# Development, validation and application of a GC-MS method for the simultaneous detection and quantification of neutral lipid species in *Trypanosoma cruzi*

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## Abstract

The development and validation of an analytical method for the simultaneous analysis of five neutral lipids in *Trypanosoma cruzi* epimastigotes by GC-MS is presented in this study. The validated method meets all validation parameters for all components and the chromatographic conditions have been optimized during its development. This analytical method has demonstrated good selectivity, accuracy, within-day precision, recovery and linearity in each of the established ranges. In addition, detection and quantification limits for squalene, cholesterol, ergosterol and lanosterol have been improved and it is worth highlighting the fact that this is the first time that squalene-2,3-epoxide validation data have been reported. The new validated method has been applied to epimastigotes treated with compounds with *in vitro* anti-*T.cruzi* activity. This new methodology is straightforward and constitutes a tool for screening possible sterol biosynthesis pathway inhibitors in *Trypanosoma cruzi*, one of the most studied targets in Chagas disease treatment. Therefore, it is an interesting and useful contribution to medicinal chemistry research.

**Keywords:** *Trypanosoma cruzi*, squalene, squalene-2,3-epoxide, ergosterol, lanosterol, GC-MS

## 1. Introduction

The protozoan parasite *Trypanosoma cruzi* (*T. cruzi*) is the causative agent of Chagas disease (CD). CD has been classified as one of the 17 neglected tropical diseases by the World Health Organization (WHO) [1]. This parasitosis is endemic in 21 Latin American countries and as a result of migration flows, the number of cases has increased in non-endemic countries such as the USA, Canada, some European countries, and Japan and Australia [2, 3]. It is estimated that approximately 5.7 million people are infected by CD [4] and the disease causes approximately 7000 deaths annually, thereby becoming a serious public health problem [5]. The therapeutic arsenal against CD remains inadequate and insufficient. Currently, the only two drugs available for CD are nifurtimox and benznidazole. Both of them have issues related to toxicity

and low efficacy in the chronic phase; neither one has been approved by the Food and Drug Administration (FDA) [6]. This scenario justifies the urgent need to develop safe and cost-effective therapeutic alternatives that can help fight against CD [1].

Advances in the study of the biochemistry and molecular biology of *T. cruzi*, and the complete sequencing of its genome have allowed identifying important molecular targets and specific metabolic pathways of *T. cruzi* that could be attacked by new drugs [7].

Sterols are a group of lipids classified as polycyclic steroids that have a structural function because they form part of the cell membrane. The presence of these sterols is essential for maintaining the viability and functionality of the plasma membrane in all eukaryotic cells. The different types of sterols are synthesized through a complex biochemical pathway called sterol biosynthesis pathway (SBP). This biochemical route is one of the most studied targets in the anti-*T. cruzi* drug discovery process [8]. Blocking SBP causes changes in the composition of the membrane [9], which causes defects in its ultra-structure [10]. These changes modify the physical properties and the activity of the membrane enzymes, leading to cell lysis [11].

Cholesterol is the main sterol in the mammalian system while in trypanosomatids, such as *T. cruzi*, the main sterols are ergosterol and 24-alkylsterols [12]. The *de novo* endogenous sterols biosynthesis in *T. cruzi* epimastigotes, unlike in mammals [11], would mainly take place in mitochondria [13] and glycosomes, and would reach the membrane through transporters [14]. This biochemical pathway (**Figure 1**) consists of two main stages, isoprenoids biosynthesis pathway (IBP) and SBP. The IBP starts with the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA and after several enzymatic reactions, squalene is obtained. The following stage corresponds to the SBP itself and involves the cyclization of squalene and its transformation into ergosterol. It is in this second phase where the differences between parasites and mammals can be found due to the presence of specific and characteristic enzymes [11, 15].

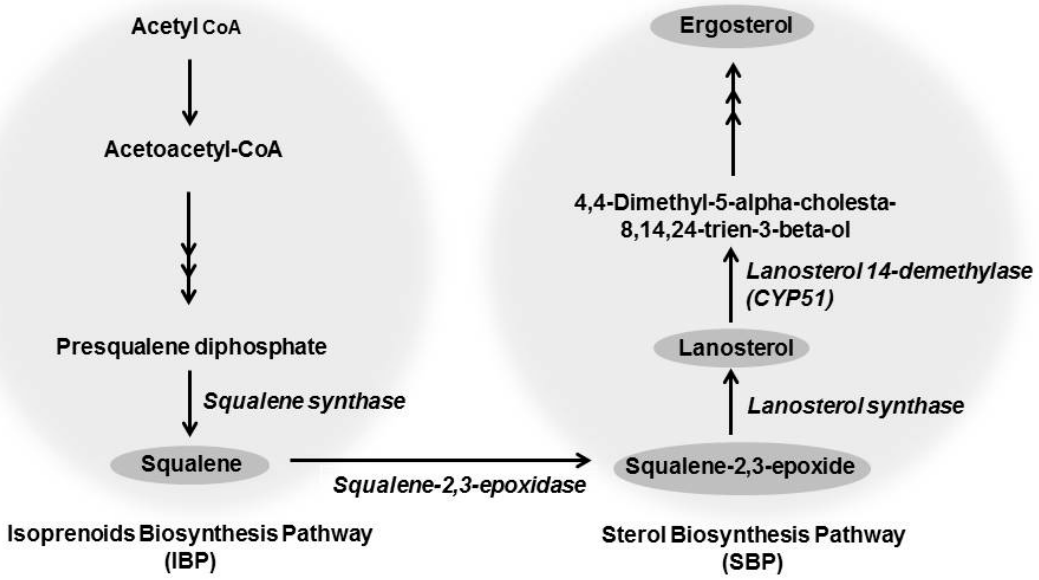


**Figure 1.** Sterol biosynthesis pathway in *T. cruzi* epimastigotes

Advances made in lipidomics have made it easier to study the lipid profile of trypanosomatids; it has been determined that $C_{28}$ and $C_{29}$ sterols are the most plentiful [16]. T. cruzi sterol composition varies according to the parasite stage during its life cycle. In epimastigote form, it has been observed that approximately 40% is consisted of C28 sterols such as ergosterol, 30% of C29 sterols, and a small percentages of C30 sterols such as lanosterol and isoprenoids such as squalene and squalene epoxide [17, 18]. In addition, it is known that approximately 10-50% ischolesterol derive from host or serum present in the culture medium [16, 17]. Despite this, *T. cruzi* needs to synthesize its endogenous sterols, essential for maintaining its viability and cell proliferation capacity in its different life stages [8].

The lipid composition and changes in the profile of *T. cruzi* epimastigotes have been studied by different research groups using various chromatographic methods, differing in the extraction, separation, detection, identification, quantification procedures and in the number of different types of lipids that can be evaluated simultaneously [13, 14, 18-35]. Among these methodologies, simple methods with Thin-Layer Chromatography (TLC) have been used, providing qualitative and semi-quantitative results [20-23]. In addition, methodologies with chromatography gas with mass detector (GC-MS) and FID detector have been used, allowing simultaneous determination of lanosterol, ergosterol and cholesterol, among other sterols [18-20]. Gerpe *et al*. presented a study using HPLC in which the detection and quantification limits determined for squalene were 0.6 and 2.1 µg/mL, respectively. With regard to ergosterol, a minimum value of 2.7 µg/mL was reported [23]. Urbina's group has spent many years researching the changes in the *T. cruzi* lipid profile after treatment with sterol biosynthesis pathway inhibitors (SBIs). In their different studies, using GC-MS with FID detector methodology, they reported minimum values of 1.9, 2.5, 1.8 and 6.1 % for squalene, lanosterol, ergosterol and cholesterol, respectively. These assays were carried out in SCAN conditions and lipid composition was determined for the library of compounds; values were expressed in mass percentage [13, 14, 24-31]. Overall, the described methodologies for the determination of the components of SBP in biological matrices are varied [32-34]. Nevertheless, some authors have considered GC-MS as one of the most efficient and accessible alternatives for lipid quantification in biological samples due to its high resolution and the existing economic and environmental advantages over other methods. Therefore, we decided to use this method in our study [35].

Different methods for the quantification of sterols have recently been validated in different biological matrices using GC-MS; however, no methods have been found that allow the simultaneous quantification of squalene, squalene-2,3-epoxide, lanosterol, ergosterol and cholesterol in *T. cruzi* epimastigotes. Furthermore, there is little information regarding the determination of squalene-2,3-epoxide in this biological matrix. Within this context, our work provides the development and validation of a new method capable of simultaneously analyzing five components of SBP by GC-MS in selected-ion monitoring conditions (SIM) in this biological matrix. The development and validation of this method is a useful analytical tool and an important contribution to

the field of medicinal chemistry as a possible method for identifying SBIs in the drug discovery process for the treatment of CD.

In order to study the applicability of the new validated method, some compounds with interesting *in vitro* activity against *T. cruzi* were assessed to determine their possible inhibitory effect against SBP.

## 2. Materials and methods

### *2.1. Chemicals*

The sterols reference standards squalene ([111-02-4], ≥ 98%, [Wisconsin, US]), 2,3-oxidosqualene ([7200-26-2], ≥ 92%, [Bengalore, India]), cholesterol ([57-88-5], ≥ 99%, [Missouri, US]), ergosterol ([57-87-4], ≥ 95%, [Buchs, Switzerland]), and lanosterol ([79-63-0], ≥ 93%, [Missouri, US]) were supplied by Sigma-Aldrich. Methanol was purchased from AppliChem Panreac (Barcelona, Spain), hexane was obtained from Merck KGaA (Germany) and chloroform was purchased from Quimipur (Madrid, Spain). Sodium chloride and sodium phosphate for buffer solution were obtained from Panreac (Barcelona, Spain).

### *2.2. Biological material*

*T. cruzi* epimastigote Tulahuen 2 and Y strains were obtained from the Grupo de Química Medicinal-Laboratorio de Química Orgánica, Facultad de Ciencias Universidad de la República de Uruguay.

### *2.3. Standard solutions preparation*

An individual stock solution was prepared in hexane for each concentration for each reference standard, correcting the stock concentration based on the purity data obtained; the solutions were stored a -20ºC. Working solutions were freshly prepared prior to each assay by diluting each reference standard in the same solvent.

### *2.4. Sample preparation method for membrane sterols*

Epimastigote culture from Tulahuen 2 (DTU Tc VI) and Y (DTU Tc II) strains of *T. cruzi* were used to obtain membrane sterols. A negative control (NC) was assessed with epimastigote culture without compound, and Terbinafine (Tbf) was used as positive control at a dose of $IC_{50}$= 44.7 µM. The selected compounds ($IC_{50}$ concentration) were incubated with epimastigote culture at 10 million per mL for 72 h at 28 ºC. After incubation time, the epimastigote culture was centrifuged at 3000 rpm for 10 min. The obtained supernatant was discarded. The pellet was re-suspended in 6 mL of 0.05 M sodium phosphate buffer solution pH 7.4 and then centrifuged again at 3000 rpm for 10 min (Step A). The supernatant was discarded and the pellet was re-suspended in 5 mL of chloroform:methanol mixture 2:1. The suspension was then kept for 12 h at 4 ºC. Next, 5 mL of saturated NaCl solution was added and the mixture was extracted with 3 mL of chloroform and with 3 mL of hexane, taking special care to avoid collecting any aqueous phase (Step B) [22]. The extracted volume was evaporated with nitrogen gas. The sample was re-dissolved in 600 µL of hexane and then injected

into the GC-MS equipment at the development conditions (Step C). OJO (arriba en amarillo): “from” versus “of”. ¿El cultivo esta HECHO DE (= “of”) esas dos cepas o SE SACA el cultivo DESDE esas 2 cepas, como en “provenir de” (= from). Yo CREO que debe ser “of”, no te parece? Ya me dirás. Se cultivan epimastigotes de esas dos cepas por lo tanto yo creo que from

*2.5. GC-MS analysis and conditions*

Chromatographic separation was performed on an Agilent 6890 gas chromatograph (GC) coupled to a 5973 quadrupole mass spectrometer (MS) from Agilent Technologies, USA. The injector was programmed at 250 ºC and a splitless injection mode was used. Aliquots of 2 µL of the sample dissolved in hexane were injected into GC-MS. Separation of the sterols was carried out using HP-5-MS 30 m x 0.25 mm i.d. x 0.5 µm column (Agilent Technologies, USA). The carrier gas was helium, at a constant flow rate of 1 mL $min^{-1}$. The GC oven temperature started at 150 ºC, and then was ramped to 250ºC at 40 ºC $min^{-1}$. Next, the temperature was raised to 300 ºC at 5 ºC $min^{-1}$ and held there for 3.5 min. The source temperature was fixed at 280 ºC and MS worked in electron impact mode (EI, 70 eV). In order to avoid overloading the GC-MS with hexane, a solvent delay of 4 min was implemented. The mass spectra were acquired over the mass-to-charge (*m*/*z*) range of 50-600. The SIM conditions for the neutral lipids were determined after the identification of the most abundant and characteristic ions in SCAN conditions. Integration was performed with Chemstation integrator.

*2.6. Method validation parameters*

The validation was based on the criteria established by current regulations for bioanalytical methods. Selectivity, stability, limit of detection (LOD), limit of quantification (LOQ), range, linearity, precision, accuracy and recovery were studied [36, 37].

*2.7. Calibration curve*

Calibration samples were prepared by spiking matrix, previously prepared with extraction dissolvent in step B. First, five different individual concentrated solutions were prepared: squalene (850 µg/mL), squalene-2,3-epoxide (100 µg/mL), cholesterol (500 µg/mL), lanosterol (200 µg/mL) and ergosterol (200 µg/mL). Next, the corresponding volume was measured to prepare a stock solution containing the mixture of these 5 components at 101, 48, 48, 32, 16 µg/mL, respectively. Serial dilutions were made from this stock solution in order to obtain the solution containing the mixture for each concentration level.

*2.8. Application of the GC-MS method to real samples*

The new validated GC-MS method was applied to obtained extracts (step C) from treated epimastigotes with reference drug Tbf (positive control) and with our anti-*T. cruzi* compounds; untreated epimastigotes were used as negative control (NC). The quantitative determination was performed following the previously explained

experimental procedure. The calibration curves were prepared in triplicate daily. The concentration of lipids after the treatment with the compounds (S) was determined in µg/mL. With these obtained values, we calculated the accumulation percentage (A%) or depletion percentage (D%) of lipid using the following equation:

% = [(S*100/Total lipid concentration obtained from the NC)-100].

A positive result indicates accumulation (A) and a negative result indicates depletion (D).

## 3. Results and discussion

### *3.1.Optimization* of *GC-MS analysis and conditions*

In order to optimize the chromatographic separation of the five neutral lipids, the abundances of fragmented ions obtained and the different temperature gradients were assessed. As a result, the methodology described in section 2.5 was developed. **Table 1** shows monitoring ions and retention times for each lipid. The elution order was: squalene, squalene-2,3-epoxide, cholesterol, ergosterol and lanosterol (**Figure 1**).

**Table 1. GC-MS SIM conditions for the studied sterols.**

| Sterol | RT (min) | SIM (*m/z*) |
|---|---|---|
| Squalene | 9.03 | 81, 137*, 341, 410 |
| Squalene-2,3-epoxide | 10.14 | 69, 81, 121, 135, 426 |
| Cholesterol | 12.09 | 275, 301, 353, 368, 386 |
| Ergosterol | 13.01 | 337, 363, 396 |
| Lanosterol | 14.59 | 393, 411, 426 |

RT: Retention time, (Characteristics ions).

*Extracted ion (137) was used for quantification of squalene.

### 3.2.*Validation method*

The selectivity of the method allowed an adequate separation between squalene, squalene-2,3-epoxide, cholesterol, ergosterol and lanosterol. As shown in **Figure 2**, no interference peaks were detected in the retention times of the neutral lipids that were analyzed. Moreover, no interaction was observed among the sterols nor between sterols and the matrix.

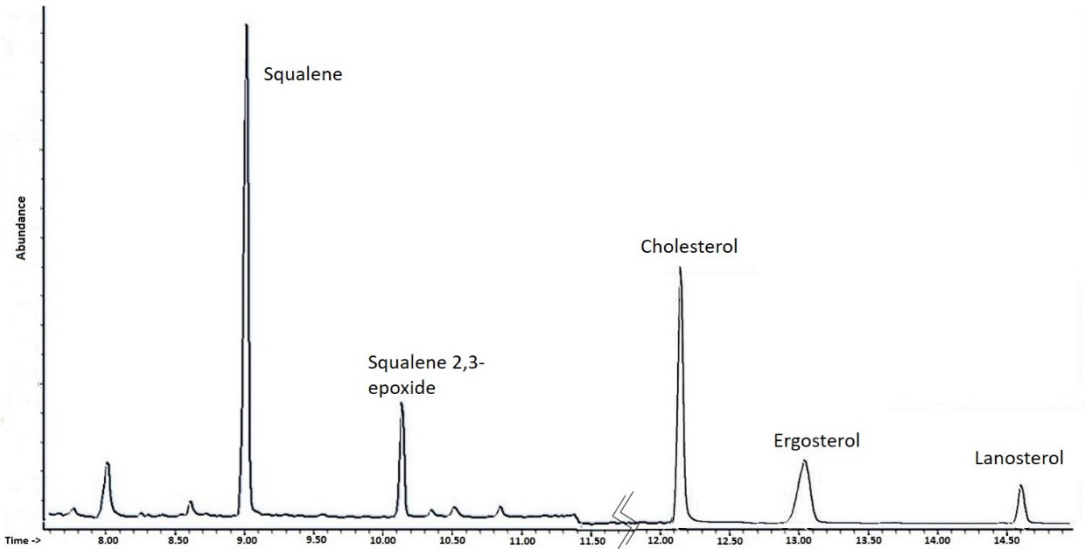


**Figure 2. Chromatogram obtained at the optimized conditions of the validated method.**

In this study, stability of the sterol samples was evaluated at two different storage temperature conditions. The sample was considered to be stable when it presented a recovery percentage over 90%. First, stability was assessed at room temperature every 12 h in a 60-h period. It was also measured after 24 h of freezing at -20ºC. The stability study at room temperature revealed that the 5 studied sterols were stable up to 36 h except for squalene-2,3-epoxide, which remained stable for 24 h (**Figure 3 and supplementary data**). Thus; the stability of the 5 neutral lipids at room temperature for 24 h is guaranteed.

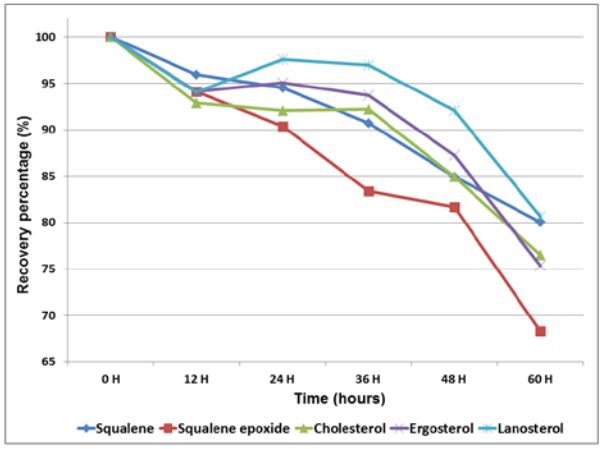


**Figure 3.** Stability study at room temperature. Samples were evaluated in triplicate and have shown RSD<15%.

Linearity was determined using the three spiking samples at a minimum of 5 concentration levels each, within the ranges shown in **Table 2**. The average obtained from the three spiking samples at each level of concentration met the established RSD in all concentrations. The range of all lipids was set considering LOQ as the lower limit, and 12 times LOQ as the upper limit for squalene-2,3-epoxide, 30 times LOQ for ergosterol and 100 times LOQ for squalene, cholesterol and lanosterol. While squalene-

2,3-epoxide displayed a unique linear range, the other lipids exhibited two linear ranges (**Table 2**). The obtained linear regression for each lipid reported good linear determination coefficient ($R^2$>0.98). Furthermore, it has been confirmed that the intercept is not statistically different from the zero value and that the slope is significantly different from the zero value with a 95% confidence interval. As for residuals analysis, it was observed that their behavior is random and does not reflect a definite trend.

LOD and LOQ were determined in the three spiking samples, at their respective concentration levels, according to AEFI by the signal-to-noise (SN) method. LOQ for squalene, squalene-2,3-epoxide, cholesterol and lanosterol was in the range of 0.13-3.10 µg/mL. In addition, obtained concentrations met the accuracy criteria (%error<20%), precision (RSD<15%), and recovery percentages (80-120%), and their values were higher than 10 times the SN. With respect to the LOD of the five lipids, they were within a concentration range of 0.05-1.86 µg/mL, and these concentrations exhibited values greater than 3 times the SN (**Table 2**).

To determine the accuracy and precision (within-day and between-days), three spiking samples corresponding to high, medium and low concentrations within the ranges for every neutral lipid were evaluated. At precision within-day analysis, all lipid RSD values were in the range of 1.94-14.99%. The values obtained satisfied the established parameters (RSD≤15%) at the three levels tested.

Precision between-days were measured on three different days. Squalene (range: 6.54-84.15 µg/mL), squalene-2,3-epoxide (range: 3.10-39.89 µg/mL) and ergosterol (2.09 and 3.49 µg/mL) had a RSD≤15%. Meanwhile, the other lipid concentrations and the lowest concentration range of squalene exhibited RSD>15%. Taking into account that all lipids were precise within-day, but at several concentrations levels did not present an acceptable precision between-days, a daily calibration curve was prepared to evaluate the samples.

With regard to accuracy, it was proven that the method met the requirement set for this parameter. The method gave values of percentage error in a range of 3.35-19.28% (%error allowed ± 20%) for the lower concentrations and a range of 0.36-14.65% (%permissible error ± 15%) for the rest of the concentrations (**Table 2**).

**Table 2.** Validation method data: Linearity, range, precision and detection and quantification limits.

| Sterols | RT (min) | SIM (*m/z*) | Range (µg/mL) | Linearity y = bx ± a | Determination coefficient ($R^2$) | Fortification level (µg/mL) | Mean | Accuracy (% Error) | Precision (%RSD) | | LOD (µg/mL) | LOQ (µg/mL) (%RSD) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Within-day | Between-days | | |
| **Squalene** | 9.03 | 81, 137*, 341, 410 | 0.85 – 6.54 | y = 180880.13x - 47122.95 | 0.9884 | 0.85 | 1.01 | 19.28 | 1.94 | > 15 | 0.31 | 0.85/ 1.94 |
| | | | | | | 2.36 | 2.02 | 14.11 | 14.89 | > 15 | | |
| | | | | | | 6.54 | 5.59 | 1.96 | 2.04 | 14.97 | | |
| | | | 6.54 – 84.15 | y = 189769.93x + 99777.38 | 0.9958 | 6.54 | 6.67 | 14.65 | 2.04 | 14.97 | | |
| | | | | | | 30.29 | 33.34 | 10.07 | 5.17 | 10.85 | | |
| | | | | | | 84.15 | 83.42 | 0.87 | 2.48 | 12.18 | | |
| **Squalene-2,3-epoxide** | 10.14 | 69, 81, 121, 135, 426 | 3.10 – 39.89 | y = 1152017.41x - 850168.65 | 0.9977 | 3.10 | 2.99 | 3.60 | 3.13 | 13.31 | 1.86 | 3.10/ 3.13 |
| | | | | | | 8.62 | 8.03 | 6.80 | 4.96 | 12.59 | | |
| | | | | | | 39.89 | 39.66 | 0.57 | 3.54 | 14.98 | | |
| **Cholesterol** | 12.09 | 275, 301, 353, 368, 386 | 0.40- 1.87 | y = 203170.23x - 21519.56 | 0.9960 | 0.40 | 0.42 | 3.35 | 6.85 | > 15 | 0.24 | 0.40/ 6.85 |
| | | | | | | 1.12 | 1.07 | 2.15 | 14.90 | > 15 | | |
| | | | | | | 1.87 | 1.89 | 1.16 | 6.99 | > 15 | | |
| | | | 1.87- 39.98 | y = 429645.90x - 326847.43 | 0.9990 | 1.87 | 1.60 | 14.07 | 6.99 | > 15 | | |
| | | | | | | 8.64 | 8.12 | 5.97 | 3.80 | > 15 | | |
| | | | | | | 39.98 | 39.84 | 0.35 | 4.02 | > 15 | | |
| **Ergosterol** | 13.01 | 337, 363, 396 | 0.75- 3.49 | y = 331790.17x - 222286.57 | 0.9829 | 0.75 | 0.90 | 19.17 | 13.38 | > 15 | 0.16 | 0.75/ 13.38 |
| | | | | | | 2.09 | 2.07 | 1.07 | 7.97 | 13.50 | | |
| | | | | | | 3.49 | 3.53 | 1.17 | 14.99 | 11.38 | | |
| | | | 3.49- 26.94 | y = 309500.10x - 85786.37 | 0.9967 | 3.49 | 3.35 | 4.13 | 14.99 | 11.38 | | |
| | | | | | | 9.70 | 10.42 | 7.43 | 14.66 | > 15 | | |
| | | | | | | 26.94 | 26.79 | 0.55 | 14.20 | > 15 | | |
| **Lanosterol** | 14.59 | 393, 411, 426 | 0.13- 0.62 | y = 140037.54x - 9134.59 | 0.9904 | 0.13 | 0.15 | 10.67 | 5.63 | > 15 | 0.05 | 0.13/ 5.63 |
| | | | | | | 0.37 | 0.35 | 6.14 | 14.61 | > 15 | | |
| | | | | | | 0.62 | 0.63 | 1.90 | 9.26 | > 15 | | |
| | | | 1.03- 13.30 | y = 230785.03x - 24253.01 | 0.9991 | 1.03 | 0.95 | 8.55 | 7.51 | > 15 | | |
| | | | | | | 4.79 | 4.99 | 4.29 | 3.07 | > 15 | | |
| | | | | | | 13.30 | 13.25 | 0.36 | 5.17 | > 15 | | |

RT: Retention time, SIM: Selected ion monitoring (Characteristics ions). *Extracted ion (137) was used for quantification of squalene.

It is noteworthy that the developed method has improved LOD and LOQ for squalene, cholesterol, ergosterol and lanosterol in this biological matrix with respect to the method described in literature up to now [23]. The validated method has allowed the simultaneous quantification of squalene, cholesterol, ergosterol and lanosterol. Therefore, this method is proposed as a fast screening method for the identification of SBIs with activity against *T. cruzi*.

*3.3. Application of the method to real samples*

A possible target for the discovery of antichagasic drugs is the SBP. The inhibition of any of the enzymes involved in this pathway would cause accumulation of its substrate and the depletion of the corresponding product which could cause the death of the parasite.

The inhibitory capacity of anti-*T. cruzi* compounds on some enzymatic reactions of the SBP was evaluated by the GC-MS method in Tulahuen 2 and Y strains. Squalene, lanosterol and ergosterol were quantitatively determined in real samples. Although squalene-2,3-epoxide has been detected, it has not been possible to quantify its concentration because it was below its LOQ

**Table 3** shows the results obtained by the new validated GC-MS method in samples of untreated Tulahuen 2 strain epimastigotes (NC), those treated with the reference drug Tbf and those treated with 8 compounds with *in vitro* anti-*T. cruzi* activity. Tbf showed squalene and lanosterol accumulation and ergosterol depletion as expected because Tbf is a known SBI [38]. These results seem to corroborate the inhibitory activity of terbinafine on the enzymes squalene-2,3-epoxidase and CYP51 described in the literature [8, 39].

Five of the eight compounds (**1**, **2**, **4**, **5** and **7**) showed ergosterol depletion. Compounds **4** and **5** stood out for presenting the highest percentages of depletion, especially compound **4** because it showed the same depletion percentage as Tbf. Taking into account the SBP (**Figure 3**), these results appear to indicate that compounds **4** and **5** could be causing an inhibitory effect on Lanosterol 14-demethylase (CYP51) because a marked accumulation of lanosterol is observed in *T. cruzi* Tulahuen 2 strain epimastigotes.

**Table 3.** Squalene, Lanosterol and Ergosterol quantification in *Tulahuen 2* strain

| Code | Squalene | | | Lanosterol | | | Ergosterol | | |
|---|---|---|---|---|---|---|---|---|---|
| | µg/mL | A% | D% | µg/mL | A% | D% | µg/mL | A% | D% |
| **NC** | 1.15 | | | 0.13 | | | 1.30 | | |
| **Tbf** | 1.28 | 11.30 | - | 0.20 | 53.85 | - | 0.90 | - | -30.77 |
| **1** | 1.17 | 1.74 | - | 0.42 | 223.08 | - | 1.23 | - | -5.38 |
| **2** | 1.27 | 10.43 | - | 0.21 | 65.54 | - | 1.21 | - | -6.92 |
| **3** | 1.21 | 5.22 | - | 0.35 | 169.23 | - | 1.33 | 2.31 | - |
| **4** | 1.16 | 0.87 | - | 0.21 | 61.54 | - | 0.90 | - | -30.77 |
| **5** | 1.09 | - | -5.22 | 0.29 | 123.08 | - | 1.04 | - | -20.00 |
| **6** | 1.32 | 14.78 | - | 0.46 | 253.85 | - | 1.81 | 39.23 | - |
| **7** | 1.57 | 36.52 | - | 0.17 | 30.77 | - | 1.29 | - | -0.77 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **8** | 1.26 | 9.57 | - | 0.13 | - | - | 1.43 | 10.00 | - |

NC: Negative control, Tbf: Terbinafine, %A: accumulation percentage; %D: depletion percentage.

**Table 4** presents the results obtained in the Y strain *T. cruzi* epimastigotes. In this strain, Tbf showed the same behavior as in the Tulahuen 2 strain. **Table 4** shows that all compounds exhibited ergosterol depletion. Compounds **1**, **3**, **4**, **5**, **6**, **7** and **8** showed the greatest ergosterol depletion percentages, similar or even higher than those of Tbf. Compounds **1** and **4** produced squalene and lanosterol accumulation with consequent depletion of ergosterol. This behavior may suggest a Squalene-2,3-epoxidase and CYP51 inhibitory activity, enzymes involved in the squalene and lanosterol transformation. Compounds **3** and **8** showed squalene accumulation causing lanosterol and ergosterol depletion. This appears to indicate a Squalene-2,3-epoxidase inhibition, enzyme involved in the squalene transformation. Compounds **5** and **6** showed lanosterol accumulation, so they could be acting as possible CYP51 inhibitors. Compound **7** showed ergosterol depletion without squalene or lanosterol accumulation, suggesting a possible inhibition of prior enzymes to the squalene biosynthesis.

**Table 4.** Squalene, Lanosterol and Ergosterol quantification in Y strain**.**

| Code | Squalene | | | Lanosterol | | | Ergosterol | | |
|---|---|---|---|---|---|---|---|---|---|
| | **µg/mL** | **A%** | **D%** | **µg/mL** | **A%** | **D%** | **µg/mL** | **A%** | **D%** |
| **NC** | 1.03 | | | 0.24 | | | 1.82 | | |
| **Tbf** | 1.29 | 25.24 | - | 0.36 | 50.00 | - | 1.31 | - | -28.02 |
| **1** | 1.31 | 27.18 | - | 0.32 | 33.33 | - | 1.10 | - | -39.56 |
| **2** | 1.21 | 17.48 | - | 0.23 | - | -4.17 | 1.70 | - | -6.59 |
| **3** | 1.13 | 9.71 | - | 0.24 | - | - | 1.01 | - | -44.51 |
| **4** | 1.35 | 31.07 | - | 0.52 | 116.67 | - | 1.21 | - | -33.52 |
| **5** | 1.02 | - | -0.97 | 0.37 | 54.17 | - | 1.01 | - | -44.51 |
| **6** | 0.96 | - | -6.80 | 0.37 | 54.17 | - | 1.04 | - | -42.86 |
| **7** | 0.97 | - | -5.83 | 0.20 | - | -16.67 | 0.97 | - | -46.70 |
| **8** | 1.26 | 22.33 | - | 0.13 | - | -45.83 | 1.41 | - | -22.53 |

NC: Negative control, Tbf: Terbinafine, %A: accumulation percentage; %D: depletion percentage.

The Y strain SBP seems to be more sensitive to the tested compounds than the Tulahuen 2 strain. This could be explained by the genetic differences between both strains [40].

## 4. Conclusions

In this paper we have reported a validated methodology for the quantification of neutral lipids in *T. cruzi* epimatigotes using GC-MS in SIM conditions. The new method achieved an important improvement in LOD and LOQ for squalene (0.31 and 0.85 µg/mL), squalene-2,3-epoxide (1.86 and 3.10 µg/mL), cholesterol (0.24 and 0.40 µg/mL), ergosterol (0.16 and 0.75 µg/mL) and lanosterol (0.05 and 0.13 µg/mL), respectively. In addition, the method is linear in two ranges for all the neutral lipids studied except for squalene-2,3-epoxide which has a single linear range, thereby

enhancing the results published so far. The novelty of this method is that it provides a linear range for the quantification of squalene-2,3-epoxide. However, the squalene-2,3-epoxide concentration was below its LOQ in real samples. Hence, it is suggested that the concentration of epimastigotes in the culture medium should be increased in order to obtain quantifiable values. The method has been proven to be selective within-day, precise, accurate and with good recovery values. The innovation of this research is its applicability within the field of medicinal chemistry because simultaneous quantification of 4 neutral lipids essential for the viability of *T. cruzi* has been achieved.

This new method facilitates the identification of possible SBIs, being a rapid screening method for determining the possible mechanism of action of active compounds against the parasite. Therefore, its suitability as a screening methodology in the identification of possible SBIs in the discovery process for new drugs for treating CD has been demonstrated.

## Notes

The authors declare no competing financial interest.

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