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CHEMICAL SCIENCES

A green method for determination of ethanol in homeopathic medicines using thermal infrared enthalpimetry

KAROLINA C. SCHLOSSER, ALESSANDRA S. OLIVEIRA, MARIANE B. FAGUNDES, ROGER WAGNER, RENIUS O. MELLO, JULIANO S. BARIN & FABIANA E.B. DA SILVA

Abstract: In this work, a simple and fast method is proposed for the determination of ethanol in homeopathic medicines using thermal infrared enthalpimetry (TIE). Samples containing alcohol in a wide concentration range (from 5% to 95% v/v) were used. Purified water or absolute ethanol was added directly into homeopathic medicine and increase of temperature was monitored using an infrared camera. Total volume, stirring speed and dispensing rate of solutions were the most significant parameters studied for method optimization. A response surface methodology (RSM) was used for optimization of the experimental conditions. The method was validated in the following parameters: selectivity, linearity, linear range, precision (repeatability and intermediate precision), limit of detection and quantification, accuracy and robustness. Linear range was obtained from 4% to 55% (ethanol, v/v). The proposed method showed accuracy in agreement with the conventional one. The proposed method it was demonstrated a good alternative for determination of ethanol in homeopathic medicines, presenting low cost, fast analysis and agreement with the principles of green analytical chemistry.

Key Words: alcoholic solutions, green chemistry, thermography, pharmaceutical.

INTRODUCTION

Homeopathy is a complementary medical system developed in Germany more than 200 years ago by physician Samuel Hahnemann. In the processing of homeopathic liquid formulations for internal use, the active ingredients are subjected to successive dilutions and vigorous agitation with alcoholic solutions in accordance with Agência Nacional de Vigilância Sanitária (ANVISA) guidelines (ANVISA 2018). These alcoholic solutions are used in different concentrations throughout the formulation process of homeopathic medicine, with higher concentrations in the initial process (mother tincture) and 30% or 5% (ethanol, v/v) for final product (oral drop or single-dose solutions, respectively) (ANVISA 2011). In this way, ethanol used in such preparations should be considered as an active pharmaceutical ingredient and submitted to quality control in final product as it may cause acute and chronic toxic effects (Ferreira 2011, Huzar & Wodnicka 2013).

In this context, suitable information and monitoring regarding the concentration of ethanol are important parameters, mainly when these pharmaceuticals are prescribed for special populations such as children, pregnant women and elderly, or patients with relevant medical history, including liver problems and alcoholism (WHO 2009, EMA 2013).

In the last years, homeopathic medicine has been the focus of several studies about consumption (Clarke et al. 2015, Relton et al. 2017,

Waisse 2017) and therapeutic action (Shang et al. 2005, Mathie et al. 2014, Dowhring & Sundrum 2016, Unlu et al. 2017). There are few studies in literature related to quality control in these pharmaceutical dosage forms (Ravishankara et al. 2001, Tumir et al. 2010, Gorlowska et al. 2015, Holandino et al. 2017, Jadhlav et al. 2016) but none of them with regard to determination of ethanol.

In general, the determination of ethanol in pharmaceutical formulations is performed according to conventional methods using distillation/density or gas chromatography (GC) approaches, which require considerable analysis time, large amounts of sample, sophisticated equipment and specialized analysts (USP-NF 2017, Costa et al. 2019). Therefore, these methods are difficult to perform in routine analyzes, especially for small-scale homeopathic medicines production (pharmaceutical compounding).

TIE analysis has been proposed recently as an alternative to conventional methods for analysis of pharmaceuticals and food, using a simple, fast and efficient approach suitable for the implementation in routine (Barin et al. 2015). The technique is based on monitoring the temperature variation of a chemical reaction by using an infrared camera. Commonly available microplates are used as reactors and reagents are added using multichannel pipettes. TIE was applied for determination of total, fixed and volatile acidity in vinegars and for simultaneous determination of acidity and salt content of pickled vegetable brine (Tischer et al. 2017a, b) obtaining good agreement with conventional method. TIE was also employed for determination of the alcoholic content in distilled beverages and wines (Oliveira et al. 2017, 2018). In these studies, sample preparation was not required, and features as shorter time for analysis and reduced energy consumption were observed in comparison with conventional method. In addition, TIE showed high sample throughput (up to 480 samples per hour), showing potential for routine analyzes.

A relevant aspect for development of new analytical methods is the reduction of toxic reagents and waste generation, dangerous to human health and/or the environment (Marco et al. 2019). Green analytical chemistry (GRAC), in turn, implements processes to reduce or eliminate the use or generation of harmful substances. It is guided by twelve principles aiming at sustainable chemistry (Galuska et al. 2013), which could be reached in a wide range by using TIE. Therefore, TIE could be considered a simple and greener alternative to conventional analytical methods described in official compendia, as pharmacopeias (Dalla Nora et al. 2019).

In the present work a green analytical method is proposed using TIE for determination of ethanol in the different stages of homeopathic medicines production, in which limitations such as time of analysis, sample volume, high cost, among others, could be overcome. A response surface methodology (RSM) based on Statistical Design of Experiments (DoEs) was used for optimization of experimental conditions. The proposed TIE method was validated and the results were compared to those obtained from conventional method.

MATERIAL AND METHODS

Instrumentation

For the TIE analysis a long-wave infrared camera (7.5–13.0 µm, FLIR E60 model, FLIR, Wilsonville, OR, USA) was used, which provided images of 320×240 pixels at a frame rate of 30 Hz. Images were processed using ResearchIR software (version 3.5, FLIR). The reactions were performed in disposable polystyrene 48-wells microplates

with internal volume of 1.6 mL (Nest Biotech, China). An electronic multichannel pipette (8 channels, 0.050-1.2 mL, Pro Research 1200, Eppendorf, Germany) was used for simultaneous addition of reagents into the wells. The homogenization of solutions was performed using a polytetrafluoroethylene-covered stir bar (3.0 x 6.5 mm) and a magnetic stirrer (Centauro, Atuba, Brazil).

Samples were also analyzed by gas chromatography instrument equipped with a flame ionization detector (GC-FID) Varian 3400 (Palo Alto, CA, USA). Separation was achieved using DB-5 (non-polar phase; cross-linked; 5% phenyl 95% dimethylpolysiloxane) capillary column with the following dimensions 30 m × 0.32 mm ID, ft = 0.25 µm (Agilent, Waldbronn, Germany).

Samples, standards, and reagents

Samples of homeopathic medicines were compounding with alcohol content of 5, 30, 70 and 95% (v/v), as showed in Table I. The different ethanol content was defined in accordance to usual concentrations found in homeopathic medicines. Low concentrations (e.g., 5%, v/v) are used in single-dose homeopathic medicines, 30% (v/v) is often found in oral drops, and high concentrations (70-95%) are used to prepare mother tinctures. Samples of homeopathic medicines without ethanol (blank sample) were used for selectivity/matrix effects studies. Absolute ethanol (LabSynth, Diadema, Brazil) and purified water (obtained in a Milli-Q system Direct-Q 3 UV, 18.2 MΩ cm, Millipore Corp., USA) were used as reagents to achieve the heat of dilution in the TIE method. Purified water was also used to dilute samples of homeopathic medicines. Hydrogen (purity > 99.9%) was used as the carrier gas in the chromatography analysis.

Table I. Homeopathic medicine samples.

Samples	Active substance	Alcoholic content (v/v)
01	*Ignatia amara*	5%
02	*Matricaria chamomilla*	
03	*Nux* vômica	
04	*Atropa belladona*	30%
05	*Baryta carbonica*	
06	Bryonia alba	
07	*Arnica montana*	70%
08	*Avena sativa*	
09	Calendula officinalis	
10	*Chelidonium majus*	95%
11	*Hamamelis virginiana*	
12	*Passiflora incarnata*	

Conventional analysis

In order to compare the results obtained with the proposed TIE method, samples were also analyzed by GC-FID in accordance with United States Pharmacopoeia – National Formulary (USP-NF) with modifications (USP-NF 2017). The method (Alcohol determination <611>, Method IIB – USP-NF) was revalidated in the following parameters: linearity, precision (repeatability and intermediate precision) and accuracy (ANVISA 2017). Butyl alcohol (0.05% v/v) was added as internal standard. Samples of ethanol (0.3%, v/v) were prepared and then injected into a split/splitless injector, operating in split mode with a 50:1 ratio at a temperature of 150 °C. Hydrogen was used as the carrier gas at a constant pressure of 10 psi. The temperature program was initially 90 °C for 2 minutes, with an increase to 230 °C at a rate of 15 °C min^{-1} and was maintained in isothermal condition for 5 minutes. The detector temperature was set at 250 °C. The quantification was based in an external calibration curve with five equidistant analytical points ranging from 0.05 to 0.75% (v/v) of ethanol.

Analytical method development

In TIE, disposable devices (e.g., microplates) were used for reactions, while an infrared camera, without contact, simultaneously monitors the temperature of multiple reactions. In this study, the microplate was positioned in front of camera (40 cm of distance) and the reagent (ethanol or purified water) was injected to eight wells simultaneously. The temperature was monitored before and after the reagent injection. Using the IR camera software, a circle of 180 pixels (corresponding to 7.1 mm of diameter) was used to monitor the temperature of each well, with the average of temperatures plotted to form an enthalpogram. The difference in temperature (ΔT) was obtained from equation $\Delta T = T_f - T_i$, where T_f and T_i are the final and initial temperatures, respectively. Eight wells of the microplate were used for each experiment (n = 8).

After the construction of analytical curves, the homeopathic samples were evaluated using the same procedure. For samples with theoretical content lower than 30% (ethanol, v/v), ethanol was added as reagent to obtain ΔT (curve A). For samples with theoretical content equal or higher than 30% (ethanol, v/v), purified water was added to obtain the ΔT (curve B). The samples containing 70 to 95% (ethanol, v/v) were diluted (1:1) directly into the microplate with purified water. This dilution procedure was performed in order to work within the linear range. Q-Test (Dixon) was used to identify and exclude outliers among the replicates.

Method optimization and robustness

The proposed TIE method was optimized by RSM based on DOEs. The experiments were performed to evaluate the influence of total volume in the wells (x_1), the stirring speed (x_2) and the dispensing rate (x_3) of multichannel pipette based on rotatable Central Composite Design (CCD). Dependent variable (y) was represented by the relative standard deviation (RSD, n = 8) from calibration solution containing 40% ethanol (v/v). The design model was a quadratic fit using 18 design runs, of which 4 were repeats of the center point of the model (2^3 + 6 + 4 = 18). The rotatable CCD is listed in Table II. All experiments were performed in randomized order. Statistical analysis was performed by Statistica 7.0 software (StatSoft, Inc., 2004, USA). Estimation of experimental error and validity of polynomial models were obtained by repetition of experimental points.

RSM was also used to study method robustness. Robustness testing was performed in order to obtain information about as small changes in the procedure could affect

Table II. Central composite rotatable design arrangement.

Symbol	Parameters	Levels				
		-1,682	-1	0	+1	+1,682
X_1	Total volume (µL)	360	600	950	1300	1540
X_2	Stirring speed (rpm)	50	100	175	250	300
X_3	Dispensing rate (mL s^{-1})	0.36	0.41	0.5	0.63	0.71

n=8

the proposed TIE method. The parameters considered for robustness testing were: total volume from 900 to 1100 µL, stirring from 150 to 200 rpm and dispensing rate from 0.46 to 0.57 mL s^{-1}. Dependent variable was represented by RSD (n = 8) from calibration solution containing 40% (ethanol, v/v).

Validation procedure

The method was validated according to ANVISA and Instituto Nacional de Metrologia, Qualidade e Tecnologia (INMETRO) specifications, which recommend the following parameters: selectivity/matrix effect, linearity, linear range, precision (repeatability and intermediate precision), limit of detection (LOD), limit of quantification (LOQ), accuracy and robustness (INMETRO 2010, ANVISA 2017).

To determine the matrix effect were carried out two experiments: (a) analysis of solutions containing ethanol 5% (v/v) added by glycerol solutions (1 to 5%, v/v), since chemical structure of glycerol (presented in mother tinctures) is similar to ethanol; and (b) analysis of mother tincture (randomly selected) diluted without ethanol. In this situation, the ΔT of this reaction was compared to those of a reaction using only purified water.

The linearity was studied in two ranges of ethanol concentration: (a) calibration solutions containing 4-12% (ethanol, v/v) expressed as analytical curve A; and (b) calibration solutions containing 30-55% (ethanol, v/v) expressed as analytical curve B, which are shown in figure 1. The linearity was evaluated by linear regression analysis using least-square regression method. The data were evaluated by ANOVA test using independent analytical curves.

Repeatability (intra-day precision) and intermediate precision (inter-day precision) were evaluated by RSD of eight replicates using Nux vômica (ethanol 5%, v/v) and Avena sativa (ethanol 70%, v/v) samples, which were randomly selected. Intra-day precision was evaluated with the same analyst and equipment. Inter-day precision was evaluated under different conditions (different analysts on two different days).

Accuracy was evaluated by adjustment of linear regression equation obtained from the results obtained by GC-FID and TIE methods. The regression parameters evaluated by *F*-test under the following hypothesis test: H_0: β_0=0 and β_1=1 where, H_0=null hypothesis, β_0=intercept of the linear regression between the observed and the found ethanol content, and β_1=slope of line. If H_0 is not rejected, it can be concluded that the method predicts the observed values with similarity.

The detection and quantitation limits (LOD and LOQ, respectively) were based on the mean concentration and standard deviation of ten blank replicates. The detection and quantitation limits were expressed as LOD = $\hat{X}$ + 3.3σ and LOQ

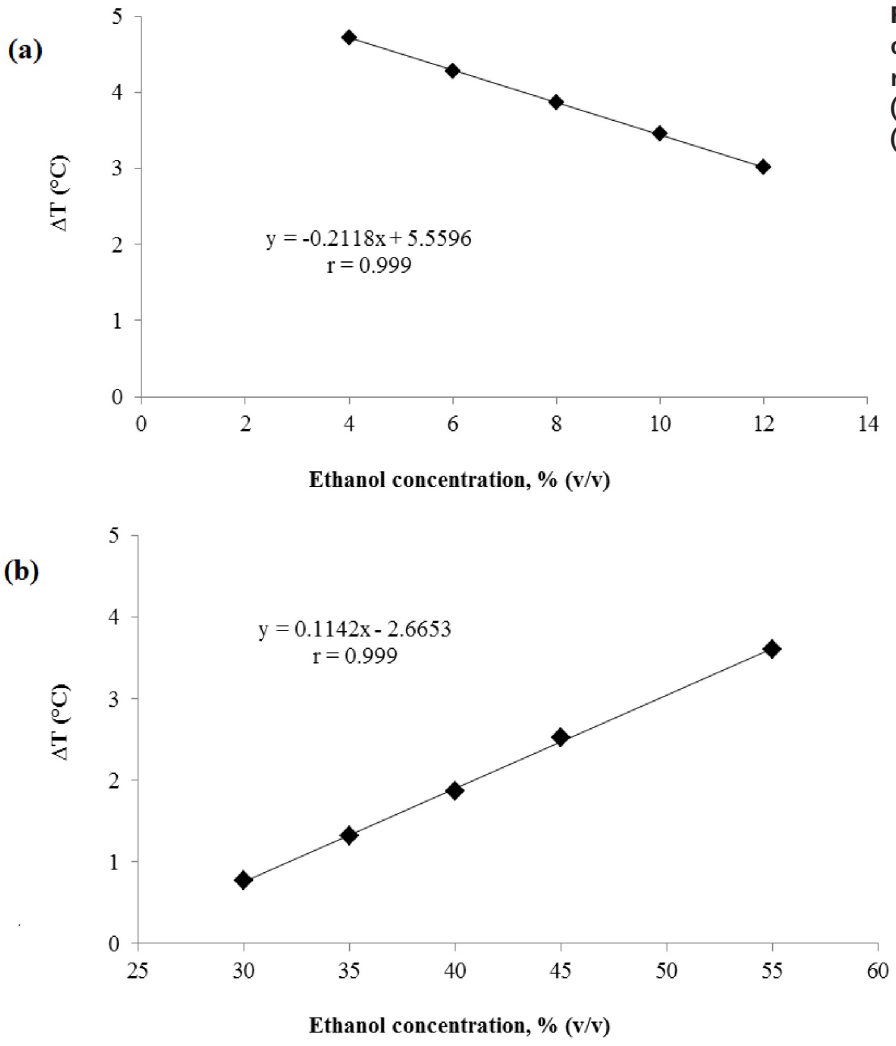

Figure 1. Analytical curves obtained using hydroalcoholic reference solutions: (a) 4-12% (ethanol, v/v) and (b) 30-55% (ethanol, v/v) (n=8).

= $\bar{X}$ + 10σ (where $\bar{X}$ = the mean concentration of blank replicates, σ = the standard deviation of the blank replicates, n=10). As curve A had a decreasing profile, it is also important to establish the maximum concentration detected. This value was obtained by the formula LOD = 3.3σ / S (where σ = the standard deviation of the blank replicates, S = the slope of the calibration curve), and considered a reasonable signal to distinguish the temperature rise from noise.

RESULTS AND DISCUSSION

Optimization of TIE

For optimization of experimental parameters, the influence of total volume (x_1), stirring speed (x_2) and dispensing rate (x_3) were investigated by RSM. Equation obtained from the model was with the form:

$$y_1 = 0.378 - 0.539x_1 - 0.025x_2 - 0.019x_3 + 0.232x_1^2 + 0.392x_2^2 + 0.164x_3^2 - 0.041x_1x_2 - 0.087x_1x_3 - 0.195x_2x_3$$

The combination effects of experimental parameters can be observed in the RSM graphs (Figure 2). For total volume vs. dispensing rate graph (Figure 2a), the best conditions were

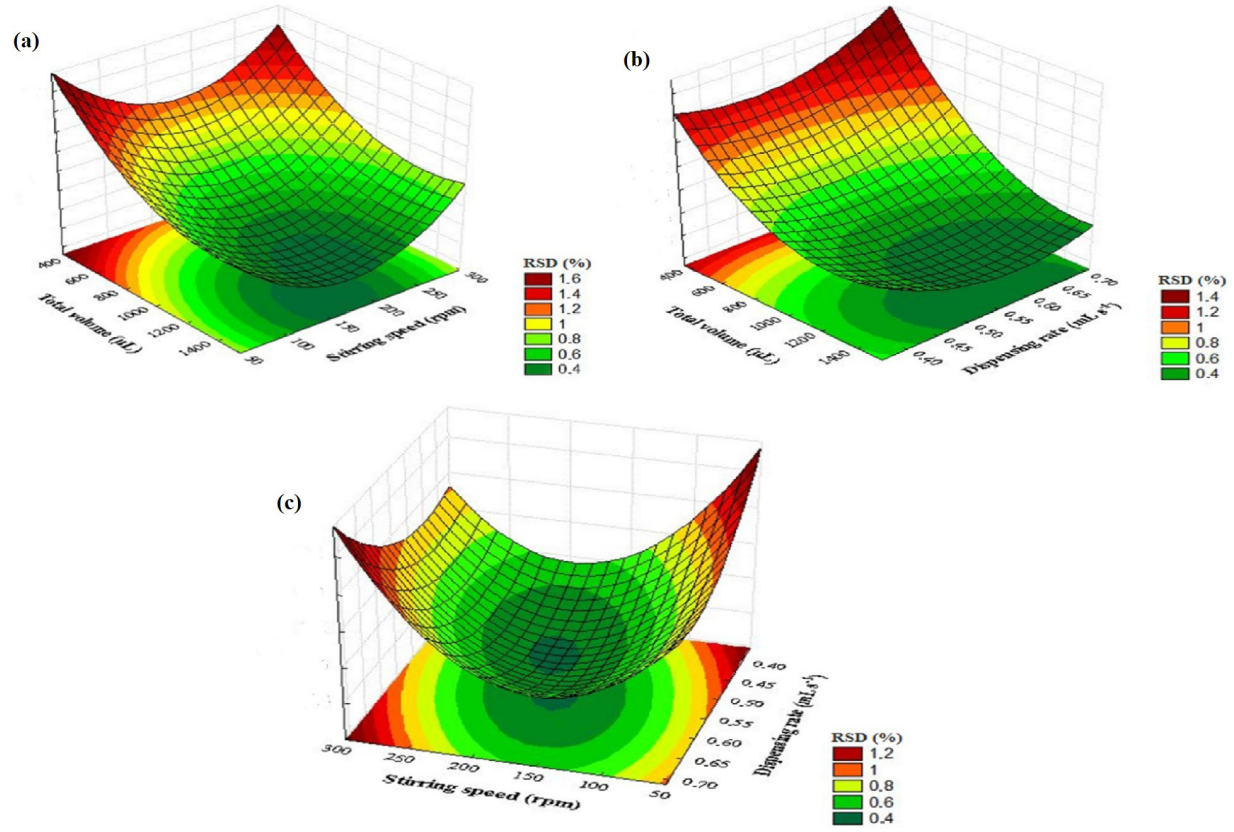

Figure 2. RSM based on DOE to the following parameters: (a) total volume vs. dispensing rate; (b) total volume vs. stirring speed; and (c) stirring speed vs. dispensing rate.

found increasing volume and dispensing rate, since lower volumes (<900 µL) presented worse analytical signals and, consequently, high RSD. Lower dispensing rates (<0.41 mL s^{-1}) impaired heat release due to slow reagent addition. Moreover, according to Oliveira et al. (2018), dispensing rate ≤0.46 mL s^{-1} increase the RSD due to the presence of the pipette tip in front of the camera during mixing of solutions. For total volume vs. stirring speed (Figure 2b), it was observed that very low (<100 rpm) stirring speeds may impair efficient homogenization of solutions and above 250 rpm increase RSD by overflow of solutions. Therefore, higher volumes (>900 µL) combined with intermediate stirring speed (100-250 rpm) are recommended. In Figure 2c, stirring speed vs. dispensing rate graph showed lower RSD in intermediate region (central position). The result obtained showed that parameters are dependent since the high level of one can be compensated by the low level of other experimental conditions. In accordance with exposed above, 1000 µL for total volume, 0.5 mL s^{-1} for dispensing rate and 175 rpm for stirring speed were considered for further experiments. The results agree with optimization parameters obtained in other studies involving TIE (Tischer et al. 2017a, b, Oliveira et al. 2017, 2018).

Figures of merit

Selectivity and matrix effect evaluation

Matrix effect was evaluated in two experiments. In the first experiment, the following solutions were analyzed: ethanol solution 5% (v/v) + glycerol solution 1% (v/v); ethanol solution

5% (v/v) + glycerol solution 2% (v/v); ethanol solution 5% (v/v) + glycerol solution 3% (v/v); ethanol solution 5% (v/v) + glycerol solution 4% (v/v); ethanol solution 5% (v/v) + glycerol solution 5% (v/v). All solutions maintained an analytical signal (ΔT) similar to ethanol solution 5% (v/v) without glycerol, demonstrating no interference in quantification of ethanol (no signal suppression or enhancement).

In the second experiment, the analysis of mother tincture showed that matrix is not detectable, since the analytical signal obtained was equal to the purified water (data no shown).

It is important to highlight that the proposed method does not require sample preparation and mother tinctures (containing concentrated components and glycerol) could result in a matrix effect. For this reason, the proposed method was considered selective for ethanol determination in homeopathic medicines because the matrix effect was not observed.

Linearity

A linear response was observed in the range of 4 to 12% (Figure 1a) and 30 to 55% (Figure 1b), with high correlation coefficient (r = 0.9998). Dissolution of ethanol in water is an exothermic process across the whole range of concentrations, probably due to decreasing total numbers of hydrogen bonds for the mixture in relation to pure solvent. For this reason, a reduced amount of water leads to lower heat generation by dilution. This way, in curve A ethanol was added as reagent and profile showed decreasing trend. Likewise, in curve B purified water was added as reagent and profile showed increasing trend.

According to ANOVA test, the data showed significant linear regression and no linearity deviation. Therefore, a linear range was feasible for determination of ethanol in homeopathic formulations within a large range of ethanol concentration.

Precision (repeatability and intermediate precision)

Precision was evaluated at two levels: (a) repeatability (intra-day, n=8) and (b) intermediate precision (inter-day, n=8). For repeatability, sample with 5% ethanol (*Nux vomica*) showed RSD value of 2.35% and sample containing 70% ethanol (*Avena sativa*) showed RSD below 0.12% (Table III). For intermediate precision, RSD values of 2.35 and 2.25% for *Nux vomica* and 0.12 and 0.21% for *Avena sativa* samples were observed for days 1 and 2, respectively. Therefore, a good agreement between the two experiments performed independently was obtained despite the use of different analyst and day of analysis under identical conditions. It is important to highlight that the proposed method presented deviations in the same level or lower than those presented by conventional (GC-FID) and TIE methods (Barin et al. 2015, Tischer et al. 2017a, b, Oliveira et al. 2017, 2018). Therefore, the proposed TIE method could be considered a suitable alternative for the determination of ethanol in these samples.

Detection and quantitation limits

Limits of detection (LOD, $\hat{X}$ + 3.3σ) and quantification (LOQ, $\hat{X}$ + 10σ) were calculated considering the mean and standard deviation of the blank replicates. For the curve A, LOD=1.77% (ethanol, v/v) and LOQ=1.93% (ethanol, v/v) were obtained. In this way, TIE can be applied in single dose homeopathic formulations, which normally present concentrations of 5.0% (ethanol, v/v). Maximum ethanol concentration detected in curve A was 28.06% (v/v).

To perform determination of ethanol in samples with alcohol content of 30.0% or

Table III. Precision (repeatability and intermediate precision) evaluated by RSD (%) (n = 8).

Samples	RSD	
	Day 1[a]	Day 2[b]
Samples with 5% ethanol		
Ignatia amara	5.77	
Matricaria camomila	3.61	
Nux vômica	2.35	2.25
Samples with 30% ethanol		
Atropa beladona	0.11	
Baryta carbonica	0.14	
Bryonia alba	0.10	
Samples with 70% ethanol		
Arnica montana	0.16	
Avena sativa	0.12	0.21
Calendula oficinalis	0.08	
Samples with 95% ethanol		
Chelidonium majus	0.10	
Hamamelis virginiana	0.25	
Passiflora incarnata	0.27	

[a]**Repeatability.**
[b]**Intermediate precision.**

above, curve B is recommended. For curve B, LOD=19.55% (ethanol, v/v) and LOQ=19.58% (ethanol, v/v) were obtained. These values were suitable for quantification of ethanol in mother tinctures and finished homeopathic medicines (oral drops).

Accuracy

Accuracy was evaluated by comparison of the results with the conventional method as showed in Table IV. *Calendula officinalis* sample showed discrepant values, and for this reason, excluded from statistical analysis. Figure 3a shows scatter plots of observed vs. predicted values (obtained by GC-FID and TIE methods) and figure 3b shows ordinary residues. A high coefficient of determination (0.9997) was observed without tendency, making acceptable the null hypothesis (H0: $\beta 1=1$). Thus, the TIE method proved to have good accuracy for quantification of ethanol in homeopathic medicines.

Table IV. Ethanol content (%, v/v) in homeopathic medicines (mean ± standard deviation) by TIE (n = 8) and GC-FID (n = 3).

Samples	TIE	GC-FID
Samples with 5% ethanol		
Ignatia amara	6.24±0.36	5.39±0.35
Matricaria camomila	5.98±0.22	5.52±0.18
Nux vômica	6.15±0.14	5.37±0.25
Samples with 30% ethanol		
Atropa beladona	28.85±0.03	29.33±1.04
Baryta carbonica	26.59±0.04	28.14±1.25
Bryonia alba	28.88±0.03	29.27±2.30
Samples with 70% ethanol		
Arnica montana	67.26±0.05	68.41±1.84
Avena sativa	68.02±0.04	68.80±1.12
Calendula oficinalis	64.52±0.03	69.98±2.37
Samples with 95% ethanol		
Chelidonium majus	95.01±0.05	94.42±2.15
Hamamelis virginiana	94.04±0.12	96.03±1.81
Passiflora incarnata	93.96±0.13	94.16±2.21

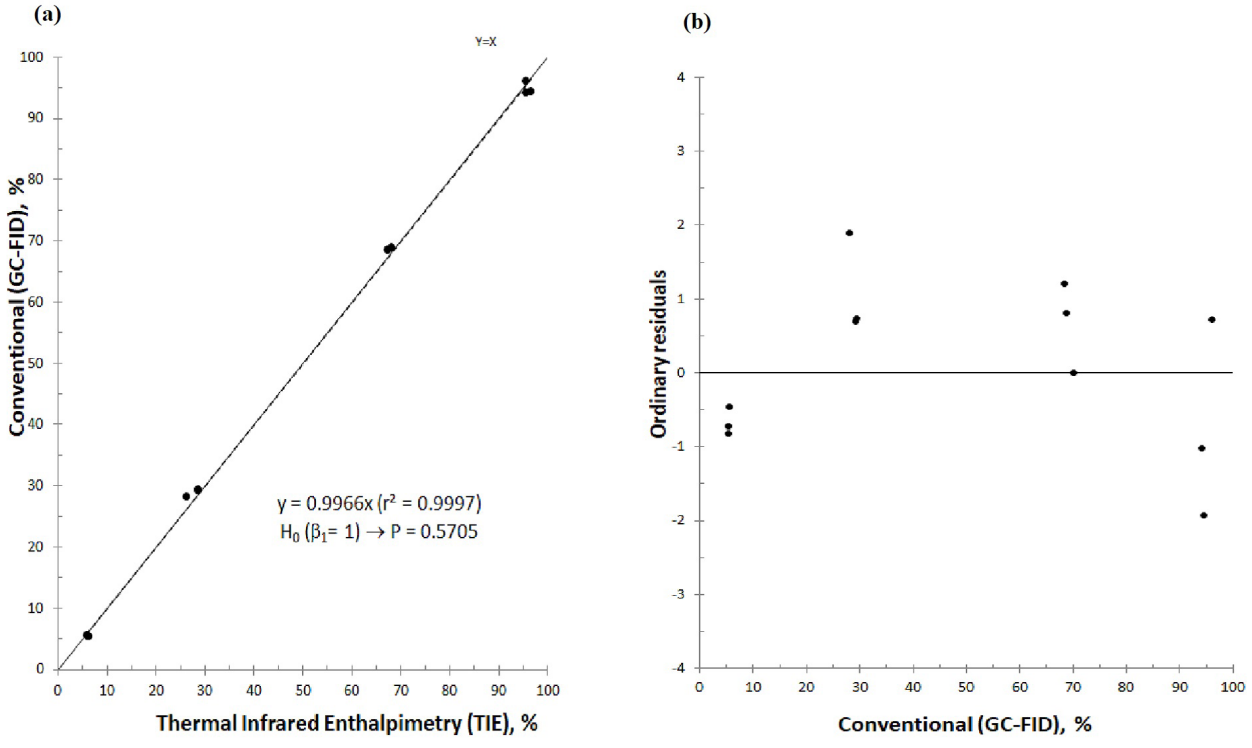

Figure 3. Scatter plots of (a) observed vs. predicted values and (b) dispersion of the ordinary residues.

Robustness

Robustness was investigated using the RSM approach (Figure 2), considering smaller variations to provide an indication of its reliability during routine use. The results indicated no relevant difference within the parameters studied, since RSD values did not exceed 0.6% (considered suitable for this study). Therefore, it can be concluded that TIE method is robust within the ranges evaluated: total volume for 900 to 1100 µL (Figure 2a), stirring speed for 150 to 200 rpm (Figure 2b) and dispensing rate from 0.46 to 0.57 mL s^{-1} (Figure 2c). In this way, it was possible to determine the robust domain (tolerable variations) using RSM.

CONCLUSION

The proposed TIE method could be considered as a promising tool for fast ethanol determination in homeopathic medicines and it could be used in routine analyzes of these pharmaceuticals in a wide range (from 5% to 95% v/v). In addition, it presents low cost, quick analysis and agreement with the principles of green analytical chemistry, contrarily to the official methods.

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KAROLINA C. SCHLOSSER[1]
https://orcid.org/0000-0002-7348-1664

ALESSANDRA S. OLIVEIRA[2]
https://orcid.org/0000-0003-1820-0126

MARIANE B. FAGUNDES[2]
https://orcid.org/0000-0001-7210-2290

ROGER WAGNER[2]
https://orcid.org/0000-0002-6176-7913

RENIUS O. MELLO[2]
https://orcid.org/0000-0002-5934-7784

JULIANO S. BARIN[2]
https://orcid.org/0000-0002-8351-3287

FABIANA E.B. DA SILVA[1]
https://orcid.org/0000-0002-1185-0671

[1]Programa de Pós-graduação em Ciências Farmacêuticas/ PPGCF, Universidade Federal do Pampa, Campus Uruguaiana, Prédio 700, Br 472, Km 585, Caixa Postal 18, 97500-220 Uruguaiana, RS, Brazil

[2]Universidade Federal de Santa Maria, Departamento de Tecnologia e Ciência de Alimentos/DTCA, Centro de Ciências Rurais/ CCR, Av. Roraima, 1000, 97105-900 Santa Maria, RS, Brazil

Correspondence to: **Fabiana E.B. da Silva**
E-mail : fabianasilva@unipampa.edu.br

Author contributions

Karolina C. Schlosser, Alessandra S. Oliveira and Mariane B. Fagundes carried out the TIE studies and drafted the manuscript. Roger Wagner carried out the GC studies. Renius O. Mello participated in the design of the study and performed the statistical analysis. Karolina C. Schlosser, Juliano S. Barin and Fabiana E.B. da Silva conceived of the study, participated in its design, coordination and helped to draft the manuscript. All authors read and approved the final manuscript.