Solid phase extraction-high performance liquid chromatography-tandem mass spectrometry three metabolic regulators in food: clomiphene, trimetazidine, and meldonium

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Abstract

Clomiphene, trimetazidine, and meldonium are three common clinical drugs, and long-term consumption will cause damage to human health. Currently, animal-derived food contamination is a major concern, and these compounds have a high probability of entering the human body and remaining there for a long time. In this study, a liquid chromatography-tandem triple quadrupole mass spectrometry method was developed to determine the residues of three metabolic regulators in food. With 0.1% formic acid water-acetonitrile solution (25:75, V/V), samples were extracted ultrasonically. After MCX SPE Column Cleanup, the Shiseido PC HILIC column was used for separation, with positive ion multiple reaction monitoring mode monitoring and isotope internal standard method used for quantifying. In this optimized condition, both clomiphene and trimetazidine had detection limits of 0.25 μg/kg and quantification limits of 0.5 μg/kg. Meldonium had a detection limit of 2.5 μg/kg and a quantification limit of 5.0 μg/kg. Recovery rates ranged from 80.1–119.9%, and precision RSD was 1.95–15.4% (n = 6). This method is suitable for detecting food containing clomiphene, trimetazidine, and mildronium.

1 Introduction

Clomiphene, trimetazidine, and meldonium are three common clinical drugs, of which clomiphene is used to stimulate the development of follicles in females, induce ovulation, and treat symptoms of endocrine infertility(Ghazeeri, Kutteh, Bryer-Ash, Haas, & Ke, 2003; Kocak, Caliskan, Simsir, & Haberal, 2002; Legro et al., 2007; Nestler, Jakubowicz, Evans, & Pasquali, 1998). In clinical practice, trimetazidine is primarily used to protect against different types of myocardial ischemia and cardiac dysfunction(Detry, Sellier, Pennaforte, Cokkinos, Dargie, & Mathes, 1994; Tuunanen et al., 2008). Meldonium, also known as mildonium, is used as an anti-ischemic drug in the treatment of coronary artery disease and concomitant arterial hypertension, myocardial infarction, and chronic heart failure (Denys & Ihor, 2019; Liepinsh et al., 2006; Pupure et al., 2010). Long-term consumption of foods containing clomiphene, trimetazidine, and meldonium, however, can cause metabolic disorders, liver damage, and respiratory and nervous system disorders. Ingestion of clomiphene may result in adverse effects such as blurred vision and cardiovascular problems. Pregnant and lactating women should not use or consume this drug since it might cause teratogenic effects and fetal toxicity(Eiden et al., 2020; Zahid, Arshad, Zafar, & Al-Mohannadi, 2016). The ingestion of trimetazidine may cause or aggravate Parkinson's symptoms, neurological disorders such as dizziness, headaches, and sleep disturbances, as well as gastrointestinal disorders such as abdominal pain, diarrhea, indigestion, nausea, and vomiting(Minners, van den Bos, Yellon, Schwalb, Opie, & Sack, 2000; Wisel et al., 2009). Ingestion of meldonium may result in insomnia, belching, dreaminess, and stimulation of the nervous system(Jaudzems et al., 2009; Shaforostova, Gureev, Volodina, & Popov, 2022; Sjakste, Gutcaits, & Kalvinsh, 2005; Vilskersts et al., 2009; Voronkov, I.Shkurat, & Lutsak, 2008; Zakharenko, Petrovsky, & Putilov, 2018).

Currently, there is a high risk of animal-derived food contamination, and these three types of compounds are likely to enter the human body and remain in the human body during the food chain cycle (Wang et al., 2022). In a study by Euler Luisa et al., the egg production rate of chickens was significantly enhanced

by taking clomiphene oral liquid, but the residues of clomiphene were found in their eggs(Euler et al., 2022). To prevent human health from being affected by the ingestion of clomiphene, trimetazidine, and meldonium, a detection method is required to screen out the preparations containing these compounds. However, the current international food safety national and local standards do not include trimetazidine, clomiphene, or meldonium. Nowadays, liquid chromatography, liquid chromatography-tandem mass spectrometry, and so on are used to determine clomiphene, trimetazidine, and meldonium(Hmelnickis, Pugovics, Kazoka, Viksna, Susinskis, & Kokums, 2008; Peng et al., 2010; Temerdashev, Azaryan, & Dmitrieva, 2020). Due to the complexity of food matrixes and the interference of sample matrixes, liquid chromatography results are inaccurate and are prone to false positives(Pirok, Stoll, & Schoenmakers, 2019). Chromatography-mass spectrometry has the advantages of high sensitivity, strong anti-interference ability, and wide analysis range, which is commensurate with the current demands of food safety testing(Gilar, Bouvier, & Compton, 2001; Jiang et al., 2020; Van Thuyne, Van Eenoo, & Delbeke, 2006). Currently, no method has been reported to detect trimetazidine, clomiphene, and meldonium simultaneously in common foods.

In this experiment, acetonitrile or a water-acetonitrile solution containing 0.1% formic acid was used (25:75, V/V) for extraction. After purification by MCX solid phase extraction column, separation was performed by Shiseido PC HILIC column (150 mm × 2.0 mm, 5 μm). Then the three metabolic regulators, including trimetazidine, clomiphene, and mildronate in food, were identified using high-performance liquid chromatography-tandem mass spectrometry. This method has good purification, high sensitivity, and good separation, making it suitable for determining metabolic regulators accurately on a qualitative and quantitative basis.

2 Materials And Methods

2.1 Chemicals and reagents

An Agilent 6470 triple quadrupole tandem mass spectrometer equipped with an Agilent 1290 liquid chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA) was employed; centrifuges; Millipore Milli-Q Plus water purification system (Millipore, Brussels, Belgium); Gl-88 vortex mixer was obtained from Qilinbeier company and KQ-5200 ultrasonic cleaning instrument was provided by Suzhou Kunshan Ultrasonic Instrument.

The acetonitrile and methanol are HPLC grade; All solutions were prepared with ultrapure water obtained from a Milli-Q Element water system (18 M Ω.cm or higher); The standards of Clomiphene, Clomiphene-D_5, Trimetazidine, Trimetazidine-D_8, Mildronate, Mildronate-D_7 were purchased from Alta Technologies; The MCX solid phase extraction column (200 mg/6cc) was provided by Waters, USA.

Source of the sample were purchased from different local supermarkets in Beijing. The author's unit inspected the sample.

2.2 Preparation of standard solution

The standard stock solutions were prepared in chromatographic grade methanol and stored in amber glass bottles at -18 ^{o}C. The mixed working solutions were obtained by diluting the stock solutions with chromatographic grade methanol.

2.3 Sample preparation

Solid and semi-solid samples

1.0 g of sample was weighed into a 10 ml centrifuge tube with a cap. A 20 μL internal standard solution was added, mixed well, and then allowed to stand for 30 min. To dissolve the sample, 5 mL 0.1% formic acid-acetonitrile solution was added. After a vigorous shake for 30 seconds, a 10-minute ultrasonic extraction, a centrifuge at 10,000 r/min for 10 min at 4°C. Transfer the supernatant to a new centrifuge tube. The residue was then added to 5 mL of extraction solution, and the extraction cycle was repeated. Pooled supernatants were then analyzed and purified.

liquid sample

1.0 g of sample was weighed into a 10 ml centrifuge tube with a cap. A 20 μL internal standard solution was added, shaking vigorously for 10 seconds. 9 mL of 0.2% formic acid-acetonitrile solution (25:75, V/V)was added to dissolve the sample, shaking vigorously for 30 s, ultrasonic extraction for 10 min; Centrifuge at 10,000 r/min for 10 min at 4°C. The supernatant was taken and then analyzed and purified.

Grease and its products

1.0 g of sample were weighed and placed into a 10 ml glass graduated tube (the solid sample was first placed in a water bath at 60°C for approximately 5 minutes, with occasional shaking to dissolve the fat). A 20 μL internal standard solution was added, mixed well for 10 seconds, and then 5 mL of 0.1% formic acid-acetonitrile solution (25:75, V/V)was added to dissolve the sample, shaking vigorously for 2 min. As soon as the supernatant was layered, it was transferred to a second centrifuge tube. Add 5 mL of extract to the residue, repeat the extraction once, combine the supernatant, centrifuge at 10,000 r/min at 4°C for 10 minutes, and remove the supernatant again for further purification.

table sugar and table salt

1.0 g of sample was weighed into a 10 ml glass graduated tube. Add 20 μL internal standard solution and 3 mL of hot water around 50°C, vortex for 10 s until sample fully dissolved. 5 mL of 0.2% formic acid acetonitrile was added and vortex for 2 min. After layering, the supernatant is transferred to another centrifuge tube. The residue was then added with 5 mL of extraction solution, and the extraction was repeated once for further purification. Pooled supernatants and waited for purification.

Purification: Transfer the obtained extract to an MCX solid phase extraction column activated in sequence with 3 mL of methanol and 3 mL of water while maintaining a flow rate of 2–3 mL per minute. After loading all samples, the cartridge was washed with 2 mL of 0.1% formic acid-acetonitrile solution

(25:75, V/V), the effluent was discarded, and the cartridge was eluted with 5 mL of a methanol solution containing 2% ammonia water. The eluate was collected, dried at 37°C under a weak nitrogen flow, dissolved in 70% methanol aqueous solution, and made to a volume of 1 mL, then passed through a 0.22 mm filter membrane and analyzed using LC-MS/MS.

2.4 Chromatography -mass spectrometry conditions

In liquid chromatography, the following conditions were used: column: PC HILIC column (2.0 mm × 150 mm, 5 μm) (Shiseido, Tokyo, Japan) at 25°C column temperature. The flow rate was 0.3 ml min^{-1} with an injection volume of 2 μl. A 20 mmol/L ammonium acetate aqueous solution (containing 0.05% formic acid) and acetonitrile (70:30, V/V) were used as the mobile phases.

Mass spectrometry conditions: electrospray ion source (ESI); positive ion mode scan; drying gas temperature was 350°C with 5 L/min; drying gas was N_2; atomizing gas pressure was 275.8 kPa; multi-reaction detection method. The mass spectrometric parameters of the compounds are shown in Table 1.

Table 1
Mass spectrometry parameters of the compounds

Analyte	Precursor ion(m/z)	Product ion(m/z)	Fragmentation voltage(V)	Collision energy(eV)
Clomiphene	406.1	100*;58.1	160	30;30
Clomiphene -d_5	411	100	160	30
Trimetazidine	267.2	181.1*;166.1	100	15;35
Trimetazidine -d_8	275.1	181.1	100	15
Mildronate	147.1	58.1*;59.2	60	40;20
Mildronate -d_3	150.1	61.2	60	40
*Quantitative ion				

3 Results And Discussion

3.1 Optimization of sample pretreatment

Animal-derived foods contain more fat and animal protein, so methanol and water can easily extract fat and protein simultaneously, which has a detrimental effect on the extraction of the target compound and subsequent purification. As a result, acetonitrile was selected as the primary extraction reagent. A comparison was made between the effects of 50% acetonitrile in water, 75% acetonitrile in water, and 100% acetonitrile as extracts on clomiphene, trimetazidine, and mildronate. The results showed that: when acetonitrile was used as the extraction solution, mildronate extraction efficiency was less than 40%;

when 50% acetonitrile aqueous solution was used as the extraction solution, clomiphene extraction efficiency was less than 50%, indicating that clomiphene, trimetazidine, and mildronate were polarized differently. Thus, it is essential to find a solution that can simultaneously extract all three compounds. Repeated tests have revealed that 75% acetonitrile can extract clomiphene, trimetazidine, and mildronate, and the extraction effect does not differ significantly. As a result of the differences in sample water content and requirements for subsequent purification steps, 0.1% formic acid water-acetonitrile solution (25:75, V/V) was employed as the extraction reagent for solid samples; 0.2% formic acid acetonitrile was employed for liquid samples.

In this method, the recovery rate of beef, seasonings, fish, edible vegetable oil, and other food extracted once and twice was investigated. 1.0 g of the sample should be added to 100 L of mixed standard working solution, and then extraction should be performed as indicated above. The results showed that the recovery rate of one extraction was not ideal, the recovery rate of the matrix was about 75%~88%, and the total recovery rate could reach 89% when the extracts after two extractions were combined with the first extracts. It is therefore determined that solid and semi-solid foods should be extracted twice during the extraction process, and the two extracts should be combined for purification.

For liquid foods with high water content, such as beverages, yogurt, and liquid milk, the sample matrix is uniform. Following the above-mentioned optimized experimental steps for extraction, the recovery rate after one extraction can be as high as 90.7%~97.3%.

The purification effects of PRiME HLB (6cc/200 mg) solid-phase extraction column, MCX (3cc/60 mg) solid-phase extraction column and WAX (3cc/60 mg) solid-phase extraction column were compared. 5 mL 100ng/mL quaternary ammonium compound mixed working solution was applied to the column for analysis respectively. The experimental results showed that the recoveries of the target compounds of MCX (3cc/60 mg) solid phase extraction column and PRiME HLB (6cc/200 mg) solid phase extraction column were all > 90%, the anion ion exchange solid phase extraction column is not suitable for the purification of these three metabolic regulators. In addition, the experiment found that the PRiME HLB solid phase extraction column has a poor purification effect on matrices such as seasonings, soy sauce, and brown sugar; A solid-phase extraction column equipped with MCX (3cc/60 mg) has dual retention capabilities of reversed-phase and cation exchange, as well as good poly reactivity for basic compounds such as clomiphene, trimetazidine, and mildronate, making it an excellent choice for purifying alkaline compounds from complex food matrixes. Therefore, in this method, MCX (3cc/60 mg) solid phase extraction column is selected as the purification column for the sample. According to the packing characteristics of MCX (3cc/60 mg) solid phase extraction column, 2% ammonia solution in methanol was selected as the elution solvent of this type of solid phase extraction column. This method compared the recovery of target compounds with elution volumes of 1 mL, 2 mL, 3 mL, 4 mL, 5 mL, 6 mL, 7 mL and 8 mL of elution solvent. As the elution solvent volume increased to 3 mL, the recovery rate of the target compound was close to 90%. After that, with the increase of the elution volume, the recovery rate of the target compound did not increase anymore, indicating that the target compound could be completely eluted with 3 mL of elution solvent. Considering that the target compound was sufficiently eluted and the

maximum volume of the solid-phase extraction column was 3 mL, 5 mL of 2% ammonia in methanol was chosen to elute the target compound on the solid-phase extraction column. In addition, the experiment found that: in seasoning, soy sauce, and chicken matrix samples, when water and methanol are used as eluents, mildronate will be lost, so this method uses 2 mL of 0.1% formic acid-acetonitrile solution (25:75, V/V) as eluent.

3.2 Optimization of mass spectrometry conditions

According to the chemical ionization properties of clomiphene, trimetazidine, and mildronate and their internal standards, ESI^+ was selected as the ionization mode, and the mass spectrometer conditions were optimized by continuous injection with a flow syringe pump. The experimental results show that the target compound can obtain a higher abundance of precursor ions in the ion source ESI^+ ionization mode. The secondary mass spectrometry analysis was carried out employing production scanning, and the product ions were optimally selected to determine quantitative ions and auxiliary qualitative ions. By optimizing the mass spectrometry parameters such as fragment, Cell Accelerator Voltage, collision energy, and Dell, the ion pair intensity generated by the quasi-molecular ion and characteristic fragment ion of the target compound can be maximized. Referring to the relevant regulations in EU Resolution No. 2002/657/EC, 2 pairs of characteristic transitions with higher response values were selected for each target as quantitative and qualitative transitions, and finally, various mass spectrometry parameters were optimized in the multiple reaction monitoring modes. The relevant parameters are shown in Table 1.

3.3 Optimization of chromatographic conditions

3.3.1 Selection of Column. In this experiment, Shiseido PC HILIC column (150 mm × 5 mm, 2.0 μm), Hypersil GOLD C18 column (50 mm × 4.6 mm, 1.9 μm) and Agilent Poroshell 120 PFP column (100 mm × 4.6 mm, 2.7 μm) were used to separate the target compounds. As a result of the above three columns, three target compounds were effectively separated. Due to mildronate's strong polarity, PC HILIC columns were selected as the analytical columns for this method in order to fully separate mildronate from complex matrices. PC HILIC column was selected as the analytical column for this method.

3.3.2 Selection of chromatographic separation conditions. The choice of mobile phase has a certain influence on the ionization of the target and the sensitivity of mass spectrometry detection. PC HILIC columns generally use acetonitrile and aqueous ammonium acetate as mobile phases. The pH can affect the separation and retention of target compounds and the results of mass spectrometry ionization. The separation effects of the flow relative to the target compound were compared separately: 20 mmol\L ammonium acetate aqueous solution (containing 0% formic acid) + acetonitrile=30:70, (V/V) (mobile phase 1), 20 mmol\L ammonium acetate aqueous solution (containing 0.025% formic acid) + acetonitrile=30:70, (V/V) (mobile phase 2), 20 mmol\L ammonium acetate aqueous solution (containing 0.05% formic acid) + acetonitrile=30:70, (V/V) (mobile phase 3). The results showed that the peak shape broadening of the first mobile phase clomiphene could not be eluted (see Figure 1); The separation of the three target compounds was similar between the second mobile phase and the third mobile phase, but the effect of the third mobile phase target compound was higher (see Figure 2-3).

3.4 Selection of quantitative methods

Matrix effect refers to the phenomenon that other components in the sample other than the target analyte affect the measured value of the analyte, inhibiting or enhancing the response of the target compound. Although the sample has been cleaned up to a certain extent, matrix effects cannot be completely eliminated. It was found in the test that there was a strong matrix inhibition effect of mildronate after the sample treatment, as shown in Table 2. The commonly used method to eliminate the matrix effect is to add an isotopic internal standard because the food matrix is complex. Therefore, for accurate quantification, this experiment selects the isotopic internal standard to eliminate the matrix effect of the sample.

Table 2
Matrix effects of three metabolic conditioners

Analyte	trimetazidine	clomiphene	mildronate
liquid milk	0.81	0.75	0.73
beef	0.89	0.82	0.11
soy sauce	0.92	0.67	0.29
seasoning	0.83	0.90	0.30

3.5 Linearity range and detection limit

The contents of trimetazidine and clomiphene were 0.500, 1.00, 2.00, 5.00, and 10.0 μg/L, respectively, and the contents of mildronate were 5.0, 10.0, 20.0, 50.0, and 100.0 μg/L, respectively. Standard series with internal standard concentrations of 2 μg/L, 2 μg/L, and 50 μg/L for trimetazidine, clomiphene, and mildronate, respectively. A standard curve was drawn as the peak area ratio (y) versus the concentration ratio (x) of the target compound and the corresponding internal standard. At the same time, a blank sample was selected, the standard solution was quantitatively added, and the processing and determination were carried out according to the above-mentioned experimental method. When the qualitative ion signal-to-noise ratio of the spectrum obtained by the test solution is greater than 3, the addition amount is set as the minimum detection limit. When the quantitative ion signal-to-noise ratio is greater than 10 and the qualitative ion signal-to-noise ratio is greater than 3, the addition amount is set as the minimum quantitative limit, and the results are shown in Table 3.

Table 3
Linear equation, linear relationship, correlation coefficient, limit of quantification and limit of detection of three metabolic conditioners

Analyte	Linear equation	Linear range(μg/kg)	Correlation coefficient(r)	Limit of quantitation (μg/kg)	Limit of detection(μg/kg)
trimetazidine	Y = 9.45X-0.3315	0.5–10.0	0.9998	0.5	0.2
clomiphene	Y = 12.28X + 0.6012	0.5–10.0	0.9993	0.5	0.2
mildronate	Y = 0.90X-0.0068	5.0-100	0.9991	5.0	2.0

3.7 Recovery rate and precision of the method

Representative samples were selected for recovery and precision tests. Eleven matrices, including beef, fish, chicken, eggs, milk, brown sugar, peanut oil, seasonings, soy sauce, sauce, and sausage, were selected to test the recovery and precision of standard additions. To better investigate the recovery rate and precision of the method, this method is based on selecting a negative matrix to carry out equal-fold, 2-fold, and 10-fold standard recovery tests. The average spiked recovery at different concentrations was 80.1-119.9%, and the precision RSD was 1.95–15.4% (n = 6).

4 Conclusion

In this study, a liquid chromatography-tandem mass spectrometry method was established for the simultaneous determination of clomiphene, trimetazidine, and mildronate in food. The method adopts ultrasonic extraction with acetonitrile-formic acid aqueous solution, MCX (3cc/60 mg) solid-phase extraction column purification, positive ion multiple reaction monitoring mode monitoring, and internal standard method for quantification. At different concentration levels, the method recoveries ranged from 85.1–113.1%, and the average relative standard deviations ranged from 2.57–9.84% (n = 6). The sample pretreatment of the method is simple and easy, the analysis is rapid, the recovery rate is high, the limit of quantification and precision can meet the requirements of residue detection, and it is suitable for accurately determining the clomiphene, trimetazidine, and mildronate residues in food.

Declarations

Author contributions

Hao Wang: methodology, investigation, formal analysis, and writing the original draft. Yu Wang: formal analysis, methodology, and writing review & editing.

Mingyuan Shao: writing review & editing. Li lin: methodology and writing – review & editing. Tongna Mu: supervision and writing review & editing. Yanqin Liu: supervision and writing review & editing.

Conflict of Interest

All authors declare that they have no conflicts of interest.

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Data Availability

All data generated or analyzed during this study are included in this published article.

Ethics Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate: Not applicable.

Informed Consent: Not applicable.

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Figures

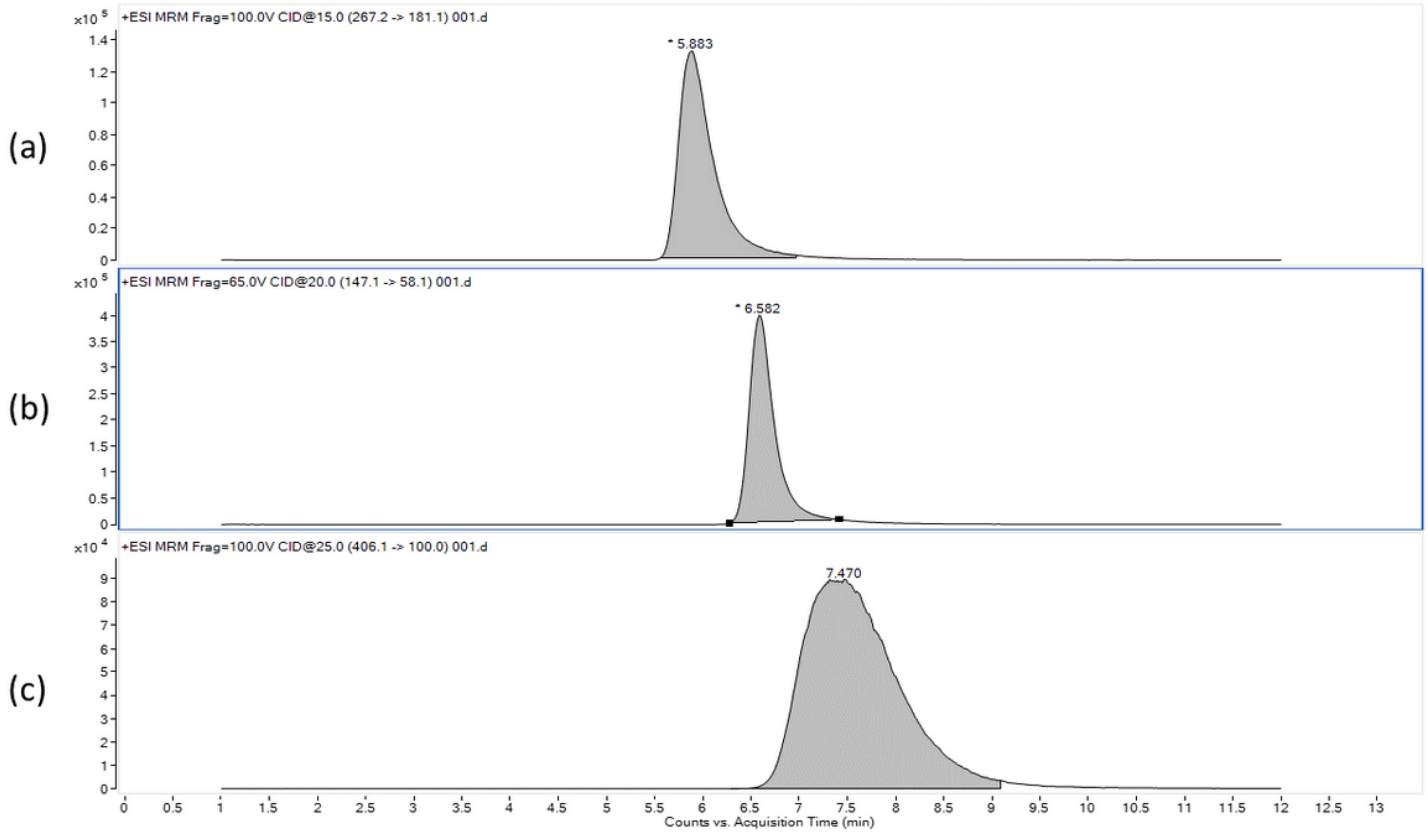

Figure 1

Standard solution MRM chromatograms of three metabolic conditioners in mobile phase 1. (a) trimetazidine, (b) mildronate, (c) clomiphene.

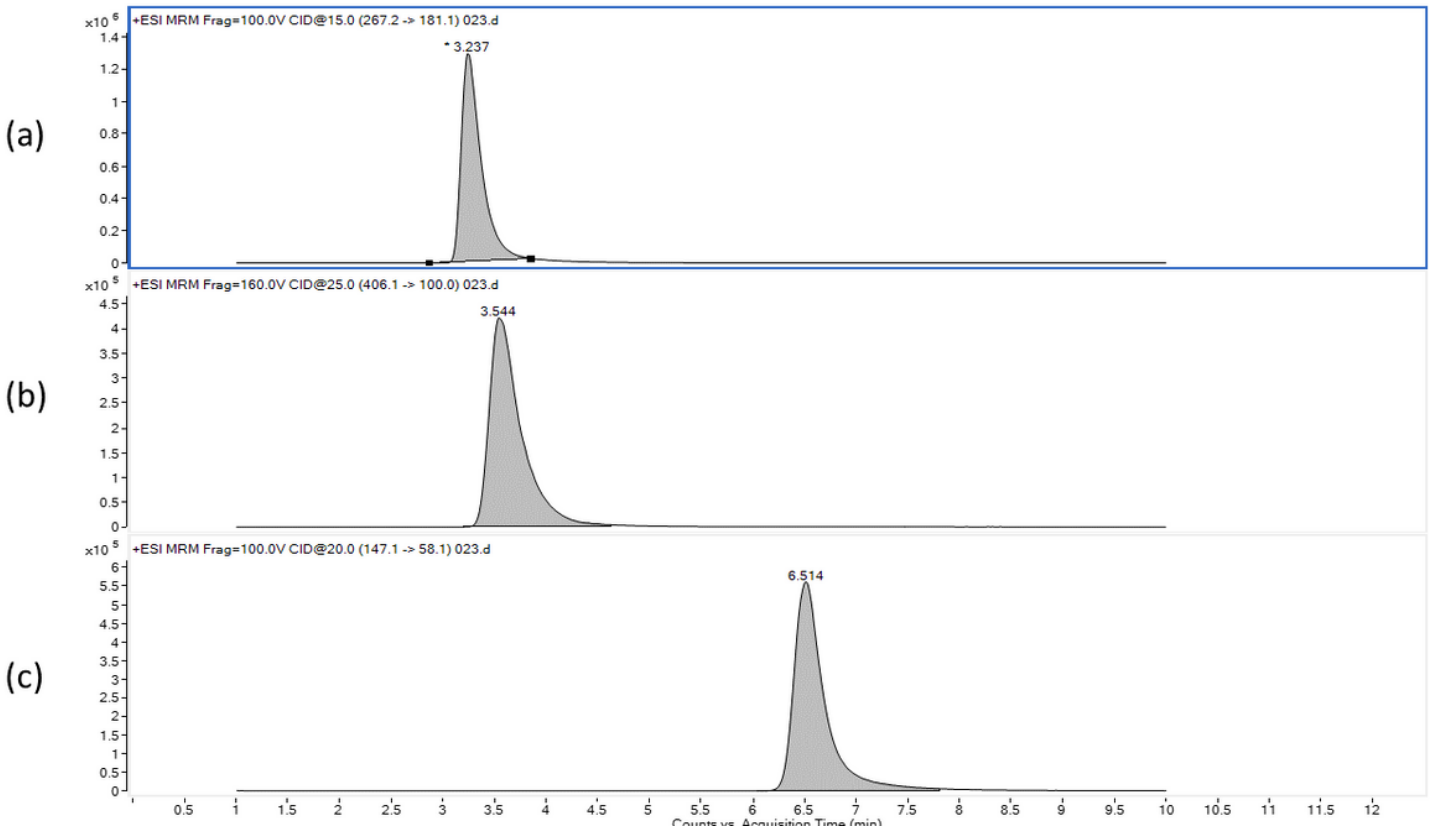

Figure 2

Standard solution MRM chromatograms of three metabolic conditioners in mobile phase 2. (a) trimetazidine, (b) mildronate, (c) clomiphene.

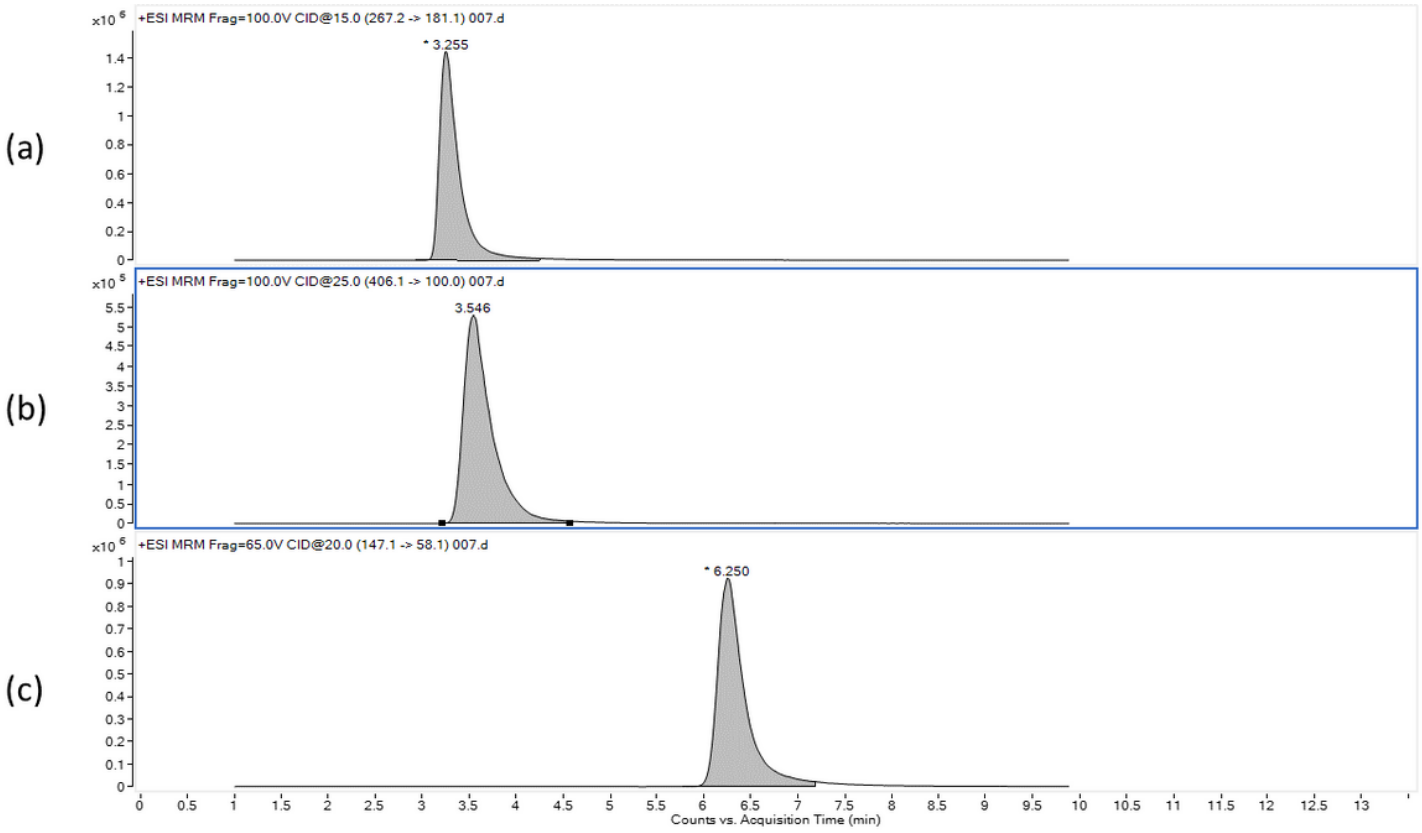

Figure 3

Standard solution MRM chromatograms of three metabolic conditioners in mobile phase 3. (a) trimetazidine, (b) mildronate, (c) clomiphene.