

Desirability Function in analytical method development for determination of glitazones and metabolites employing HF-LPME

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Thiazolidinedione, often shortened to TZD or glitazone, helps lower insulin resistance, which is the underlying problem for many people with type 2 diabetes. The two most known glitazones are pioglitazone (PGZ), with the brand name medicine Actos[®], and rosiglitazone (RSG), which is Avandia[®]. This study presented a multivariate optimization in the microextraction procedure employing Fractional Factorial Design (FFD) combined with Desirability Function (DF) to determine TZD and metabolites in biological samples. Microextraction requires several parameters to be optimized; however, most of them still use univariate optimization. Finding optimum conditions by simple response is relatively simple, but the problems, in case of microextractions, are often more complex when it has more responses. For example, changing one factor that promotes one response may suppress the effect of the others. Thus, this multivariate optimization was applied for two bioanalytical methods for determination of TZD and metabolites, one by HPLC and other by CE, both using Hollow Fiber Liquid-Phase Microextraction (HF-LPME). The results establish the optimal values and elucidate how the factors that affect HF-LPME procedure perform in extraction efficiency for TZDs. Additionally, this study demonstrates that DF can be an important tool to optimize microextraction procedures.

Keywords: Glitazones. Hollow-fiber liquid-phase microextraction. Fractional Factorial Design. Desirability Function.

INTRODUCTION

Thiazolidinediones (TZDs), commonly referred to as glitazones, are a class of oral agents used to treat Type-2 diabetes mellitus. The mechanism of action of these hypoglycemic agents involves reducing plasma glucose concentrations by enhancing the insulin sensitivity through the activation of the peroxisome proliferator-

activated receptor (PPAR) γ . In addition, TZDs are the most expensive class of hypoglycemic medications and are indicated either as monotherapy or in combination with metformin or with sulphonylureas. Two thiazolidinediones (rosiglitazone – RSG [Avandia] and pioglitazone - PGZ [Actos]) are the most known TZD medications, but are marketed only in some countries, including United States. Several countries banned these drugs after an investigation concluded the benefits of glitazone no longer outweighed the risks (Inzucchi, 2002).

PGZ is metabolized in the human liver into several products, designated M-I to M-VI. Four primary (M-I, M-II, M-IV, and M-V) and two secondary metabolites (M-III and M-VI) have been described, with M-III (keto derivative of PGZ) being a product of M-IV (hydroxyl

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derivative of PGZ). The main PGZ metabolites found in human serum are M-III and M-IV, both pharmacologically active (with anti-hyperglycemic potency of about 40–60% of PGZ) (Muschler *et al.*, 2009; Shen *et al.*, 2003). The two major metabolites of RSG are *N*-desmethyl rosiglitazone (*N*-Dm-R) and ρ -hydroxy rosiglitazone (ρ -OH-R) (Cox *et al.*, 2000). The specific P450 enzymes involved in the metabolism of RSG are primarily cytochrome 2C8 (CYP2C8), with minor contributions from CYP2C9 (Baldwin, Clarke, Chenery, 1999).

Hollow Fiber Liquid-Phase Microextraction (HF-LPME), described for the first time in 1999, is a sample preparation technique for high-performance liquid chromatography (HPLC), capillary gas chromatography (GC), and capillary electrophoresis (CE). This microextraction has several advantages over other extraction methods: (1) it is very simple and inexpensive, (2) it provides excellent clean-up, (3) it leads to very high preconcentration, (4) carry-over problems are eliminated, (5) it is inexpensive, (6) it has low consumption of organic solvent, and (7) by applying a multi-stirrer, many samples could be extracted simultaneously (Rasmussen, Pedersen-Bjergaard, 2004; de Oliveira *et al.*, 2008).

HF-LPME is an equilibrium process, which is influenced by important factors, such as the type of organic solvent, volume of donor and acceptor solution, extraction time, stirring rate, sample and donor pH, sample temperature, and the addition of salt. Several papers have optimized the HF-LPME conditions by employing successive variations in variables such as a one-factor-at-a-time (OFAT). Although it is well accepted, the process is relatively time-consuming and expensive for a large number of variables and frequently fail to predict the optimum condition. The major drawback of the OFAT approach is the lack of inclusion of the interactive effects among variables (Rasmussen, Pedersen-Bjergaard, 2004; de Oliveira *et al.*, 2008; Magalhães *et al.*, 2010; Magalhães *et al.*, 2008; de Santana, Bonato, 2008; Cai *et al.*, 2016). Experimental design allows estimation of the effect of several variables simultaneously. Fractional Factorial Design (FFD) enables identification of interactions between factors more accurately and can determine the effect of one factor among the other factors evaluated (Prado, Garrido, Periago, 2004; Nejad *et al.*, 2010).

Derringer and Suich found the solution to optimize multiple responses by developing the Desirability Function (DF), which converts multiple responses into a single one, combining the individual responses into a composite function followed by its optimization. Therefore, in the case of multiple response optimization, DF can be employed, as it is the most critical and most widely applied multi-criteria methodology in analytical procedures (Candiotti *et al.*, 2014; Asgharinezhad, Ebrahimzadeh, 2016). However, only Asgharinezhad and Ebrahimzadeh (2016) have employed this function to optimize HF-LPME. Here, we aim to compare the FFD combined with DF in two different bioanalytical methods for glitazones: 1) RSG employing HPLC, and 2) PGZ employing CE, both using HF-LPME as sample extraction. Then, these results were compared with results already obtained experimentally (Calixto, Bonato, 2013; Calixto, Bonato, 2010).

MATERIAL AND METHODS

Chemicals and reagents

PGZ and its metabolites (Keto-P and OH-P), and RSG and its metabolites (*N*-Dm-R and ρ -OH-R) were all obtained from Toronto Research Chemicals (North York, ON, Canada).

The reagents (analytical grade) employed in the capillary electrophoretic method were phosphoric acid, sodium dihydrogen phosphate, and sodium hydroxide, obtained from Labsynth (Diadema, SP, Brazil), Merck (Darmstadt, Germany) and Spectrum (New Brunswick, NJ, USA), respectively. All solutions used as CE running buffer, and in the CE, rinse cycle procedure were filtered through a Millex-HV 0.45 μ m disk filter from Millipore (Bedford, MA, USA) and degassed by ultrasound for 5 min. Water was purified with a Milli-Q plus system (Millipore).

For the analytical procedure, HPLC-grade acetonitrile was purchased from Tedia (Fairfield, OH, USA) and acetic acid (HPLC-grade) was obtained from J. T. Baker (Phillipsburg, NJ, USA). Water was distilled and purified using a Millipore Milli-Q-system (Milford, MA, USA).

For the HF-LPME procedure, *n*-octanol from Sigma-Aldrich (St. Louis, MO, USA) was used as extraction solvent. Sodium hydroxide and perchloric acid were from

Spectrum (New Brunswick, NJ, USA) and Nuclear (São Paulo, SP, Brazil), respectively. Hydrochloric acid was acquired from Mallinckrodt (Xalostoc, Mexico). Sodium dihydrogen phosphate and sodium hydrogen phosphate were obtained from Merck (Darmstadt, Germany).

Instrumentation and analytical conditions

CE procedure for PGZ and metabolites determination

CE experiments were performed on an Agilent CE system (Agilent Technologies, Waldbronn, Germany), model G1600A, consisting of an autosampler, a photodiode array detector (set at 190 nm), a temperature controlling system (4– 60 °C) and a power supply able to deliver up to 30 kV. A CE ChemStation software was used for instrument control, data acquisition and data analysis. The uncoated fused silica capillary (Microsolv, Eatontown, NJ, USA) employed had 50 μm I.D., 48 cm total length, and 40 cm effective length. The electrophoretic conditions were described in a previous work (Calixto, Bonato, 2013).

HPLC procedure for RSG and metabolites determination

Analyses were carried out using a chromatographic system from Shimadzu Corporation (Kyoto, Japan). This equipment consists of two LC-10 AD_{VP} pumps, an SPD-M10 A_{VP} UV/Vis detector (operating at 245 nm), a system controller SCL-10 A_{VP}, and a CTO-10 AS_{VP} column oven. Class VP software was used to control the LC system and for data acquisition. Injections were performed manually through a 20 μL loop with a Rheodyne model 7725i injector (Cotati, CA, USA). This study used, the X-Terra RP-18 column (100 mm \times 3.9 mm, 3.5 μm) with water: acetonitrile: acetic acid (85:15:0.5, v/v/v) as mobile phase, at 22 °C, under isocratic conditions, flow rate of 1.0 mL/min and detection at 245 nm. The chromatographic conditions were described in a previous work (Calixto, Bonato, 2010).

HOLLOW-FIBER LIQUID-PHASE MICROEXTRACTION PROCEDURE

Each extraction device consisted of two gel-loading round tips (Sorenson Bioscience, Salt Lake City,

UT, USA) connected to a 15 cm piece of Accurel PP Q3/2 porous polypropylene hollow fiber (Membrana, Wuppertal, Germany) and assembled in a polyurethane snap cap to avoid leakage during agitation. The inner diameter of the hollow fiber was 600 μm , the thickness of the wall was 200 μm , and the pore size was 0.2 μm (Calixto, Bonato, 2013; Calixto, Bonato, 2010).

To perform the HF-LPME extraction, the organic solvent was first impregnated in the membrane for 15 s, and the excess solvent was removed by 15 s of ultrasonication in water. Then the acceptor phase was introduced into the lumen of the membrane using a microsyringe and the device was dipped into the sample. The extraction was carried out using an Ika Vibrax VXR basic stirrer (Staufen, Germany). This system allows the extraction of 36 samples simultaneously. The biological samples optimized in this study consists of microsome preparation for metabolism studies. After extraction, the acceptor solution was withdrawn from the membrane using a microliter syringe and analyzed according to the method (Calixto, Bonato, 2013; Calixto, Bonato, 2010).

Multivariate optimization

To extract PGZ and RSG and their metabolites, basic analytes, a three-phase liquid-phase microextraction was selected. Experimental designs were conducted to evaluate the parameters that can interfere in the HF-LPME extraction. Based on the previous work already published, hydrochloric acid was selected as acceptor phase and octanol as organic solvent (Calixto, Bonato, 2013; Calixto, Bonato, 2010).

PGZ

FFD with four factors and two levels (eight experiments) was used to optimize the other parameters that can interfere in the HF-LPME extraction: donor phase pH (Low level: 6.0 – High level: 10.0), acid concentration (mmol/L) in the acceptor phase (Low level: 10.0 – High level: 100.0), extraction time (min) (Low level: 15 – High level: 45), and sample agitation (rpm) (Low level: 1000 – High level: 1500).

RSG

FFD with five factors and two levels (eight experiments) was used to optimize other parameters that can interfere in the HF-LPME extraction: donor phase pH (Low level: 6.0 – High level: 8.0), acid concentration (mMol/L) in the acceptor phase (Low level: 2.0 – High level: 50.0), extraction time (min) (Low level: 20 – High level: 40), sample agitation (rpm) (Low level: 1000 – High level: 1750), and sodium chloride addition (% *w/v*) (Low level: 0 – High level: 10).

The influence of each factor and their interaction with PGZ, RSG, and their metabolites for both analytical methods were evaluated by design space counter plot, and the desirability function were determined. These results were performed in Minitab 17.1.0 statistical program (State College, PA, USA).

RESULTS AND DISCUSSION

Selection of separation conditions

The electrophoretic conditions employed in this study were: capillary dimension of 47.5 cm (40 cm effective length) and 50 μm I.D., capillary pre-conditioning for 7 min (0.1 mol/L NaOH for 2 min, water for 2 min, and running buffer for 3 min), applied voltage of 30 kV, capillary temperature of 35 $^{\circ}\text{C}$, and detection at 190 nm. Sample introduction was carried out by hydrodynamic injection (50 mbar, 15 s). After eight runs, the running buffer was changed due to buffer depletion. This process presented in the Figure 1 and based on in a previously published work (Calixto, Bonato, 2013).

The chromatographic conditions employed in this study were: mobile phase consisting of water, acetonitrile, and acetic acid (85:15:0.5, *v/v/v*). The acetic acid was added after it was degassed ultrasonically, at a flow rate of 1.0 mL/min and detection at 245 nm, with the running time of 12 min, as presented in the Figure 2, and based on a previous work (Calixto, Bonato, 2010).

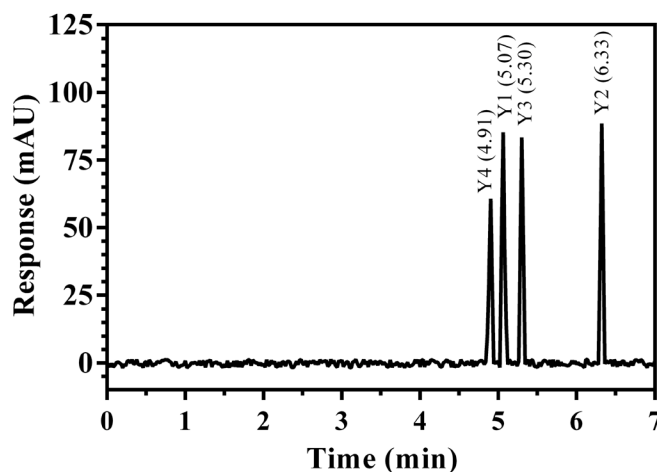


FIGURE 1 - Representative electropherogram of PGZ (Y1), Keto-P (Y2), OH-P (Y3), and RSG (Y4). RSG is the internal standard (IS). The optimized electrophoretic conditions were: uncoated fused silica capillary, 47.5 cm (40 cm effective length) \times 50 μm id.; hydrodynamic injection (50 mBar, 15 s), running buffer of 50 mmol/L phosphate buffer solution (pH 2.5); UV detection at 190 nm; capillary temperature of 35 $^{\circ}\text{C}$ and applied voltage of 30 kV.

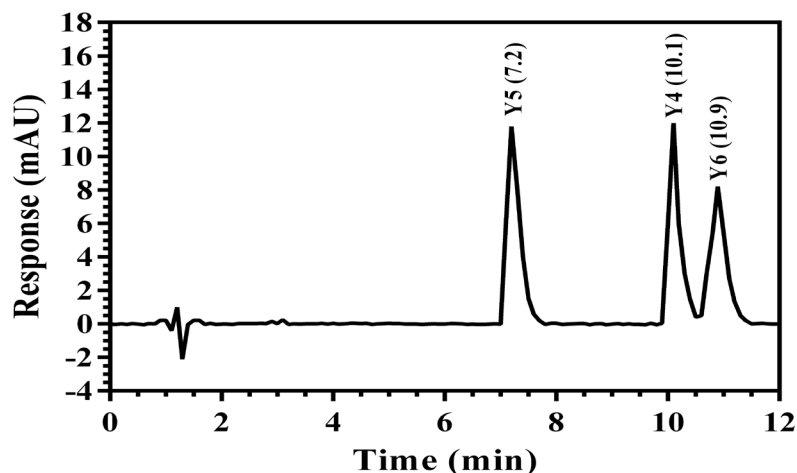


FIGURE 2 - Representative chromatogram of RSG (Y4), N-Dm-R (Y5), and p-OH-R (Y6). Chromatographic conditions: column X-Terra RP-18 column (100 mm × 3.9 mm, 3.5 μm) using water: acetonitrile: acetic acid (85:15:0.5, v/v/v) as mobile phase, at 22°C, under isocratic conditions, flow rate of 1.0 mL/min and detection at 245 nm.

Multivariate optimization

Fractional Factorial Design

Full Factorial, Fractional Factorial, and Plackett-Burman designs, all of them at two levels for each factor (k), are the most widely used to select the factors because they are economic and efficient. FFD allows the evaluation of a relatively large number of factors in a small number of experiments (Candiotti *et al.*, 2014). FFD is often used to select the most important input factors and discard the insignificant ones. In addition, FFD can obtain the main effects plots, which are useful to understand how each factor affects the response of interest (Fukuda *et al.*, 2018). In the context of this work, the main effects plots

were used to set up the analytical conditions that provide higher recovery values for each of the analytes.

The results obtained for the FFD with four factors and two levels (eight experiments) for PGZ and metabolites are presented in the Table I. Those for RSG and metabolites with five factors and two levels (eight experiments) are in the Table II.

A model contour plot (2D plot) for HL-LPME optimization for PGZ and its metabolites as well as RSG and its metabolites were presented in the Figures 3 and 4, respectively. The contour plot is a 2D response representing the impact of the factors that influenced the HF-LPME extraction, and the response evaluated was the efficiency extraction (above 20%) for all analytes.

TABLE I - Independent (X1, X2, X3, and X4) and recovery factor extraction (%) - dependent variables (Y1, Y2, Y3, and Y4), fractionate factorial design matrix, and results of experimental design for pioglitazone (PGZ) and its metabolites (Keto-P and OH-P)

Independent variables				Dependent variables			
X1	X2	X3	X4	Y1	Y2	Y3	Y4
pH	HCl (mmol/L)	Agitation (rpm)	Time (min)	PGZ	Keto-P	OH-P	RSG
6	10	1000	15	2.59	24.51	5.99	15.11

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TABLE I - Independent (X1, X2, X3, and X4) and recovery factor extraction (%) - dependent variables (Y1, Y2, Y3, and Y4), fractionate factorial design matrix, and results of experimental design for pioglitazone (PGZ) and its metabolites (Keto-P and OH-P)

Independent variables				Dependent variables			
X1	X2	X3	X4	Y1	Y2	Y3	Y4
pH	HCl (mmol/L)	Agitation (rpm)	Time (min)	PGZ	Keto-P	OH-P	RSG
10	10	1000	45	4.98	4.25	5.30	4.53
6	100	1000	45	37.89	43.56	28.18	20.22
10	100	1000	15	0.00	7.77	21.83	20.31
6	10	1500	45	4.09	40.42	10.21	26.30
10	10	1500	15	22.94	13.88	16.09	10.53
6	100	1500	15	27.22	42.95	17.46	19.82
10	100	1500	45	0.00	16.73	29.38	28.52

TABLE II - Independent (X1, X2, X3, X4, and X5) and recovery factor extraction (%) - dependent variables (Y4, Y5, and Y6), fractionate factorial design matrix, and results of experimental design for rosiglitazone (RSG) and its metabolites (*N*-Dm-R and *p*-OH-R)

Independent variables					Dependent variables		
X1	X2	X3	X4	X5	Y4	Y5	Y6
pH	HCl (mmol/L)	Agitation (rpm)	Time (min)	NaCl (%)	RSG	<i>N</i> -Dm-R	<i>p</i> -OH-R
6	50	1750	20	0	50.43	13.06	26.93
8	50	1750	40	10	63.88	31.45	38.57
8	2	1750	40	0	8.50	3.44	3.12
6	50	1000	40	0	14.61	2.69	2.87
8	2	1000	20	0	15.74	9.75	8.26
6	2	1750	20	10	1.33	0.35	0.00
6	2	1000	40	10	28.28	15.28	20.58
8	50	1000	20	10	27.54	7.38	4.93

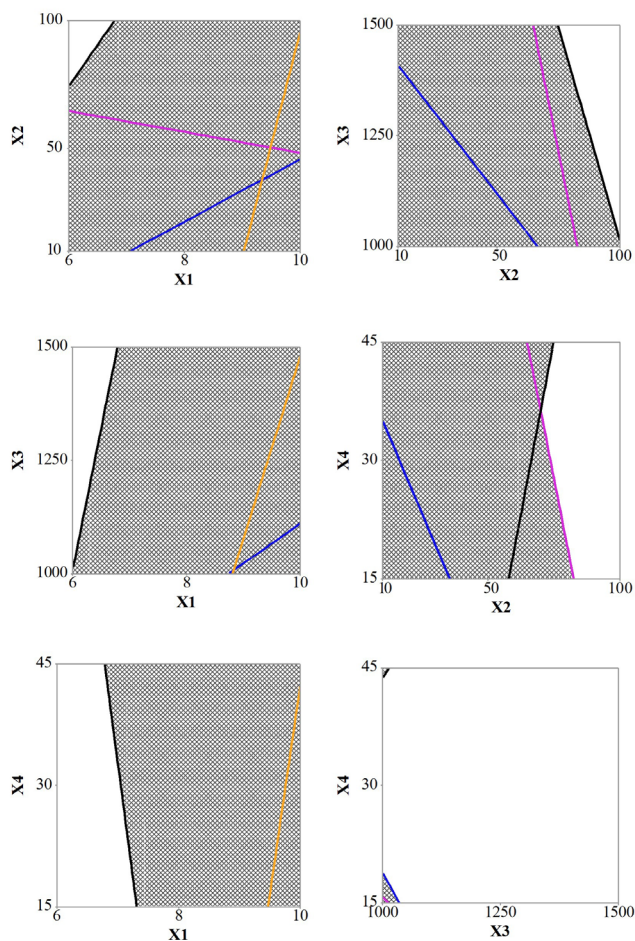


FIGURE 3 - Design space contour plot of PGZ ($Y1 \geq 20\%$), Keto-P ($Y2 \geq 20\%$), OH-P ($Y3 \geq 20\%$), and RSG ($Y4 \geq 20\%$) as function of pH ($X1$, 6 to 10), HCl concentration ($X2$, 10 to 100 mmol/L), agitation speed ($X3$, 1000 to 1500 rpm), and extraction time ($X4$, 15 to 45 min).

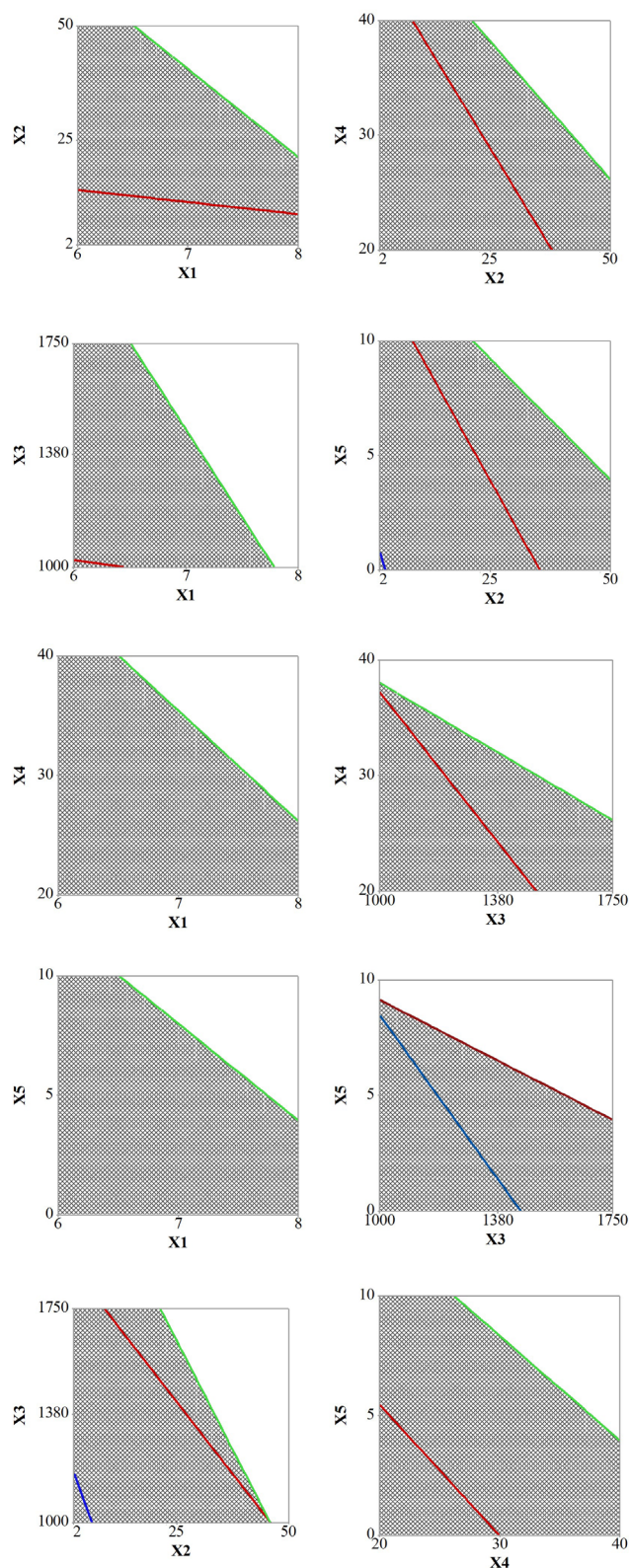


FIGURE 4 - Design space contour plot of RSG ($Y4 \geq 20\%$), N-Dm-R ($Y5 \geq 20\%$), and p-OH-R ($Y6 \geq 20\%$) as function of pH ($X1$, 6 to 8), HCl concentration ($X2$, 2 to 50 mmol/L), agitation speed ($X3$, 1000 to 1750 rpm), extraction time ($X4$, 20 to 40 min), and NaCl concentration ($X5$, 0 to 10%).

HF-LPME is basically a nonexhaustive extraction technique. In other words, the technique does not extract the total amount of analyte present in the sample but is based on a chemical equilibrium (Calixto, Bonato, 2010). For PGZ (black contour) and metabolites (Keto-P: orange contour and OH-P: pink contour), chemical equilibrium, under the optimized conditions, was reached in less than 15 minutes. The contour plot does not present significant change in the extraction efficiency when the extraction time is increased. There is no significant difference in the contour plot when extraction time and agitation are evaluated together. On the other hand, the pH and HCl concentration play an important role in extraction efficiency.

For RSG (blue contour) and metabolites (*N*-Dm-R: green contour and *p*-OH-R: brown contour), as the pH value increases, the extraction efficiency rose, mainly for *N*-Dm-R. The pH 8.0 permitted reduction of the extraction time, salt addition, agitation speed, and HCl concentration compared to pH 6.0, and with the same extraction efficiency. The same behavior was noted for HCl concentration. As the HCl concentration increased, the analytes protonation increased, and consequently, they presented lower affinity for organic solvent, increasing their concentration in the lumen of the membrane and resulting in higher extraction efficiency. Thus, we could obtain the same extraction efficiency, with reduced extraction time, added salt, agitation speed, and pH, when the HCl concentration increased from 2 to 50 mM.

DF is based on the obtainment of separate response surface models for each analyzed response. Their predicted theoretical values are used to calculate the individual desirabilities that are then transformed to a geometric mean (the overall desirability). The scale of

the individual DF ranges between 0 and 1, while the most desirable response is the Desirability Value (D) that is equal to 1, and a completely undesired value D is 0 (Novaes *et al.*, 2016). Figure 5 and 6 presents the D value for PGZ and its metabolites, and RSG and its metabolites, respectively. Even insignificant increase of extraction time, agitation speed, NaCl addition, and HCl concentration, promoted greater extraction efficiency for both methods.

RSG presented log D 2.43 at pH 6.0; 1.77 at pH 8.0; and 1.15 at pH 10.0; while for PGZ, the log D value was 3.33 at pH 6.0; 2.27 at pH 8.0; and 1.64 at pH 10.0. The same behavior was observed for their metabolites. This explains the higher efficiency extraction for lower pH values in CE procedure for determination of PGZ and metabolites, when evaluated with pH 6.0 and 10.0.

However, the same behavior for pH was not observed in HPLC procedure for determination of RSG and metabolites. It was assumed that RSG and its metabolites are close to the isoelectric point (6.42) in the pHs evaluated (pH 6.0 and pH 8.0). Considering that log D is not very different, this behavior could be explained according to the charge of the molecules. RSG presented charge of +0.43 at pH 6.0, and -0.94 at pH 8.0, and the same behavior was observed for its metabolites. The acceptor phase had HCl, a highly protonated medium, which could explain why higher efficiency was observed at pH 8.0 compared to pH 6.0. These values were obtained from Chemicalize.

Comparing to the works already published, extraction time and HCl concentrations were different, along with NaCl addition for the HPLC method, for the optimized factors obtained here, which is because the chosen factors were not just based on extraction efficiency (Calixto, Bonato, 2013; Calixto, Bonato, 2010).

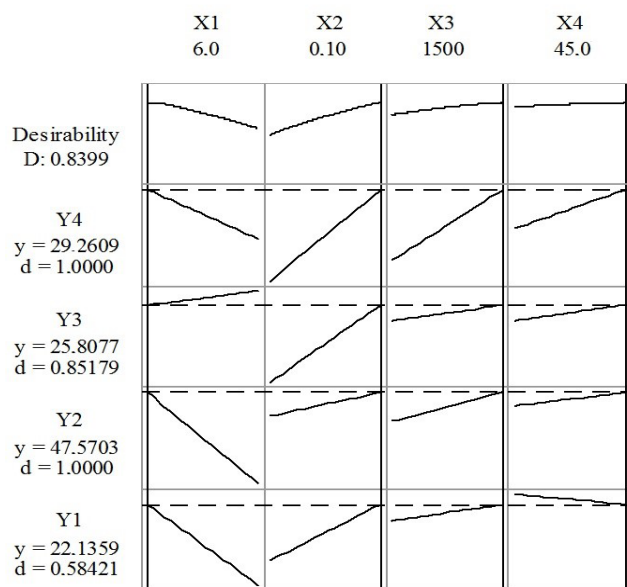


FIGURE 5 - Optimization plot of PGZ (Y1), Keto-P (Y2), OH-P (Y3), and RSG (Y4) as function of pH (X1, 6 to 10), HCl concentration (X2, 10.0 to 100.0 mmol/L), agitation speed (X3, 1000 to 1500 rpm), and extraction time (X4, 15 to 45 min).

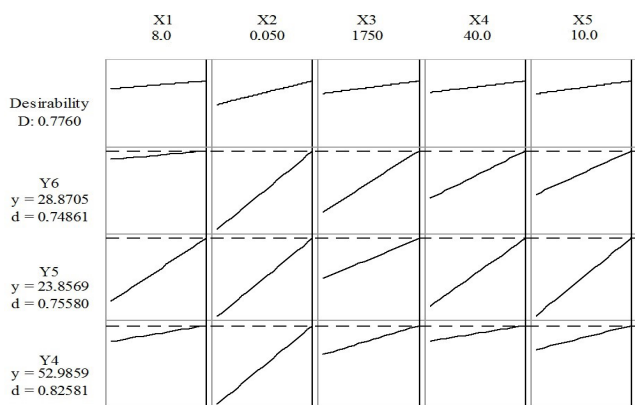


FIGURE 6 - Optimization plot of RSG (Y4), N-Dm-R (Y5), and p-OH-R (Y6) as function of pH (X1, 6 to 8), HCl concentration (X2, 2.0 to 50.0 mmol/L), agitation speed (X3, 1000 to 1750 rpm), extraction time (X4, 20 to 40 min), and NaCl concentration (X5, 0 to 10%).

CONCLUDING REMARKS

Microextractions have several parameters that must be optimized to achieve higher efficiency; however, most of works published still prefer to use univariate optimization, even knowing that multivariate optimization provides a better understanding of the factors involved.

Thus, this work presented an excellent option to optimize microextraction techniques, considering that only one work has employed DF for HF-LPME optimization. FFD in combination with the DF presents an excellent alternative for optimization of sample preparation.

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