African Journal of Biotechnology Vol. 10(48), pp. 9804-9811, 29 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1352
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Application of numerical modeling for optimization of selective hot water extraction of taxifolin from 'milk thistle' seeds

Hala El-Adawi[1], Yasser Abdel-Fattah[2] and Abeer Abd El-Wahab[1]*

[1]Medical Biotechnology Department, GEBRI, Mubark City for Scientific Research and Biotechnology Applications, Universities and Research Center District, New Borg El-Arab, P.O. Box 21934 Alexandria, Egypt.
[2]Bioprocess Development Department, GEBRI, Mubark City for Scientific Research and Biotechnology Applications, Universities and Research Center District, New Borg El-Arab, P.O. Box 21934 Alexandria, Egypt.

Accepted 1 April, 2011

The term flavonoid is used for a class of plant chemicals known for their activity as highly potent antioxidants and accordingly their ability to protect the body against oxidative and free radical damage. Taxifolin has attracted our attention because it constitutes the flavonoid moiety in 'milk thistle' seeds. In this research, a novel optimization-extraction method of taxifolin from 'milk thistle' seeds has been developed. Total antioxidant content was measured to monitor the efficiency of the extraction under different experimental conditions (solid- liquid ratio, extraction time and extraction temperature /pressure). Response surface methodology based on numerical modeling was adopted for optimization of extraction procedures. High pressure liquid chromatograph (HPLC) analysis was applied to identify taxifolin and some other phenolics in the 'milk thistle' extract. Using this method allowed the development of an empirical polynomial model for the production of antioxidants compounds from 'milk thistle' seeds. Application of such models is of great importance for pharmaceutical industries.

Key words: Milk thistle, extraction, numerical modeling, antioxidant, high pressure liquid chromatograph (HPLC).

INTRODUCTION

There is an increase in the use of complementary and alternative medicine (CAM), especially herbal therapy, among patients with liver disease. The most commonly used herbal agent is the seeds of the plant *Silybum marianum*. The seeds of the plant contain the highest concentrations of flavanolignans, a class of compounds that display hepatoprotective properties (Psotova et al., 2002; Skottová et al., 2003; Sobolová et al., 2006). The flavanolignans, along with one dihyroflavanol are collectively referred to as silymarin. Flavanolignans include silychristin, silydianin and the diastereomers silybinin A and B. Taxifolin, a dihydroquercitin, is a molecular precursor to the flavanolignans and is also included in the silymarin complex, as are the diastereomers isosilybinin A and B (Tittel and Wagner, 1977).

Taxifolin is one of the most effective natural antioxidants (Lee et al., 2007). The therapeutic benefits of Taxifolin were demonstrated in several studies, which showed the Taxifolin (Dihydroquercetin) acts in several ways to help avert cardiovascular disease. Scientists have demonstrated that dihydroquercetin inhibits lipid peroxidation, a process that often leads to atherosclerosis (Van et al., 2003, Potapovich and Kostyluk, 2003, Kravchenko et al., 2003 and Casaschi et al., 2004). A conjugated form of dihydroquercetin known as astilbin inhibits the same enzyme targeted by popular

*Corresponding author. E-mail: abdelwahababeer@gmail.com. Tel: +203- 4204985. Fax: + 203-4593407.

Abbreviations: CAM, Complementary and alternative medicine; **MAE,** microwaves assisted extraction; **SFE,** supercritical fluid extraction; **ASE,** accelerated solvent extraction; **PAH,** polycyclic aromatic hydrocarbons; **PCB,** polychlorinated biphenyl; **FTC,** ferric thiocyanate method; **BBD,** Box-Behnken experimental design; **TAC,** total antioxidant content; **HWE,** hot water extract.

cholesterol-lowering statin drugs such as Lipitor®, Zocor®, and Pravachol® (Chen et al., 2001). Recently, Vladimirov et al., 2009 proved that taxifolin (Dihydroquercetin) inhibits the free radical formation at key stages of apoptosis.

In the move to reduce or eliminate the use of organic solvent and improve the extraction process, newer sample preparation methods such as microwaves assisted extraction (MAE), supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE) have been introduced for the extraction of medicinal components of plant materials (Huie, 2002; Zygmunt and Namiesnik, 2003; Rohner et al., 2004). Water is non-flammable, non-toxic, readily available and an environmentally acceptable solvent. It has not yet received much attention as an analytical extraction solvent for plant materials because water is too polar to efficiently dissolve most organics that are associated with botanicals. It has been demonstrated that, raising the temperature with enough pressure to maintain the liquid state allows it to quantitatively extract a wide variety of organic solutes from many different matrixes. The ability of water to extract non-polar organic such as polycyclic aromatic hydrocarbons (PAH) and polychlorinated biphenyl (PCB) is linked to the fact that, the dielectric constant (polarity) of water can be reduced significantly with increasing temperature (Miller and Hawthrone, 1998; Lundstedt et al., 2000; Smith, 2002; Ong et al., 2006; Teo et al., 2008).

It was aimed in this study to optimize the water extraction conditions of taxifolin under high pressure and temperature by implementation of statistical experimental design based on response surface methodology. This method represents a numerical model correlating relationship between the tested variables and various possible responses such as total extracted phenolics and the antioxidant activity. To the best of our knowledge, this is the first report on maximizing the recovery yield of taxifolin using numerical modeling in order to reduce the operation cost as well as avoiding degradation of the desired product. The measured responses are indicative on the efficacy of the extraction method on quantitative basis.

MATERIALS AND METHODS

Reagents

High performance liquid chromatography (HPLC) grade solvents, including hexane, methanol and phosphoric acid 85% were obtained from Merck (Darmstadt-Germany). Linoleic acid, ammonium thiocyanate, ferrous chloride and ascorbic acid were obtained from Sigma-Aldrich, Germany. The silymarin standard was obtained from Mobaco-Co., Egypt and used as received.

Pre-extraction sample preparation

'Milk thistle' seeds were obtained from Mobaco-Co., Egypt. Seeds were crushed in a coffee grinder for 2 min with intermittent stops to prevent samples' heating. The sample were wrapped and stored at-18°C until the extractions were performed.

In order to get rid of fats of the sample, 40 g of crushed seeds were soaked in 300 ml of hexane overnight. For extraction of defatted seeds they have been exposed to different physical treatments (trials) as implemented in the experimental design.

Determination of antioxidant activity by the ferric thiocyanate method (FTC)

The FTC method was adopted from Osawa and Namiki (1981) for determination of antioxidant activity. Samples (20 $mgml^{-1}$) dissolved in 4 ml of 95% (w/v) ethanol were mixed with linoleic acid (2.51%V/V) in 99.5% (w/v) ethanol(4.1ml), 0.05 M phosohate buffer pH 7.0 (8 ml) and distilled water (3.9) and kept in screw-cap containers at 40°C in the dark. To 0.1 ml of this solution was then added 9.7 ml of 75% (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyante precisely, 3 min after the addition of 0.1 ml of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture. The absorbance at 500 nm of the resulting red solution was measured every 24 h until maximum absorbance value of the control was attained. The percent inhibition of linoleic acid per oxidation was calculated as:

(%) inhibition= 100-((absorbance increase of the sample/absorbance increase of the control) X100)

All tests were run in duplicate and analysis of all samples were run in triplicate and averaged.

HPLC analysis

The analysis was done using a Beckman C18 column (100 × 4.6 mm, 5 µm particle size), equipped with an autosampler, quaternary pump and a UV/Visible multi wavelength detector. The solvents were 0.5% phosphoric acid in 20% methanol (A) and 0.5% phosphoric acid in 80% methanol (B). The column was eluted at 1.0 ml min^{-1}.

Response surface experimental design

Optimization of extraction method was carried out according to response surface methodology (Box and Behnken, 1960). Box-Behnken experimental design (BBD) implementing response surface methodology was conducted to locate the true optimum conditions for extraction by examining the following variables: Solid-liquid ratio (X_1), autoclaving extraction time (X_2) and autoclaving pressure/temperature (X_3). The major measured response was the total antioxidant content (TAC %), while other minor responses as extract's weight and phenolic compounds were concomitantly measured. In this instant, a design matrix based on 15 trials involving three center points was constructed. Each variable was tested in three levels, coded as follows: -1, 0 and +1 for low, middle and high levels, respectively. The levels of each factor along with the coded are given in Table 1. The experimental results of the RSM were fitted with the second-order polynomial equation by the multiple regression technique.

$$Y = \mathrm{B}_0 + \sum_{i=1}^{k} Bixi + \sum_{i=1}^{k} Biixixj + \sum\sum_{i<j} Bijxixj \quad (1)$$

Where, Y is the predicted response, B_0, B_i, Bii, Bij are constant coefficients; x_i and x_j are the coded independent variables or factors. The quality of fit of the second-order model equation was expressed by the coefficient of determination R^2 and its statistical

Table 1. Box-Behnken experimental design and results.

Trial no.	Variables			TAC%
	Solid/liquid ratio X_1	Exposure time X_2	Temperature/ pressure X_3	
1	0 (1:7.5)	0 (30)	0 (121/1.2)	20.5
2	1 (1:11)	0 (30)	-1 (118/0.9)	8.4
3	0 (1:7.5)	1 (40)	1 (127/1.5)	17.8
4	1 (1:11)	0 (30)	1 (127/1.5)	7.6
5	-1 (1:4)	1 (40)	0 (121/1.2)	5.5
6	-1 (1:4)	0 (30)	1 (127/1.5)	38.3
7	0 (1:7.5)	0 (30)	0 (121/1.2)	20.5
8	1 (1:11)	-1 (20)	0 (121/1.2)	0
9	0 (1:7.5)	-1 (20)	-1 (118/0.9)	0.57
10	-1 (1:4)	-1 (20)	0 (121/1.2)	0.76
11	-1 (1:4)	0 (30)	-1 (118/0.9)	32.4
12	0 (1:7.5)	0 (30)	0 (121/1.2)	20.5
13	0 (1:7.5)	1 (40)	-1 (118/0.9)	2.1
14	1 (1:11)	1 (40)	0 (121/1.2)	3.6
15	0 (1:7.5)	-1 (20)	1 (127/1.5)	16.3

Values between brackets represent the actual values of variables; TAC%, total antioxidant content (%).

significance was determined by an *F*-test. The significance of the regression coefficients was tested by a *t*-test.

RESULTS AND DISCUSSION

Due to the high polarity of compounds present in the extract and for fine measurement methodology, HPLC-UV was used to identify those compounds. Figure 1 illustrates the extraction chromatogram on using (a) ethanol and (b) water as extraction solvents. In case of water extraction, the two major extractable compounds were taxifolin and silychristin; whereas minor amounts of silybinins were detected. On the contrary, major peaks of silybinins and minor taxifolin peak were measured on using ethanol extraction.

Apparently, the mono-structure of taxifolin seams to be more water soluble because of the five OH groups. Although the silybinins contains six OHs, it has two heavy molecular and more hydrophobic parts residing in the aromatic moieties that make it more extractable with ethanol than water. The better extractability of taxifolin by hot water over Soxhlet extraction was previously reported (Barreto et al., 2003).

Other peaks in the chromatogram were not identified due to the lack of standards. Where they are assumed to be phenolic compounds in a sense, they contributed significantly to the total phenolics in the extract, since total measured phenolic concentrations were much higher than the sum of the individual phenolic concentration identified and quantified by HPLC (data not shown).

In order to optimize the operation conditions namely: Extraction exposure time, pressure and solid-liquid ratio, Box-Behnken experimental design was implemented measuring the percentage of total antioxidant content as response (TAC %). The choice of TAC as response came from the fact that the potential of biological activity of taxifolin is due to its antioxidant activity (Rogovskiĭ et al., 2010). Statistical experimental designs methodology was previously implemented on extraction studies of *S. marianum* seeds (Liu et al., 2009; Zheng et al., 2009).

Fifteen trials (including three replicates at the center points) experiment was randomized as detailed in Table 1, to maximize the TAC% through standard regression analysis. Table 1 represents the design matrix of this experiment along with the experimental results. TAC% was determined according to Osawa and Namiki (1981) and considered as the response in the optimization process.

Multiple regressions were performed to fit the experimental response data to the theoretical polynomial model (see materials and methods) with the experimental data. The analysis of variance for the three variables (solid-liquid ratio, extraction time and pressure) indicated that, antioxidant activity can be well described by a polynomial model with a relatively high coefficient of determination (R^2 = 0.80). The value of the determination of coefficient suggests that, only about 20% of the total variations are not explained by the model. The statistical analysis of the full model in Table 1 shows that, extraction pressure has a positive influence on the measured antioxidant activity, whereas solid-liquid ratio had a negative significant effect. Interactions between tested variables, although not significant, had negative effect on the measured antioxidant activity. The polynomial model correlating the relationship between the three independent variables and the total antioxidant content percentage

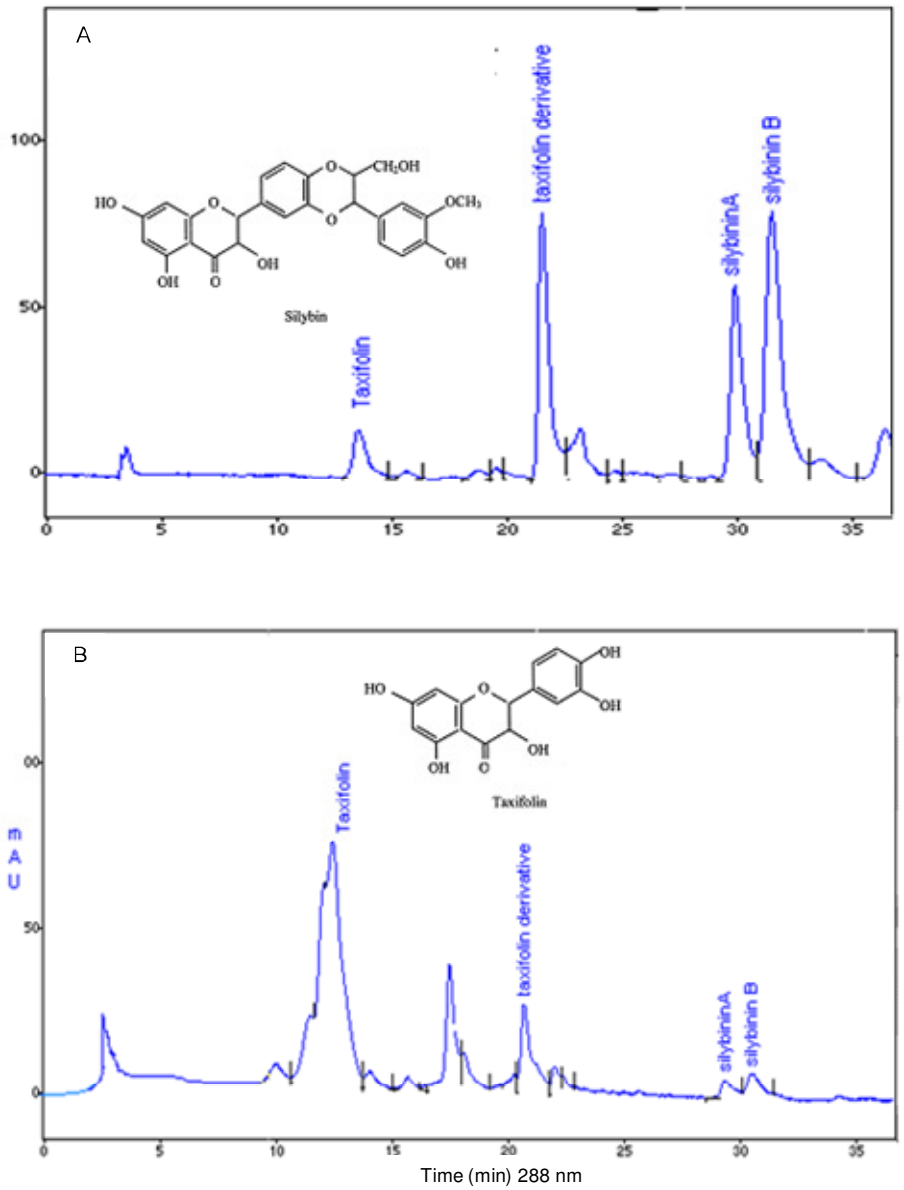

Figure 1. HPLC gradient elution chromatogram and UV analysis of 'milk thistle' seeds extraction. (A) Ethanol extraction; (B) hot water and high pressure extraction.

could be described as follows:

$$Y_{TAC\%}=20.5-7.7X_1+1.4X_2+5.1X_3-0.29X_1X_2-2.75X_1X_3-0.008X_2X_3-2.2X_1^2-15.8X_2^2+4.5X_3^2$$

Where, X_1, X_2 and X_3 represent codified values for solid/liquid ratio, extraction time and temperature /pressure, respectively. The residual analysis of the data (not shown) concludes that, the model accurately represents data in the experimental region (Strobel and Sullivan, 1999).

The optimum level of each variable was predicted using the non-linear optimization algorithm of Microsoft excel solver tool. As represented by coded levels, the optimum point that brings maximum total antioxidant content (38.4%) was obtained using the following conditions: Solid/liquid ratio at -1 (1:4), extraction time at 0.054 (30 min) and temperature/pressure at +1 (1.5 psi).

Based on the model, the effect of each factor can be predicted separately and presented graphically as a partial-effect function. Partial-effect plot describes how the response moves as the level of that variable changes. The over-layed partial-effects of variables on antioxidant content are presented in Figure 2, where solid/liquid ratio has significant effect on TAC%. With extraction time, the measured response increased gradually until the coded value of extraction time reached 0 and decreased after the extraction time level became higher than its coded

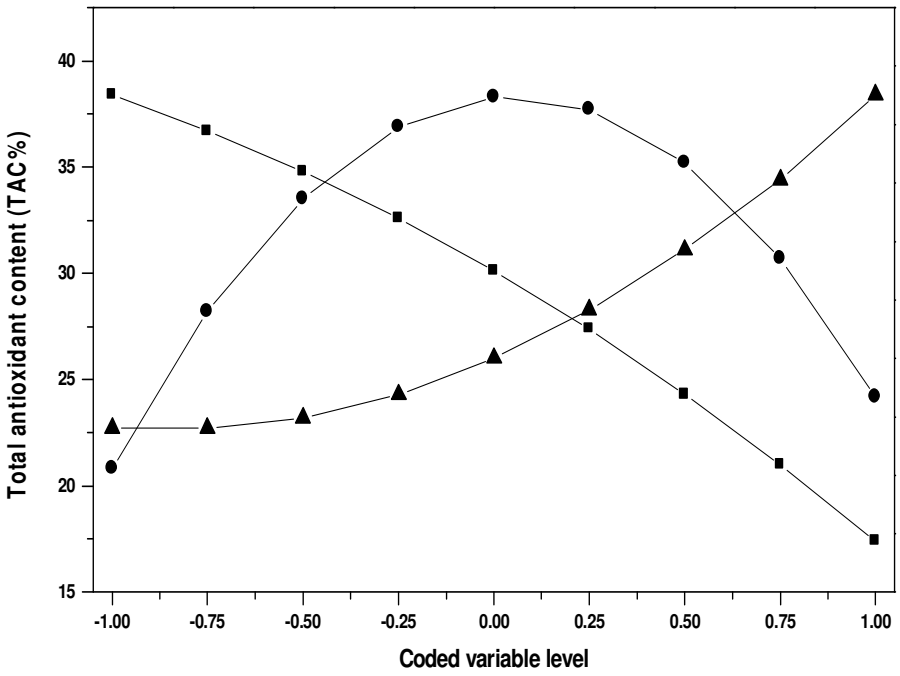

Figure 2. Partial-effects plot as a function of solid/liquid ratio (■), extraction time (•) and temperature/pressure (▲) coded levels (–1, 0 and +1).

level of 0. In addition, the difference between responses at minimum and maximum extraction time levels is relatively big (20.8 and 38.3%). Increasing the levels of temperature/pressure and decreasing the solid/liquid ratio results in decreasing the total antioxidant content.

Three-dimensional response surfaces presented in Figure 3 for the independent variables were obtained by keeping two variables constant, which indicated the changes in total antioxidant content under different extraction conditions. Figure 3b shows non-additive effects of solid-liquid ratio and pressure due to the significant interaction between them. In Figure 3a and c, it is to be seen that the effects of pairs of factors were additive since there are no interactions except the solid-liquid ratio and pressure interaction. By additively of the two-factor effects, it is meant that the effect of one factor on the response does not depend on the level of the other factor. In Figure 3a, it is obvious that maximum response was attained at moderate levels of extraction time (30 min) and 1:4 solid-liquid ratios (lower level). Figure 3c illustrates that, increasing pressure value to 1.5 at moderate extraction time led to maximum antioxidant activity. The optimum point deduced from Figure 3 is in accordance with the mathematically calculated optimum point.

Increasing temperature results in increasing the solubility of flavonoids especially on using water as solvent (Kwon et al., 2003). This is also in agreement with Zheng et al. (2009) findings, as they concluded optimum silymarin extraction in a microwave-assisted extraction with ethanol.

Validation of optimum point

The adequacy of the model was examined by an additional experiment using the derived optimal conditions. The predicted value was 38.4% and the experimental value was 39%. This is approximately 95% of the predicted value, which indicates that, the generated model is an adequate prediction of the antioxidant activity.

Conclusion

The recovery of 'milk thistle' compounds generally involves two steps, de-fatting and extraction. The current work removed the lipids by hexane, as shown in the pre-extraction sample preparation step. This step is important as it has been proven by Wallace et al. (2003), where they recorded that the defatted material yields twice the extract concentration of the whole seeds' extract.

Traditionally, the second step involves extraction using organic solvents, which was replaced in our study by water. Hot water attracted the attention of Duan et al. (2004) who were the first group to use it instead of organic solvents. Their approach was to search for a milder and greener solvent. They succeeded in reducing the extraction time from 200 to 55 min upon increasing the temperature from 100 to 140°C. Unfortunately, at 140°C, severe degradation of unprotected silymarin compounds was recorded. In the present study, to avoid the earlier mentioned degradation, the autoclave was run

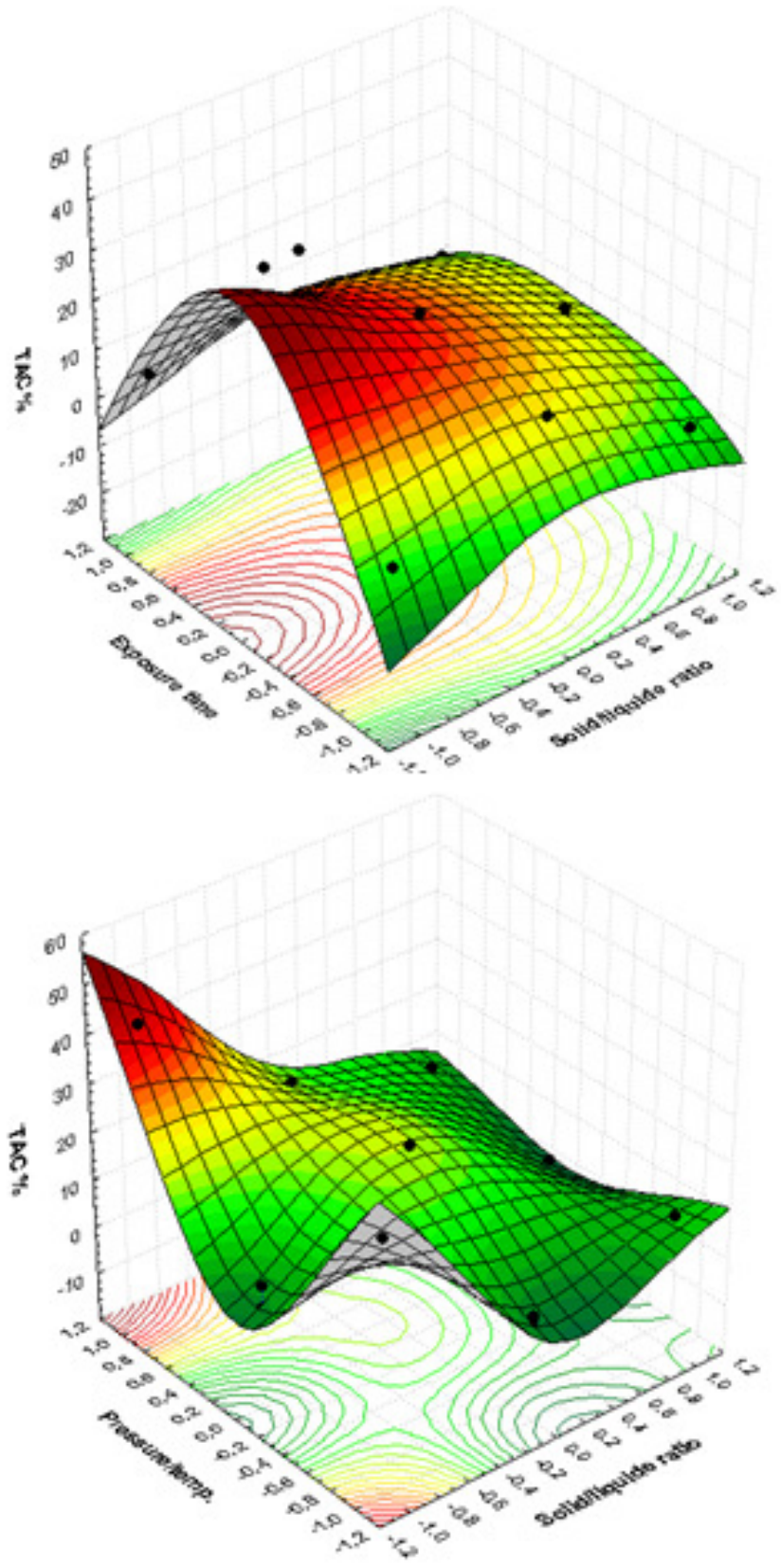

Figure 3. Three dimensional response surfaces for the effect of extraction conditions on TAC%.

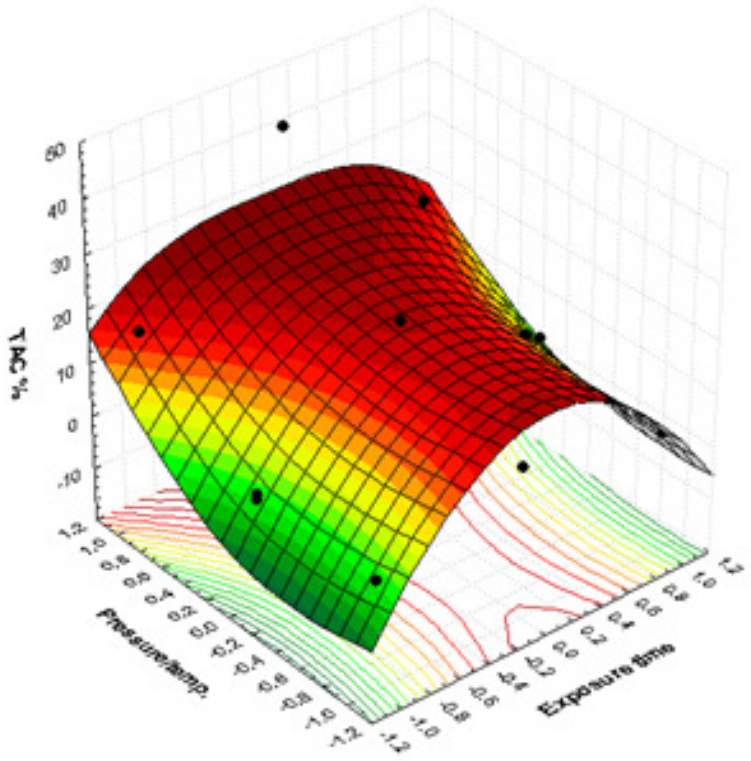

Figure 3. Contd.

at temperatures ranging from 118 to 127°C. Consequently, the extraction time decreased to 30 min, as shown in the results. The results of Wallace et al. (2007) support the use of water in place of organic solvents for flavonoids' extraction. Conclusively, the study proved that the hot water extract (HWE) could be used to extract flavonolignans from 'milk thistle' without the cytotoxic effects associated with using the traditional organic solvent ethanol.

The response surface methodology allowed the development of an empirical polynomial model for the production of antioxidant compounds from 'milk thistle' seeds using water extraction method under high pressure. The model was able to foresee confidently the antioxidant activity by changing provided physical parameters. Application of such models is of great importance for industrial applications.

REFERENCES

Barreto JFA, Wallace SN, Carrier DJ, Clausen EC (2003). Extraction of natraceuticals from milk thistle. Appl. Biochem. Biotechnol. 108: 881-889

Box G, Behnken D (1960). Some new three-level designs for the study of quantitative variables. Technometrics 2: 455–475.

Casaschi A, Rubio BK, Maiyoh GK, Theriault AG (2004). Inhibitory activity of diacylglycerol acyltransferase (DGAT) and microsomal triglyceride transfer protein (MTP) by the flavonoid, taxifolin, in HepG2 cells: potential role in the regulation of apolipoprotein B secretion. Atherosclerosis. Oct; 176(2): 247-53.

Chen TH, Liu JC, Chang JJ, Tsai MF, Hsieh MH, Chan P (2001). The in vitro inhibitory effect of flavonoid astilbin on 3-hydroxy-3-methylglutaryl coenzyme A reductase on Vero cells. Zhonghua Yi Xue Za Zhi (Taipei). 64(7): 382-387.

Duan L, Carrier DJ, Clausen EC (2004). Silymarin Extraction from milk thistle using hot water. Appl. Biochem Biotechnol.113-116(12): 559-568

Huie CW (2002). A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. Anal. Bioanal. Chem. 373: 23-30

Kostyluk VA, Kraemer T, Sies H, Schewe T (2003). Myeloperoxidase /nitrite-mediated lipid peroxidation of low-density lipoprotein as modulated by flavonoids. FEBS Lett. 537(1-3): 146-150.

Kravchenko LV, Morozov SV, Tutel'yan VA (2003). Effects of flavonoids on the resistance of microsomes to lipid peroxidation in vitro and ex vivo. Bull Exp Biol Med. 136(6): 572-575.

Kwon JH, Belanger JMR Pare JRJ, Yaylayan VA (2003). Application of the microwave-assisted process (MAP™) to the fast extraction of ginseng saponins. Food Res.Int.36: 491-498

Lee SB, Cha KH, Selenge D, Solongo A, Nho CW (2007). The chemopreventive effect of taxifolin is exerted through ARE-dependent gene regulation. Biol Pharm Bull., 30(6): 1074-1079.

Liu H, Du X, Yuan Q, Zhu Lei (2009). Optimization of enzyme assisted extraction of silybin from the seeds of silybum marianum by Box-Behnken experimental design. Phytochem anal. 20: 475-483

Lundstedt S, van Bavel B, Haglund P, Tysklind M, Oberg L (2000). Pressurised liquid extraction of polycyclic aromatic hydrocarbons from contaminated soils J Chromatogr A. Jun 23; 883(1-2):151-62.

Miller DJ, Hawthorne SB (1998). Method for determining the solubilities of hydrophobic organics in subcritical water. Anal Chem. Apr 15; 70(8): 1618-1621.

Ong ES, Cheong JSH, Goh D (2006). Pressurized hot water extraction of bioactive or marker components in botanical and medicinal plant materials. J.Chromatogr. A. 1112: 92-102.

Osawa T, Namiki M (1981). A novel type of antioxidant isolated from leaf wax of Eucalptus leaves. Agric. Biol. Chem. 45: 735-739.

Potapovich AI, Kostyuk VA (2003). Comparative study of antioxidant properties and cytoprotective activity of flavonoids. Biochemistry 68(5): 514-519.

Psotova J, Chlopcíková S, Grambal F, Simánek V, Ulrichová J (2002). Influence of silymarin and its flavonolignans on doxorubicin-iron induced lipid peroxidation in rat heart microsomes and mitochondria in comparison with quercetin. Phytother Res. Suppl 1: S63-67.

Rogovskiĭ VS, Matiushin AI, Shimanovskiĭ NL, Semeĭkin AV, Kukhareva TS, Koroteev AM, Koroteev MP, Nifant'ev EE (2010). Antiproliferative and antioxidant activity of new dihydroquercetin derivatives. Eksp Klin Farmakol. 73(9): 39-42.

Rohner CA, Matsler AL, Siebenmorgen TJ (2004). Comparison of three extraction systems for determining surface lipid content of thickness-fractionated milled rice. Cereal Chem. 81(9): 544-548

Skottová N, Vecera R, Urbánek K, Vána P, Walterová D, Cvak L (2003). Effects of polyphenolic fraction of silymarin on lipoprotein profile in rats fed Cholestrol-rich diets. Pharmacol Res. 47(1): 17-26.

Smith RM (2002). Extraction with superheated water: Review. J. Chromatogr. A. 975: 31-46

Sobolová L, Skottová N, Vecera R, Urbánek K (2006). Effect of silymarin and its polyphenolic fraction on cholesterol absorption in rats. Pharmacol Res; 53(2): 104-112.

Teo CC, Tan SN, Yong JW, Hew CS, Ong ES (2008). Evaluation of the extraction efficiency of thermally labile bioactive compounds in Gastrodia elata Blume by pressurized hot water extraction and microwave-assisted extraction.J Chromatogr A 22; 1182(1): 34-40.

Tittel G, Wagner H (1977) . High-performance liquid chromatographic separation of silymarins and their determination in a raw extract of Silybum marianum Gaertner (Compositae). J Chromatogr. 135(2): 499-501.

van der LB, Bachschmid M, Spitzer V, Brey L, Ullich V, Luscher T (2003). Decreased plasma and tissue levels of vitamin C in a rat model of aging: implications for antioxidative defense. Biochem Biophys. Res. Commun. 303(2): 483-487.

Vladimirov YA, Proskurnina EV, Demin EM, Matveeva NS, Lubitskiy OB, Noviko vAA, Izmailov D Yu, Osipov AN, Tikhonov VP, Kagan VE (2009). Dihydroquercetin (Taxifolin) and Other Flavonoids as Inhibitors of Free Radical Formation at Key Stages of Apoptosis. Biochemistry (Moscow), 74(3): 301-307.

Wallace S, Raible J, Carrier DJ, Vaughn K, Griffis C, Clausen E (2007). Pressurized water versus ethanol as a *Silybum marianum* extraction solvent for inhibition of low-density lipoprotein oxidation mediated by copper and J774 macrophage cells. Can. J. Physiol. Pharmacol., 85*:* 894–902.

Wallace S, Carrier DJ, Clausen EC (2003). Extraction of silymarin compounds from milk thistle (*Silybum marianum*) seed using solvent. Appl. Biochem. Biotechnol. 105-108: 891-903.

Zheng X, Wang X, Lan Y, Shi J, Xue S, Liu C (2009). Application of response surface methodology to optimize microwave-assisted extraction of silymarin from milk thistle seeds. Sep. Purif. Technol. 70: 34-40.

Zygmunt B, Namiesnik J (2003). Preparation of samples of plant material for chromatographic analysis. JCS., 39(10): 109-116.