig. 4. UV spectrum of the tryfuzole.

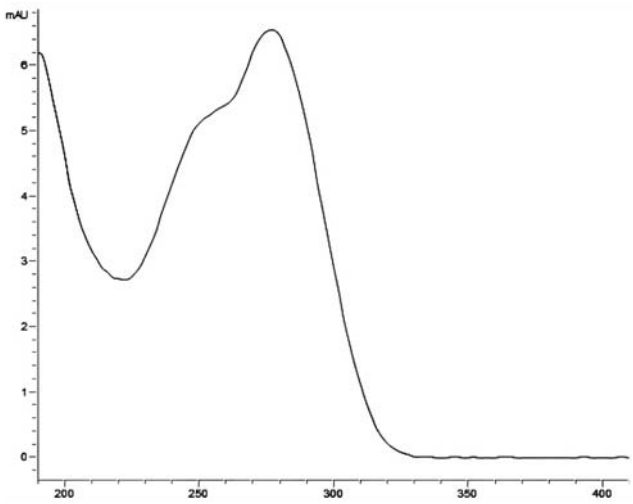


Fig. 5. UV spectrum of the IS.

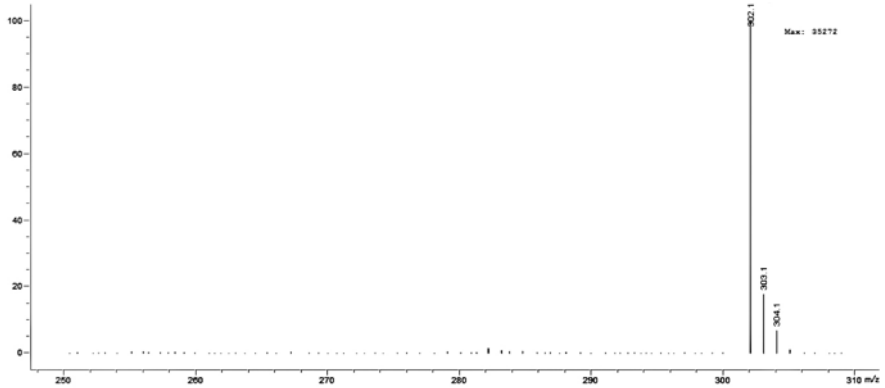


Fig. 6. Mass spectrum of the tryfuzole.

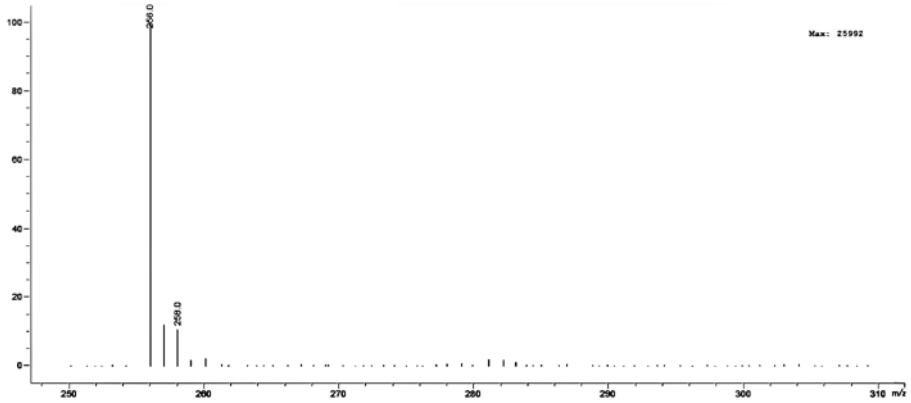


Fig. 7. Mass spectrum of the IS.

The presence of the molecules can be confirmed by the correspondent UV and MS spectra.

The composition of the mobile phase is an important factor that affects the ESI processes. A high content of organic composition in the mobile phase to reduce signal suppression. But high percentage of acetonitrile dramatically decreases retention and also selectivity of determination. We chose 30% CH_3CN. It was enough for signal intensity, selectivity and time of analysis was only 5 min in isocratic conditions.

Solid Phase Extraction development

Due to the high Log P of the tryfuzole acidic form equal 3.48±0.66 (it was calculated with ACD labs 6.0 Software), we decide that tryfuzol molecule should have good retention at non-polar phase, so we chose C18 SPE cartridge for the adequate retention. Maximal LogD exist in acidic medium from pH=2 to pH=3. So we chose 0.1% HCOOH as acidifier (pH~2.7), Log D is equal to 3.34. Also extraction of the tryfuzole from meat we produced by mixture of acetonitrile-water with 0.1% HCOOH. 3 standard solutions (0.05 mg/mL) with different concentration of the acetonitrile: 30%, 40%, 50% with 0.1% HCOOH was prepared. We used water for the washing due to small elution of analyte. Acetonitrile used for the elution of analyte due to strong elution force. Maximal recovery was at 30% CH_3CN (*Table 2*).

Table 2

Recovery dependence from acetonitrile content for SPE study trifuzole

Acetonitrile content	30% CH_3CN	40% CH_3CN	50% CH3CN
Recovery, %	58.8%	10.3%	8.57%

Sample Liquid Extraction development

Homogenized meat (100 g) was spiked by the 1 mL 1% substance of the drug «Tryfuzol», 1 mL 1% IS and mixed. It was devided into 3 samples about 30 g. The sample tryfuzole content ~3 mg. Each sample was extracted by the 45 mL acetonitrile-water mixture with 0.1 % HCOOH. Acetonitrile content was respectively 30%, 40%, 50%. Final concentration of tryfuzole and IS in the extacts was ~ 0.07 mg/mL.

We chose mixture of acetonitrile-water with 0.1% HCOOH as extragent of the tryfuzole from meat for the destruction of binding with proteins. We studied 30%, 40%, 50% acetonitrile with 0.1% HCOOH (*Table 3*). Most recovery was at 40% CH_3CN.

Table 3

Signal detector dependence from acetonitrile content for full extraction cycle (liquid extraction and SPE) tryfuzol and IS

Acetonitrile content	30% CH_3CN	40% CH_3CN	50% CH3CN
Recovery (tryfuzol), %	2.671	5.10	3.43
Recovery (IS), %	8.11	17.89	5.598

Sensitivity. LLOQ determination

LOD is about 18 µg/g of the meat homogenizate (3 times of the blank sample).

LLOQ is 30 µg/g of the meat homogenizate (5 times of the blank sample).

Selectivity and Specificity

Diode-array detection at 276 nm was quite selective. The substance of the drug «Tryfuzol» and IS were chromatographically separated with the retention time of 3.4 and 1.4 respectively. Additionally, the selectivity of the method was provided by the mass spectra substances determination. Total chromatography time was 5 min. Interference with impurities is absent (*Fig. 8*).

Linearity of Calibration Curves and LLOQ

Calibration curve was built on the basis of the depending diode-array detector response at a wavelength of 276 nm on the content of the substance in homogenate, that is performed by an external calibration standard. Calibration graph was linear for 40-475 µg/g substance in the homogenate. The satisfactory linearity was obtained. The corresponding equation was received: y=2,4198x-57,92; R^2=0,997.

Assay Precision and Accuracy

The content of the drug substance «Tryfuzol» in the QC samples were determined using external standard calibration graph equation. Accuracy and precision was determined for the substance quality control solutions (QC). Accuracy and precision of data given in the *Table 4*.

Table 4

Accuracy and precision of the substance determination method of the drug «Tryfuzol» in homogenate (n = 5) by 4 concentration levels

Analyte	The nominal concentration, µg / g	$\bar{X}$	Precision RSD (%)	Accuracy RE (%)
«Tryfuzol»	40	39.51	18.37	13.82
	70	70.65	14.10	9.906
	195	201.516	11.23	13.61
	390	391.392	10.09	9.562

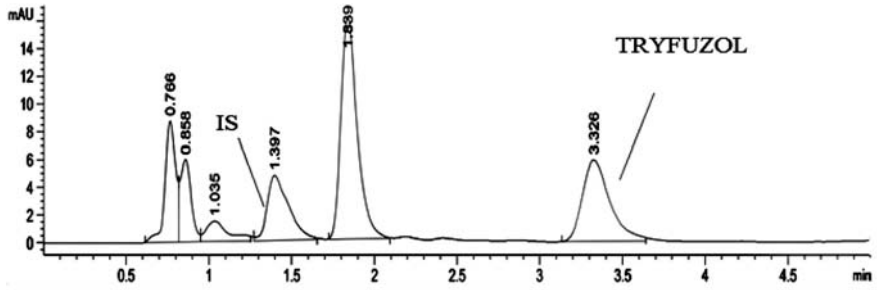


Fig. 8. LLOQ chromatograms of chiken meat spiked with two analytes and IS.

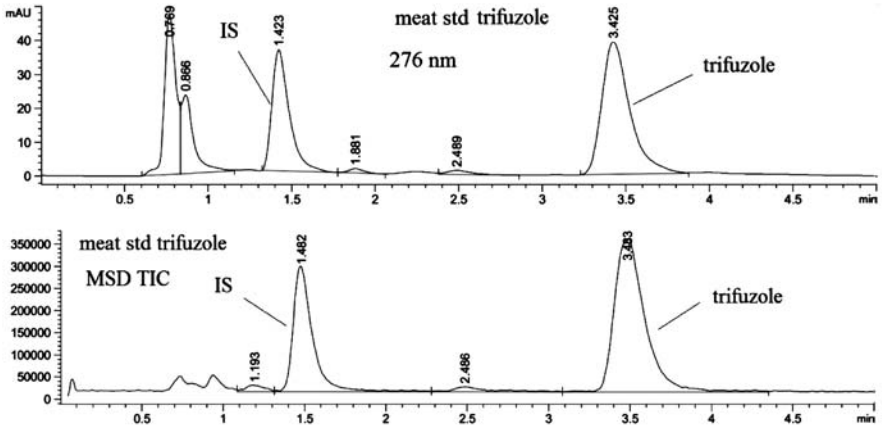


Fig. 9. Chromatograms of extracted meat standard on UV 276 nm and MSD TIC.

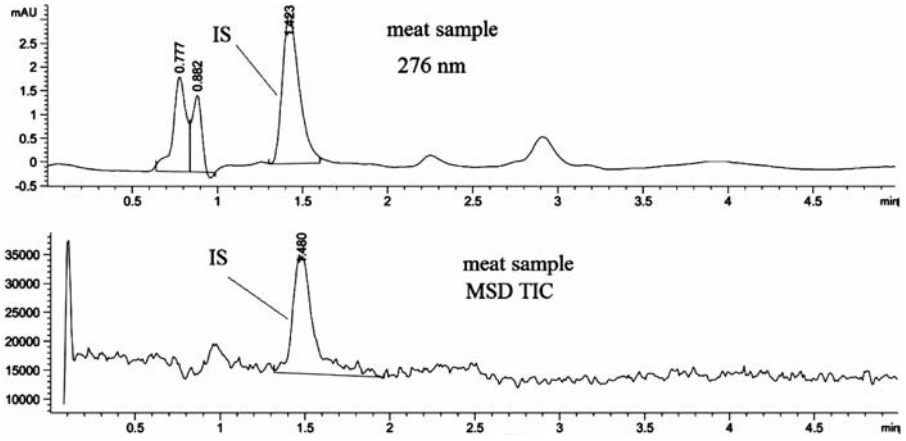


Fig. 10. Chromatograms of extracted meat sample on UV 276 nm and MSD TIC.

Application of the Analytical Method

This method was reproducible, accurate and sensitive, and can be used successfully for determination of the active ingredient of the drug «Tryfuzol» residual amounts in the poultry meat homogenate [7,8]. Samples of poultry (30 g) were treated in accordance with the method (*Fig. 2* and *Fig. 3*) and compared with an extract made from the standard substance drug «Tryfuzol» sample (*Fig. 9*). Active substance of the drug «Tryfuzol» was not detected (*Fig. 10*).

Content Calculations

Due to the better reproducibility we used only external standard calibration equation for content calculations. Internal standard we used for the extraction conditions control.

Conclusions

1. The method capable for identification «Tryfuzole» active substance residues at ≥18 μg/g and for the determination of «Tryfuzole» active substance residues in poultry meat homogenizate samples at ≥30 μg/g levels was elaborated.

2. Active substance piperidine 2-{[5-(2-furyl)-4-phenyl-1,2,4-triazoles-3- yl] thio}acetate was not found in the homogenate by elaborated method according to the experiment results when using the drug «Tryfuzol» under the scheme referred to leaflet for the first group of chiken.

Prospects for further research Elaborate methods of determination of the active substance of drug «Tryfuzol» in the blood plasma and other biological fluids.

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